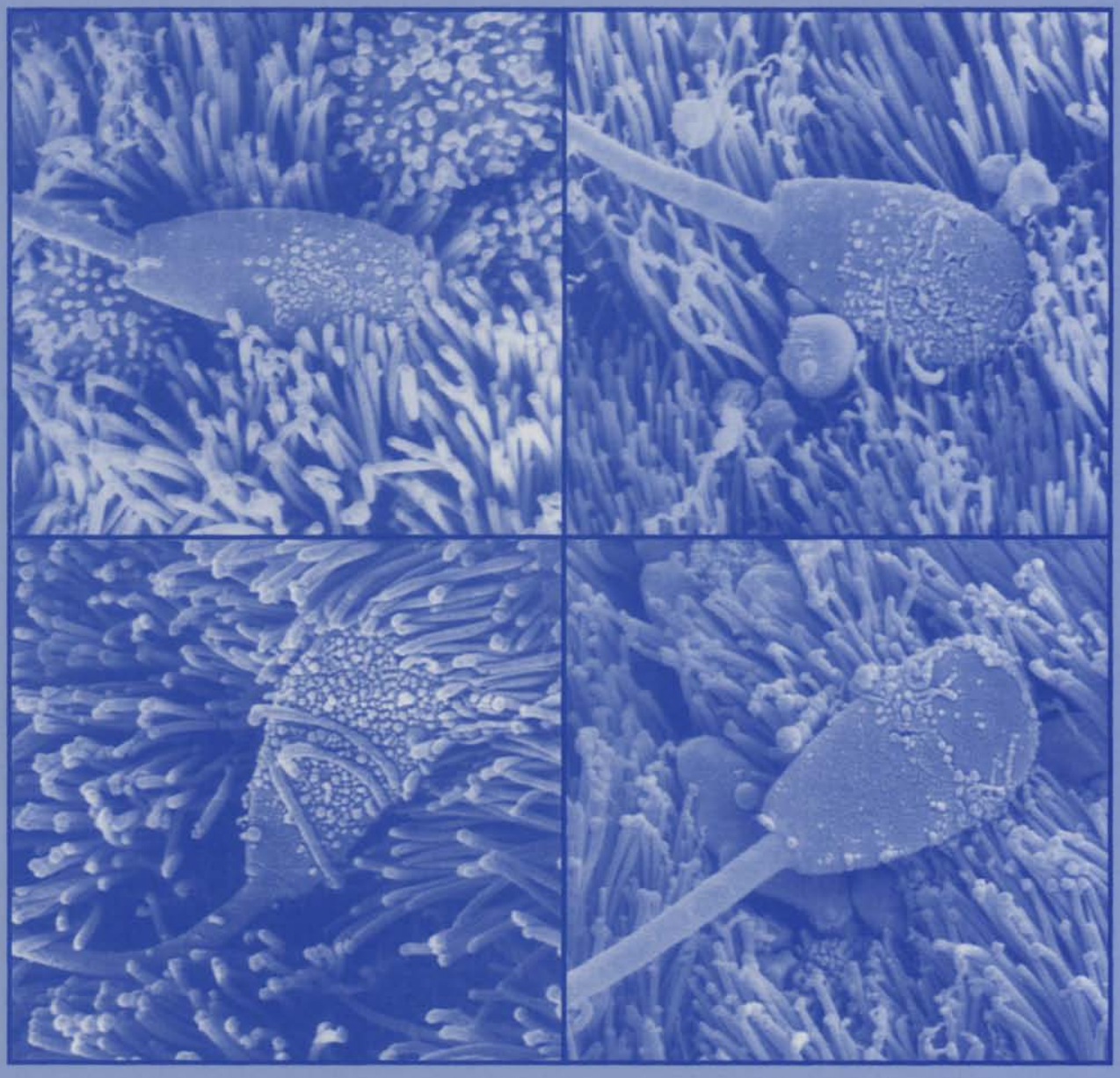
ARTIFICIAL INSEMINATION TO CLONING



Tracing 50 Years of Research

Robert H. Foote

ARTIFICIAL INSEMINATION TO CLONING

Tracing 50 Years of Research

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of Animal Science
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Artificial Insemination to Cloning

Robert Foote received his Ph.D. from Cornell University. For 50 years he has been involved in reproductive physiology research of male and female domestic animals, rabbits and ferrets, covering the development of artificial insemination to the beginning of cloning. During this time he taught courses principally in Reproductive Physiology, in Artificial Insemination, and in Animal Reproductive Biotechnologies.

Front cover: Top two photos are fresh bull spermatozoa in oviductal ampullar epithelium after 0 hours (left photo) and 3 hours (right photo) of incubation. Note the droplets already on the anterior portion of the sperm head at 0 hours and the beginning of vesiculation of the acrosomal membrane at 3 hours. The bottom two photos are frozen-thawed bull spermatozoa in oviductal ampullar epithelium after 0 and 3 hours of incubation (left to right). Note the dense coating of droplets and encasement by cilia at 0 hours and distinct vesiculation of the acrosomal membrane at 3 hours. From Suzuki and Foote, Micros. Res. Tech. 31:519. 1995, and Suzuki et al., J. Androl. 18:217. 1997, and Suzuki unpublished.

Back cover: Top photo is a bovine embryo undergoing the fourth cleavage division, with one large blastomere in the background that has not yet cleaved the fourth time. The bottom photo is a hatching bovine blastocyst with the trophectoderm cells of the blastocyst to the right and the surface of the zona pellucida revealing sperm head images to the left. Unpublished photographs by Dr. Suzuki.

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Preface and Acknowledgments

The present era is a wonderful period in which to be involved in reproductive research. Great advances in molecular biology and computer technology are unraveling the DNA double helix and the blueprints of our genetic heritage are unfolding before our eyes. As we speed forward, an awareness of the knowledge obtained earlier, which laid the tracks along which we travel, can enhance our appreciation and enjoyment of life and provide the spark for a new idea.

Our laboratory has been privileged to be in the mainstream of research on animal reproduction and biotechnology during the past 50 years. Following the establishment of the laboratory by G.W. Salisbury, this book chronicles the adventures of our laboratory as we traveled along this stream. *Imagine that you were doing this research in the context of what was known 50 years ago, a time before many of you were born. Many results obtained under less advanced research conditions at that time still are used today.* The story begins when artificial insemination (AI) was in its infancy in this country and continues to cloning. The development of bovine semen extenders, egg yolk citrate, and egg yolk-tris, and the use of the penicillin-streptomycin-polymyxin D, tested decades ago at Cornell, formed the basic preservation media for much liquid semen and, with glycerol (a British discovery) for frozen semen, throughout the world. Had these been patented, a few cents per cow would have accumulated more than $50,000,000 today.

Also, as part of developing a successful artificial insemination program, longtime studies were conducted on nutrition and management of bulls and cows. Much of this research, covering a period of 15 years, is obscure because it was published in experiment station bulletins, as was common then.

Over time, the study of spermatogenesis, sperm physiology, and sperm preservation guided our research toward the female in the areas of fertilization and early embryo development. A combination of basic investigations to provide knowledge, along with modeling and applied studies, helped to develop a successful AI program.

With the arrival of Dr. Charles R. Henderson, a mathematical genius, trained under Dr. Jay L. Lush, a symbiosis developed, combining the best techniques for identifying superior genetics with the best techniques for harvesting and dispersing these genes worldwide through sperm. Dr. Henderson's research is largely published elsewhere.

The program at Cornell was supported by a unique relationship between industry, academia, and government. Farmers formed a cooperative, and this organization, along with New York State and Cornell University established a working relationship nearly 60 years ago that still exists. The cattle research reviewed in this book would not have been possible without the generous financial support and use of facilities supplied by the New York Artificial Breeders Cooperative, Inc., which later became Eastern A.I. Cooperative and now Genex Cooperative, Inc. This AI organization provided unlimited quantities of semen, making highly replicated factorial experiments possible. Large field trials to provide the ultimate test of the value of the laboratory research were facilitated by professional inseminators who bred hundreds of thousands of cows with experimental semen, thus advancing and enhancing the overall program of breeding cattle by AI. One can summarize this era as characterized by foresight in planning and hindsight in final application.

As a laboratory-animal model to gain knowledge quickly and inexpensively, we chose the rabbit. All the procedures we wished to test with farm animals relative to semen collection, evaluation, insemination, embryo culture and transfer, and other biotechnologies could be performed and were done in the rabbit. In addition, studies establishing that all oocytes in the adult female were formed prenatally or neonatally, and other studies isolating the aging effects of oocytes, hormone secretion, blood flow and uterine composition on reproduction were published, using the rabbit model. The male rabbit was developed as a nonrodent model for studying reproductive toxicology in the male. These studies had relevance to many species, including endangered species and human beings. They were supported primarily by the National Institutes of Health.

During these 50 years we taught courses in comparative animal reproduction, artificial insemination and animal biotechnology. The staff and students were interested in many species. The comparative approach to a subject had many advantages. These interests led to comparative research, facilitated by many collaborators, and resulted in studies with cattle, horses, sheep, goats, swine, dogs, rabbits, and ferrets, as well as some in vitro studies

with human sperm. The support of approximately 20 granting agencies was crucial and is gratefully acknowledged.

The Table of Contents provides the reader with a quick road map of the organization of the book. Philosophically, the heart of the book is Chapter I, originally a paper given in response to the L.E. Casida Award. This chapter provides a bit of history of classic work done in other laboratories and the author's approach to building people and developing and sharing knowledge. It is printed essentially as it originally appeared. Physiologically, Chapters II to V are the digestive and vascular systems, summarizing the information collected, digested, and circulated through the numbered regular publications (no abstracts) from our laboratory.

Abstracts of these papers follow the chapters, providing additional information. They are reproduced essentially unchanged from the originals, representing the state of knowledge at that time, and with accepted abbreviations (HCG instead of hCG, for example). These papers are cited by number in Chapters II to V and are listed by number chronologically at the end of the book. The original articles cite more than 11,000 references, leading to a rich source of information contributed by outstanding researchers from many laboratories.

I hope that the reader will find this book interesting to peruse in a casual way, as a stream of information flowing over time (at least on the rainy days that occur periodically in Ithaca). Also, many papers can serve as a source of experimentally tested information, much published before electronic reference lists were developed.

The story here is the one I can tell best, remembering the daily contact in the lab and in the field for more than 50 years, with the 99% perspiration that followed 1% inspiration. Along with the steady stream of research were the controversies where I was asked to testify as an "expert." These included law suits relating to death of cattle embryos, the residual dibromochloropropane in the ground water in California, and the continuing debate over funding for human in vitro fertilization and related research. So there was lots of excitement in addition to the scientific discoveries. Enjoy the story.

During this journey I became more and more aware of the beauty of nature, of the monumental contributions to our understanding of nature by our forebears, of my relative state of ignorance, and of my increasing reflection on the meaning of life.

I am grateful to the many students and teachers and other researchers worldwide who contributed to this journey. The reader will recognize names of many talented people who authored papers as they moved on to distinguished careers. Several of these collectively organized an excellent symposium for me in 1992 *(Theriogenology* 38:177-374), and I felt greatly honored.

Special acknowledgment is given to my family, who supported my long hours of work in an effort to do the very best. Deloris Bevins did a superb job in helping to put the book together. Sarah Seidel and Trudie Calvert made many improvements in the manuscript. The cooperation of the staff in Media Services is much appreciated. Linda Haylor Mikula, Carol Doolittle, Richard Gingras and Gary Huddle assisted with wrapping up the manuscript in printed form.

Any errors are the responsibility of the author.

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Chapter I. Animal Scientists: Nature and Nurture[1]

Introduction

Whatever each one of us becomes depends upon how fortunate we were to receive a good measure of inheritance, a generous supply of many good things in life, and on how well we nurture and utilize these given talents by both creating and capitalizing on opportunities. I was fortunate in the "choice of my parents," in growing up on a dairy farm in the Depression when honesty, hard work, and service were mandatory, and yet the opportunity for formal education was provided, despite the shortage of money. Furthermore, I was fortunate in being a survivor of World War II after having an opportunity to grow and to learn firsthand what it meant to try to serve my country and the cause of freedom. Finally, after colliding on several occasions in the war with high-speed particles, which necessitated a change in my objective of dairy farming, I was fortunate to receive the assistance of my former undergraduate college advisers in steering me to a program of graduate study. So, I begin my presentation with an acknowledgment of great indebtedness to others. This important principle I hope I have continued to recognize throughout my scientific career, partly by appearing as the last author on papers, unless I personally did most of the work.

Dr. L. E. Casida, 25 years ago (Casida, 1966), set forth principles that are as important and appropriate today as they were when first published. He pointed out the need to learn research techniques, especially biostatistics, and to learn to think about principles and concepts. Appropriately he mentioned the importance of developing the ability to discuss, to publish, and to prepare research grant applications. He recognized the need for comparative studies and the role that laboratory animals could play in designing replicated, short-term experiments that could be applied by the same researchers to enhance understanding of the function of farm animals. He suggested a sequence of training by which the novice researcher could develop into an independent scientist. Dr. Casida's paper is an ideal one for all animal science graduate students to read and ponder. I wholeheartedly agree with his philosophy. Thoughts I will express here are examples of things I experienced which I believe were useful in attempting to achieve, in a small way, some of the objectives described by Dr. Casida.

My first contact with Dr. Casida's work was reading "The Oestrous Cycles of the Ewe; Histology of the Genital Tract," Missouri Research Bulletin 170, 1932. The University of Missouri had many excellent bulletins, especially by a series of reproductive physiologists working under the tutelage of Dr. Fred F. McKenzie. The list of references cited in Dr. Casida's bulletin includes a veritable "Who's Who" in reproductive physiology and endocrinology. The list includes Cole, Corner, Evans, Hammond, Hisaw, Leonard, Marshall, Meyer, Papanicolaou, Price, Smith, and Zondek. What a history of reproductive science course could be built around a study of these persons!

These Missouri bulletins were in the library, along with huge amounts of other interesting sources of knowledge. Cornell University has an excellent library system, and my mentors encouraged me early on to start using this resource. I was exposed to many great teachers and researchers, including Drs. Asdell, Bratton, Dukes, Loosli, Maynard, McCay, Morrison, Salisbury, and Srb. As a result of their stimulation, I decided to carry extra subjects and had concentrations in physiology, animal breeding, genetics and nutrition. Later Dr. Henderson joined the group to become acknowledged as a world leader in statistical aspects of animal breeding. These teachers were sticklers for accuracy and depth of knowledge, integration of concepts, familiarity with current research, and the proper design of experiments. I set up a notebook that soon expanded to 60 journals I found interesting in the libraries. Every month I went through the list and checked off that I had seen each number. Also, I developed a three-copy abstract card that allowed for filing a 3" x 5" author card and cross-filing by two subject headings on two 5" x 8" abstract cards. This was before the days of computer retrieval systems. *Animal Breeding Abstracts*, *Biological Abstracts* and *Chemical Abstracts* were major sources of the world literature in abstract form.

[1]Foote, R. H. Animal Scientists: Nature and Nurture. ©J. Anim. Sci. 70(Suppl. 2):51-54. 1992.

Personal Goals

I had always been indoctrinated with the ideas that one should do something worthwhile with one's life, that time was valuable, and with Ben Franklinisms such as "Never put off until tomorrow what you can do today." I've learned that not to make a decision is, in fact, a decision. Also another simple expression with powerful implications (author unknown to me) is "If it is to be, it is up to me." Thus, there was a "workaholic" element built in, which seemed essential to teach, do research, read the literature, publish, and write grant proposals, in addition to the many little things that often occupied the 8 A.M. to 5 P.M. shift. One must love science and the pursuit of knowledge, if one is to feel well rewarded through the adventures of discovery, which often are preceded by frustrations of failed experiments. However, frustrations occur in simple everyday life without this reward. J. McGavran, in The Homesteader, wrote, "Frustration is when the same snow that covers the ski slopes makes the roads to them impassable."

Another important point in working with colleagues is to recognize them as individual people, outside of the realm of science. Over my desk is a clipping from Ann Landers that reads: "Resolve to be tender with the young, compassionate with the aged, sympathetic with the striving, and tolerant of the weak and wrong. Sometimes in life you will have been all of these." I wish I could say that I have always followed this good advice, but sometimes, regrettably, I have failed.

I have always had a personal idol, who was President Lincoln, with memories of him recounted by my grandmother when I was young. We all know his Gettysburg address, but I refer here to his second inaugural address, March 4, 1865: "With malice toward none; with charity for all; with firmness in the right, as God gives us to see the right, let us strive on to finish the work we are in."

Some Attributes of a Scientist

Ideas expressed here I've borrowed from various seminars I have given and from a detailed mimeograph by N. L. VanDemark dated June 1, 1978, entitled "The Synthesis of a Scientist." Obviously, what will make a person a successful scientist are qualities that are inherent in persons successful in many walks of life. Choosing a series of traits, listed in alphabetical order, I would include the following: able, ambitious, analytical, broad in perspective, challenging, critical, dedicated, determined, enthusiastic, honest, innovative, inspired, leader (not leaner), motivator, observant, organized, patient, persistent, positive, resourceful, sincere, thinker, and understanding. That is quite a tall order, but these are traits we should cultivate. We must hunger and thirst after knowledge. We must doggedly adhere to the truth as we understand the truth to be. We must separate facts from beliefs and propaganda and attempt to minimize our biases. We should view research as (1) a privilege, (2) a responsibility, (3) a science, (4) an art, and (5) joyful work.

Research Goals

My research program is what I call "goal oriented." If there is enough information to determine how to solve a practical problem, then we can help our extension service in relaying solutions to the potential users, as well as use these examples in our classroom teaching. If knowledge is in short supply (which often is the situation), then experiments must be carefully planned to yield possible answers to specific questions or hypotheses tested.

Usually there are a host of problems to be solved so one must (1) establish a set of goals, (2) carefully assign priorities, (3) decide what to give up, and (4) get to work, as success is 1% inspiration and 99% perspiration.

Evolution of a Research Program

A few examples of how and why my research program evolved the way it did and mistakes made may be useful. In the beginning of my research career artificial insemination of dairy cattle was in its infancy. Fertility was low and what was then called *Vibrio fetus* was widespread in bull studs. The first research was aimed at better methods of preserving bull semen. One experiment was a split-ejaculate arrangement with egg yolk-citrate extender added to semen immediately after semen collection before cooling instead of the control method of cooling semen first. The fertility of the sperm was increased about 6% (Foote and Bratton, 1949). Antibiotics, penicillin, streptomycin, and polymyxin D were found to be compatible with good sperm survival,

and this combination prevented spread of *Vibrio fetus*, raising fertility several more percentage units (Foote and Bratton, 1950). Other studies involved better methods of collecting and using semen.

With fertility levels highly acceptable, our research could turn to basic studies of spermatogenesis. In order to facilitate a thorough study of the histology and kinetics of spermatogenesis, using radioisotopes, we needed a small animal model. We chose the rabbit (Swierstra and Foote, 1963, 1965). A rabbit colony was gradually established for comparative studies with large domestic farm animals. We chose the rabbit because (1) sperm could be collected repeatedly, (2) artificial insemination was simple, (3) studies with the female were simplified because of induced ovulation, and (4) a whole series of studies on fertility, embryo culture, transfer, and embryonic mortality could be modeled using a variety of techniques (Kennelly and Foote, 1965; Maurer et al., 1968; Kane and Foote, 1970; Anderson and Foote, 1975). These studies provided excellent opportunities for designing good experiments with sufficient replication, with reasonable costs and opportunities to learn many procedures and to complete theses and publish results in a few years. This contrasted to lifetime nutrition studies with bulls that covered about 15 years and were not ideal alone for thesis research or gaining tenure. At the same time studies with bulls, calves, and cows gave persons experience with large animals (Seidel and Foote, 1969; Seidel et al., 1971).

Regular cooperation with industry also was and is an important part of the research program. Research can be facilitated by this cooperation and the flow of information for practical application is expedited. This also keeps the basic research program in tune with agricultural needs. One such program was the collaboration of many major bull studs in studying testicular growth, developing simple methods of measurement, estimating heritability and the relationship to sperm production, fertility, and reproductive characteristics of the female (Hahn et al., 1969; Coulter et al., 1976). The study was repeated to measure growth for many years. This type of collaboration has been continued through the years with Eastern A.I. Cooperative, Inc., and embryo transfer studies more recently with Em Tran, Inc. Thus, a program of basic and applied research across laboratory and larger farm animal species was usually kept in reasonable balance. One problem was that we did not always control as well as was desirable the tendency to proliferate beyond our means. The resultant scattering of effort could lead to superficial research, which is poor training of researchers and money wasted when equivocal results are obtained due to inadequate replication.

Laboratory animals also can serve as models for studying reproductive function in human beings (Foote, 1989). Agents in the environment that may damage reproduction can be studied in both male and female rabbits (Foote et al., 1986a, b). The agent, dibromochloropropane (DBCP), caused infertility in male workers in the manufacturing plant in the 1970s. We were able to study the effect of DBCP in the drinking water on spermatogenesis, semen quality, and fertility, as well as to compare in vitro effects on rabbit and human sperm. These papers today serve as the basic information on quantified effects of DBCP in the drinking water on male reproduction. The data have been analyzed in detail to provide estimates of the number of animals needed in future experiments to detect treatment differences of specified magnitudes and with specified Type I and Type II errors.

Cloning

More recently, the rabbit has proven to be a useful model for production of chimeras and for cloning (Yang et al., 1990; Foote, 1991). The blastomeres of the rabbit embryo maintain totipotency, probably for a period similar to cattle. Many of the aspects of the sequence of steps in cloning cattle can be modeled in the rabbit much more rapidly and with modest costs. Simultaneously, the best procedures can then be tested with cattle. Currently both species are being used, giving undergraduate and graduate researchers, as well as postdoctorals, an opportunity to learn various skills and compare results in properly designed experiments.

Keeping a Perspective

Dwight J. Ingle gave a highly entertaining and thought-provoking speech when he was president of the Endocrine Society (Ingle, 1960). He described Percy Diorets, born in obscurity, but who received a mutated gene which "enhanced the ability of the brain to function creatively." He became a gifted

scholar with many personal problems, but he made a fascinating discovery. His professor, Professor Suopmop was about to address an "Academy of Science Writers" and he suggested he report Percey Dioret's finding. Many honors came to the professor, but little to Percy. Percy later rose to a professorship and became involved in committees and fund-raising. Later his laboratory made a startling discovery which Percy reported and which made him more famous. The lack of credit to the discoverer bothered him, but he was able to rationalize that the discovery was possible only because of the laboratory Percy had established.

Dr. Ingle goes on to point out that a human life is minuscule in terms of geologic time and space, but in the "evolution of the universe man does become important." "Man deserves respect for being alive" and creative, and Percy Diorets was an example. In "evolutionary biology we do not rant at the errors of nature or at the tooth-and-claw way that has not been completely lost from the human brain. Rather we wonder at the positive achievements of nature and its greatest gift of all--the capacity of man to control his own future even though error, chance, and risks are still a part of nature and the evolutionary process."

Teaching

While the focus of this paper is research, teaching, so that others learn, should not be overlooked. Teaching courses at the various levels stimulates discussion of research as well as feedback to the researcher of questions currently without answers. Many undergraduates become aware of research possibilities through hearing about research in courses on reproduction. Selected students can work with staff and explore closely related side projects, some of which often lead to major research efforts. Some of these able students change career objectives and apply for graduate work. The researcher who is also excited about teaching, I believe, is a better teacher because of the research. As indicated, such a teacher opens the door, exposing new horizons for many a young undergraduate.

Problems

Obviously, one person cannot be all things to all people. A person who teaches has less time for research. A person who trains graduate students cannot generate the research results that large laboratories produce with a force of postdocs. Some research groups hire only the latter. With tight grant funds, this is a serious problem. If the person willing to train graduate students is less competitive in obtaining grants, where will the graduate students be trained? There will not be the pool of postdoctorals. Universities, the federal government, and industry should provide more basic support of graduate training programs. Otherwise this somewhat "cut-throat" competition for research grants will be both discouraging and self-defeating in producing the needed ongoing supply of outstanding young scientists in this country. Although errors are a part of the evolutionary process, as Dr. Ingle pointed out, scientists must unselfishly play a very active role in advising the powerful institutions concerned with research so that science, the country, and the community of people served by research will prosper.

Acknowledgments

To all the teachers mentioned in this paper, many other colleagues and collaborators, undergraduate and graduate students, visiting scientists and other technical help, and peer reviewers who have helped to keep grant money coming, I am deeply grateful. Words are inadequate to express my deep appreciation to my family, who put up with my long hours at the laboratory.

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Chapter II. Dairy Cattle

1.0 Background and General References

The early studies on cattle reproductive technology were focused primarily on the development of artificial insemination (AI). In 1939, investigators from several universities at a meeting of the American Society of Animal Production (1) outlined studies in progress dealing with semen collection, evaluation, and preservation. A series of papers followed from Cornell on collection, bacteriology, evaluation, preservation, and insemination of semen (2-4, 7-14, 16-21). Many of the procedures adopted early in the development of AI have continued or have been improved. These are discussed in various chapters and monographs dealing with AI (132, 149, 169, 223, 294, 297, 300, 306, 369). Early contributions were made by Enos Perry in New Jersey, Paul Phillips in Wisconsin, R.E. Comstock and W.W. Green and later Edward Graham in Minnesota, John Lasley and later Harry Herman in Missouri, John Almquist in Pennsylvania, Ralph Erb in Washington, and many others, including Arthur Walton in England, Eduard Sorensen in Denmark, Yoshimasa Nishikawa in Japan, and Elie Ivanov in Russia. For additional reading suggestions consult the Appendix.

The subjects dealt with in the first part of this chapter relate to the male: semen evaluation and processing, along with fertility results. This is followed by research on managing cows for AI. Then the focus is primarily on the female, as various aspects of embryo production, culture, and biotechnology are discussed. The new biotechnologies provide opportunities for AI organizations to expand their operations (371).

In the sections that follow, general trends and conclusions are emphasized rather than details in order to facilitate reading, and recognizing that much data are readily available in the abstracts included in this book.

2.0 Objectives of the AI Program: To Obtain Sires Superior in Genetics, Sperm Production, and Fertility

The total AI program established over several years involved the essentials that are included in the title of this section plus a superior semen distribution program, highly trained inseminators, and dairy farmers with excellent programs of estrous detection and overall reproductive management. It soon became clear that all the research, as it affected progressive breeding of dairy cattle, could be quantified by simple equations published in 1956-57 (65, 69).

The terms in this equation can be subdivided into factors affecting each term. Sperm obtained per sire depends on the sire and on procedures followed in sexually preparing the bull at the time of semen collection, frequency of collection, and the number of years the animal is in service. Changes in some of these procedures also can affect fertility. The number of sperm needed to inseminate each cow to give maximal conception rates is well established. This information and the relationship between decreasing numbers of sperm cells inseminated and conception rates are discussed in a subsequent section. The

The potential genetic contribution of a sire to a population can be expressed by a simple equation:

$$\text{Contribution of each sire to genetic improvement} = [\text{Number of progeny obtained per sire}] \times [\text{Genetic superiority of the sire used}]$$

In turn, the number of progeny that could be obtained from each sire can be expressed by the following equation:

$$\text{Number of progeny obtained per sire} = [\text{Number of sperm obtained per sire}] \times [\text{Percent of potential Number of sperm inseminated/cow}] \times [\text{Percent of cows that calved}]$$

Table 1. Interrelationship of factors affecting the possible number of progeny per sire per year

[Sperm harvested per bull per year]	÷	[Number of sperm used per cow]	×	[Percent of collected sperm used]	×	[Percent of cows calving after one insemination]	=	[Number of progeny per year]
500,000,000,000		20,000,000		80		40		8,000
"		"		"		50		10,000
"		"		"		60		12,000
"		10,000,000		"		40		16,000
"		"		"		50		20,000
"		"		"		60		24,000
1,000,000,000,000		20,000,000		"		40		16,000
"		"		"		50		20,000
"		"		"		60		24,000
"		10,000,000		"		40		32,000
"		"		"		50		40,000
"		"		"		60		48,000

percentage of the sperm obtained that is used depends largely on demand. This percentage of sperm used was increased by the adoption of frozen semen. The percentage of conceptions and progeny reared are affected by many factors. About 49% of total births are females.

The quantitative outcome of the second equation is illustrated in Table 1, adapted from Foote and Bratton (69). At the present time some bulls in high demand are ejaculated very frequently. More sperm are harvested, and sperm are more highly diluted than is illustrated in the table.

The principles illustrated in this table can be used to determine the number of sires needed to breed a cow population of a specified size and the number of replacement sires needed from the progeny-testing program each year. As the number of progeny per sire increases, the percentage of those sires needed for replacements decreases in a constant-sized population. Thus, fewer and more select bulls can be saved. Our laboratory made a significant effort to determine ways to maximize values for the different components of the second equation, thus making AI as efficient as possible (131, 449). With intensive schedules of bull preparation and semen collection and a reduction in the number of sperm inseminated per cow, the number of progeny possible per year in Table 1 can be more than doubled.

3.0 Genetic Selection of Bulls

In the beginning of AI, the selection of bulls was based on the traditional pedigree analysis for milk and butterfat production and type (44, 46, 65, 89). Many authors, including Eldridge and Salisbury (44), however, reported that the multiple correlation coefficient between the estimate of butterfat production from the pedigree and the average production of daughters was low, accounting for only 14% of the variation in production among daughter groups (44). Adding information on the dams of the daughters increased the correlation coefficient to 0.7, but this contained a large common environmental component within herds. This common environment, with daughters grouped together and milked by different milkers, was a problem that reduced the value of progeny-test stations established in Denmark (77).

Henderson developed a progeny-test program and analysis system based on unselected daughters in many herds, which became the model followed throughout the AI industry (65, 69, 89). The results that he generated with a mechanical calculator and punched cards, before electronic computers, were amazing. The program had four essential points (69):

1) Obtain accurate and complete records on parents and other close relatives of the young sire to be progeny-tested.
2) Make proper use of these records in picking the sires to be tested.
3) Test several times as many bulls each year as will be required for replacements in the bull stud.
4) Progeny-test each animal properly by obtaining an adequate number of progeny, giving equal opportunities in the performance testing to the progeny of the different sires, and/or correcting records for environmental differences.

One of the problems with the progeny-test system was the long generation interval between the tested sire and the tested son. One way of reducing this interval is by banking frozen semen from young bulls and using more semen from young, highly selected bulls, based on pedigree (219). This scheme is especially attractive economically for bulls representing breeds with small populations of females, but it has not been widely adopted in the United States.

The AI organizations also were concerned about lethals and other undesirable traits controlled by single genes that might be spread by AI. Because of the large number of progeny per sire, some of these conditions were identified (65, 89), and lines carrying these genes were avoided in selecting young bulls.

Type also was considered to be important, but in the Cornell system more emphasis was given to production. The rationale was that the income on most dairy farms was derived from the sale of milk. Also, if a cow was a high producer, it was likely to have a desirable functional type. Dr. Alan Robertson presented a seminar at Cornell on the importance of production records in selecting elite cows to be dams of young sires. A listener asked him if he didn't think legs were as important. Alan replied, "When a man runs the mile in less than 4 minutes, I do not ask if he has legs."

4.0 Testes: The Sperm-Producing Factory

The paired testes obviously are the most important organs in an AI program. Clermont and others had made considerable advances in the understanding of spermatogenesis in the rat, but much less was known about spermatogenesis in domestic animals. In the 1950s we started studies on the kinetics of spermatogenesis in bulls and rabbits and presented preliminary work at a conference in 1958 (88). As similar research in bulls had been started simultaneously by Amann and Almquist in Pennsylvania, we concentrated on rabbits (98) and boars (105). The rabbit served as a useful model on which radioisotopes could be used conveniently and inexpensively.

A formula was developed to estimate daily sperm production per testis (88). A sample of testicular tissue was sectioned, and the cross sections traversed, using a microscope with a pointer mounted in the microscope optical system. Random hits on cells of different types were recorded, and from this it was possible to estimate the fraction of the testes composed of primary spermatocytes. The total volume of the testis and the proportion that comprised the parenchymal tissue were used to calculate the total number of primary spermatocytes per testis.

Using this formula, and assuming that four sperm are produced per primary spermatocyte, Kennelly and Foote (105) predicted maximal sperm production from six boars and collected 55 to 81% of the predicted number produced. The difference between the number of sperm collected and the number produced could reflect some degeneration of cells during spermatocytogenesis, systematic absorption of sperm in the epididymis, continuous loss from the urethra, and loss during semen collection.

$$\text{Estimated daily sperm production per testis} = \frac{\left[\begin{array}{l}\text{Number of primary} \\ \text{spermatocytes} \\ \text{per testis}\end{array}\right] \times \left[\begin{array}{l}\text{Percent of} \\ \text{spermatocytes} \\ \text{completing} \\ \text{spermatogenesis}\end{array}\right] \times \left[\begin{array}{l}\text{Maximum of} \\ \text{4 sperm from} \\ \text{one primary} \\ \text{spermatocyte}\end{array}\right]}{\text{Primary spermatocyte generation time in days}}$$

The component that contributed most to differences in daily sperm production among males of any species was the size of the testes. The proportion of the testes occupied by the spermatogenic components in normally functioning testes was relatively uniform. *Consequently, simply estimating the volume of the paired testes in the live animal had considerable potential in predicting what the sperm output of a particular bull or male of any species should be.* Any major difference between predicted sperm production and sperm collected should be viewed as caused by deficient testes or a deficient semen collection procedure.

Modification of this approach was suggested by Amann (see 105), and later testicular homogenates were used to estimate total sperm production. The histological method (105, 169), however, had clinical application because it could be used with testicular biopsy causing little testicular disruption.

Information on the dynamic nature of spermatogenesis has been summarized for the AI industry (120). Research techniques for studying spermatogenic function have been brought together (169). The details of spermatogenesis, with special emphasis on the high sensitivity of the spermatogenic cells to toxic agents also has been published (425).

4.1 Testes: Size, Consistency, and the Relationship to Semen Quality

A scrotal tape was developed by Hahn et al. (158, 178) to measure scrotal circumference, which gave highly repeatable measurements (r = 0.98) and was correlated with testicular weight (0.79) and sperm output (0.81). The development of testis size with age, based on 5,909 measurements of bull testes, demonstrated that testis size in the mature animal could be predicted from earlier measurements, that testis size was not highly correlated with body weight, and that there were seasonal effects on scrotal circumference (158, 237, 239, 242, 255, 256). Therefore, simple scrotal circumference measurements can be useful in selecting bulls with potential for high sperm production.

A tonometer also was developed (164) to measure testis firmness (consistency) objectively. That trait was highly significantly correlated with various estimates of semen quality and fertility. The combined measurements of testis consistency in bulls 12 to 23 and 24 to 35 months of age (255) were positively correlated with testis consistency at maturity (r=0.69). Normal values for growing bulls were established (237, 239, 256). Because these two types of simple measurements were useful in predicting semen quality (158, 164, 166) they should be useful in selecting bulls for which extensive information on semen quality is not available (178). Dr. Cranch, a Cornell engineer who helped design the tonometer, soon became president of the Worcester Polytechnic Institute, demonstrating what these types of studies can do for one's career.

Correlations between testis measurements and semen quality, or fertility are not expected to approach 1.0, particularly because most characteristics of semen and fertility, as usually measured, are not highly repeatable. Age of bulls (252), seasonal changes (26, 263, 278), frequency of ejaculation, and conditions surrounding the collection and evaluation of semen can affect estimates of semen quality markedly. Additional changes take place in sperm during transport through the epididymis (30, 120, 141).

An extensive data set consisting of 4,275 scrotal circumference and 3,859 consistency (tonometer) measurements on 1,521 Holstein sires (244) was used to calculate heritability. The heritability values for the two traits, respectively, were 0.67 and 0.34. These results clearly demonstrate that an effective way to increase the population of bulls with high sperm-producing capability in AI is to select sons of high sperm-producing sires and to measure the size and consistency of the testes of young bulls. The young bulls with the least desirable testes could be culled at an early age, particularly if semen quality also was marginal. Today, evaluation of bulls for breeding soundness, developed by theriogenologists, includes a major emphasis on testicular characteristics (323). We were often asked "How did you actually measure the testes?" Our answer was "Very carefully," as getting a kick out of this type of research can be dangerous to your health.

An added advantage of taking and using testicular measurements in a selection program is that reproductive performance of daughters is positively correlated with testis quality of the sires (324). The correlation between testis characteristics and milk production of the daughters is negligible, with a negative trend (257, 282). Selection of high producing cows should not appreciably affect the gene pool associated with large testis size.

4.2 Evaluation of Bulls for Fertility

Success of an AI program also depends on the fertility of the bulls. A multitude of factors affect fertility (177). The report by Hahn (144) that heritability and repeatability of fertility were low was consistent with the literature. When large numbers of inseminations are involved, however, the adjusted percentage of females not returning to service had a high repeatability (246, 293, 295) and gave a reliable but slightly inflated index of conception rate (265). The difference between the nonreturn rate and final conception rate depended partly on how soon the nonreturns to service were calculated (265). This difference also was reduced when antibiotics were added to semen (54, 55, 56), presumably resulting from a reduction in embryonic mortality.

Results measured by nonreturn rates continued to be accurate as long as professional inseminators performed the inseminations and reported results to the organization producing the semen. Following the development of direct sales of semen (344), the nonreturn estimates were less reliable unless systems were installed specifically to retrieve the necessary information.

With the development of young sire testing programs, sires used to produce sons not only were genetically superior but also were primarily from sires that had demonstrated good fertility in AI programs. When the sons eventually were sampled to obtain proofs (records on daughters), preliminary information was obtained on pregnancy rates resulting from use of their semen. A few young bulls were culled at this stage of the program, based on semen quality and fertility. A detailed discussion of measurements of semen quality and fertility is presented later, primarily as applied to the extensively used proven bulls.

Young sexually mature sires tend to be more fertile than old sires. Young sires with well-developed testes and high semen quality and fertility also tend to be above average in these traits when they are proven at about 6 years of age (195, 295). Semen from these bulls could be collected intensively and frozen to accumulate large banks of frozen semen that would be less expensive to maintain than feeding and caring for hundreds of bulls on an inactive status for several years while awaiting proofs in a progeny-test program (219).

5.0 Nutrition of Young Bulls

Historically most nutritional studies of dairy animals had been done with females. Because the reproductive life and performance of the bulls finally selected after progeny testing for use in AI was of great economic importance, long-term energy experiments with male calves were started at Pennsylvania State University and at Cornell University (58, 64). A large number of bulls raised on three planes of nutrition were slaughtered at different ages to perform a detailed evaluation of sexual development. Results were summarized in an experiment station bulletin (78). Another set of Holstein bulls selected for possible use in AI was raised similarly and eventually tested for fertility with approximately 82,000 first inseminations (86). A low plane of nutrition delayed onset of puberty, but when these bulls were all placed on a "normal" plane of nutrition at 80 weeks of age they caught up to the others, with no difference in fertility. Unpublished data obtained after the two bulletins were published indicated that differences in levels of energy intake on otherwise balanced diets up to 80 weeks of age had no detectable affect on reproductive performance or life span. These experiments covered a period of about 15 years, and publishing portions of the voluminous data in bulletins after 15 years was not a route that I would recommend to ensure favorable review for tenure. Maintain a second line of research involving short-term experiments.

5.1 Nutrition of Adult Bulls

When the AI programs were initiated, mature bulls were selected to produce the semen. Studies with rats had raised concern at that time that vitamin E was very important for reproduction. In the first Cornell nutrition experiment with mature bulls, Salisbury (15) reported that wheat germ oil added to a normal ration had no effect on semen quality or fertility of bulls.

Vitamin A deficiency also was thought to be a potential problem, but bulls fed average quality hay plus some concentrates were not vitamin A deficient. No clinical signs of vitamin A deficiency were detected in semen quality until after 16 months on a diet deficient in carotene and vitamin A (38). I was very glad that these studies did not last longer

because one of my jobs as a graduate student, often working alone, was to obtain jugular vein blood samples from the bulls. I had no previous training as a matador.

An experiment was conducted by Branton et al. (34) comparing 100, 120, and 140% of the recommended maintenance requirements (TDN) of dairy cows. Simultaneously 12, 16, and 20% protein were compared. In another experiment (41), concentrate mixtures containing either corn gluten feed, skim milk powder, or soybean oil meal were compared. A feeding standard was prepared with 12% protein and TDN similar to the recommended standard for dry cows. There was no advantage of including a source of animal protein in the diet, as had been advocated in some reports at that time.

Other miscellaneous studies on body weight (22, 27) were conducted. When scales are not available, the heart girth is a reliable predictor of body weight ($r = 0.98$).

6.0 Sexual Behavior of Bulls

Little information concerning preparation of bulls to obtain the best quality of semen with the artificial vagina was available when AI programs were initiated. The first quantitative study of the effect of a combination of restraint and a false mount before collecting semen was reported by Collins et al. (53). Later studies, following this pattern but with more intensive preparation, were conducted at Cornell University (61, 155, 158), and especially by Almquist and associates at Pennsylvania State University. This sexual preparation greatly increased sperm concentration and both total and motile sperm per ejaculate without decreasing subsequent libido.

Considerable differences in libido were noted by bull handlers at the local AI station, but this difference was not related to circulating concentrations of testosterone in the blood (248). This suggests that the lower levels found were above the testosterone threshold associated with normal libido. McDonald et al. (247) had observed that implants of androgens or androgens plus estradiol stimulated libido and penile development in steers. Dykeman et al. (317) observed that 200 μg of estradiol-17β (E_2) was as effective as 1,000 times this dose of testosterone in stimulating many behavioral activities of steers, including mounting. Dihydrotestosterone was much less effective. It was postulated that some testosterone was aromatized and converted to estradiol (E_2), suggesting that E_2 plays an important role in modulating sexual behavior in the bovine male. We tried to obtain an inhibitor of the aromatizing enzyme, but could not obtain enough to treat bulls. Moral: Join the rat race: even a little bull does not go a long way.

Estradiol treatment of bulls used for mounts also was found to make the mounts more attractive to bulls, as judged by the time required to elicit mounting behavior and by the increase in sperm output (452). This effect was assumed to result from an olfactory stimulation as no postural changes were observed in the E_2-treated mount bulls. Many volatile chemicals and other compounds were tested for possible olfactory and gustatory stimuli, but none of these improved the libido of the bulls (446). The most powerful olfactory stimulus was urine. In the semen collection area to which these bulls had become habituated, however, they directed more attention to the mount than to urine-soaked pads presented to them.

An unusual case was an inexperienced young Holstein bull that licked the mounts but refused to accept any assistance in mounting the teaser animal (440). The bull never mounted during months of testing. This abnormal behavior was called to the author's attention. Upon noting the licking behavior, I brushed molasses on the hind quarters and rump of the mount. The young bull licked higher and higher until his head rested on the rump of the mount. Then he mounted rapidly, semen was collected, and the bull displayed normal sexual behavior thereafter. This was in June. The bull had failed to mount in repeated tests since January, so we spuriously concluded that "the bull was slower than molasses in January, but now it was springtime." Other comments of barnyard humor followed.

7.0 Semen Collection Equipment

An artificial vagina (AV) used for many years at Cornell had a casing that enclosed the complete liner, cone and collection tube (2). This arrangement was designed to prevent extensive cooling of the collection tube during very cold weather because semen was collected outside in unheated areas. The AV accomplished the purpose for which it was designed. Eventually, as heated semen collection areas were built, a less bulky AV was used in which

the cone and collection tube were surrounded by an insulated flexible cover. This housing was appreciated by the bull handlers (including the author), who formerly had been skidded around on icy pavement by strong-nosed, high-libido bulls in the wintertime.

Several studies were conducted on the effect of the length and temperature of the AV on semen quality (155, 156), the dynamics of ejaculation (150,167) and sperm loss because of retention in the AV during routine semen collection (96, 113, 114). The parts required to make a completely transparent and nonbreakable AV were found at General Motors and other unexpected places. Of course, the outer casing of the standard AV was a car part also, being made of radiator hose. High-speed motion pictures of ejaculation revealed extensive coiling of the glans penis during rapid semen emission (0.29 seconds). This emphasized the importance of maintaining the stretched liner of the AV in a straight position to avoid possibly catching the penis in a fold of the liner and causing injury. A film by Seidel and Foote (67), showing the details of ejaculation in the bull, was distributed worldwide.

Many unpublished reports prepared annually for the forerunners of Genex Cooperative, Inc., contain studies detailing methods to minimize the loss of up to 20% of the ejaculated sperm in the AV by moistening the cone with compatible buffer before semen collection. This reduced the loss to less than 10%. Some of this information was made available to the AI industry at technical conferences (296). Another achievement was the implementation of a procedure to preweigh plastic collection tubes and the volume of semen was determined in the collection tube by weight on a top-loading balance (Foote, unpublished). A correction for the slight increase in specific gravity as sperm concentration increased was incorporated into the computer connected to the top-loading balance. The weighing procedure reduced errors by as much as 10% compared with estimating by sight the volume of ejaculates in graduated collection tubes, especially when ejaculates were small or contained bubbles.

8.0 Frequency and Management of Semen Collection

The results of years of research at Cornell University and elsewhere to obtain and use most effectively the sperm obtainable from superior sires was summarized by Foote (149, 449). With as many as six semen collections per week and good sexual preparation, approximately 2 trillion sperm can be collected per year from a mature Holstein sire. This is a major increase from nearly 50 years ago, when it was reported that semen could be collected once at 6-day intervals without reducing fertility (40). This early study was followed by extensive semen collection and fertility studies on bulls ejaculated at different frequencies without extensive sexual preparation (60) or with sexual preparation (61). These studies demonstrated that bulls could be ejaculated every 4 days for at least 1 year with no effect on fertility and with 60% more motile sperm harvested per 8 days than when semen was collected every 8 days. Also, sperm output per bull was considerably higher when sexual preparation was provided.

Subsequently Hafs et al. (76) compared daily to weekly ejaculation for 32 weeks in bulls following extensive sexual preparation. The bulls ejaculated once a week yielded 11.1 billion sperm and those ejaculated once a day yielded 33.8 billion sperm per week. Insemination of 49,244 cows resulted in no difference in fertility (a nonsignificant trend actually favored daily ejaculation). The experiment originally was tentatively planned for one year, but the bulls were still going strong with no change in performance after 8 months. By that time the exhausted bull handlers were ready to quit. Other studies at Pennsylvania and Michigan demonstrated similar beneficial effects of sexual preparation and frequent ejaculation on semen quality.

9.0 Semen Composition and Sperm Metabolism

During the initial stages of development of AI, the classical characteristics of an ejaculate of semen such as volume, sperm concentration and motility were studied. These characteristics are covered in the general references cited in Section 1.0.

The first study on chemical components of bull semen was on B vitamins (16), at a time when the role of B vitamins in metabolism of other cells and presence in the testes recently had been reported. Seminal concentrations of several B vitamins were similar to testicular concentrations, and correlation with sperm concentration suggested that these components were derived from the testicular-epididymal contribution to semen.

Osmotic pressure of bull semen was of particular interest (35) because of the importance of preparing semen diluters (later called "extenders") that were isotonic to bull sperm. The osmotic pressure of bull semen was found to be similar to that of blood and the 2.9% (v/v) concentration of sodium citrate dihydrate in the egg yolk-sodium citrate diluter was formulated on that basis.

Osmotic pressure of the diluent used to measure sperm concentration also is important. Occasionally a slight flocculent turbidity was noted in suspensions of sperm prepared to estimate sperm concentration by optical density (11). This is caused in normal semen (free from pathogenic material) by diluting the semen with anisotonic solutions (160). Most of the turbidity, associated with precipitation of the "total solids" in seminal plasma (170), is caused by protein (160). Total solids are lower in semen obtained by electroejaculation, consistent with the large volumes of fluid often resulting from this stimulation.

Catalase content of semen from several species was examined (91) because of the finding that survival of sperm was reduced by exposure to oxygen and that glycolytic pathways were used preferentially by sperm (20, 21, 28). Catalase did prolong survival of sperm stored at 5 °C. In addition, use of glucose, as affected by different semen diluters (20,21) and the suppression of oxidative mechanisms by sulfanilamide also were investigated (23, 28). These experiments led to the inclusion of sulfanilamide in liquid semen diluters.

As freezing of bull sperm became more widespread, it was suggested that the influx and efflux of sodium and potassium ions were critical to survival of cryopreserved sperm. The addition of a Tris-buffered egg yolk at room temperature caused a major efflux of sodium ions (127), but later studies with frozen sperm failed to show that this had any effect on sperm surviving freezing.

Semen collection techniques markedly affected the general characteristics of ejaculated semen (155). Studies were continued by Seidel and Foote (156, 173) to evaluate the effect of semen collection procedures on seminal components that were particularly prominent in different parts of the male reproductive system. These markers were glycerylphosphorylcholine from the epididymis, fructose from the vesicular glands and chloride ion, associated with diffusate from other areas. This permitted estimating the relative contributions to the ejaculate of different parts of the reproductive system.

A series of biochemical studies by Bredderman and Foote (187, 188, 192) established the importance of Na, K, and Ca ions, along with energy substrate (fructose) in maintaining sperm motility and volume. Ca^{2+} did not enter the sperm cell until the energy source was depleted. Fructose, CN^-, BSA, or EDTA all were beneficial in prolonging sperm motility and in stabilizing cell volume. The percentage of motile sperm was maintained at a high level for at least 24 hours at 35 °C.

The laboratory became very interested in the possible effects of steroid hormones in the reproductive fluids of the male and female after Wester et al. (205, 210) demonstrated conversion of 17β-estradiol (E_2) to estrone by bull sperm and effects of E_2, progesterone and testosterone on sperm O_2 utilization and motility. Testosterone and ouabain were antagonistic but had different binding sites. Little was known about receptors more than 25 years ago when we proposed an investigation of the binding of hormones in reproductive fluids to sperm and their effects on sperm motility and morphology. An NIH panel agreed that the results were interesting and statistically valid but felt that the proposal was risky. Funding was delayed, and without other funds this effort was abandoned. Of course, the importance of the effects of several steroid hormones with receptors on sperm have recently been well-established.

From the beginning of AI, work at Missouri and Wisconsin, in particular, demonstrated that the lipids in egg yolk protected sperm against "cold shock." Clegg and Foote (215) performed a detailed analysis of the phospholipid composition of sperm components and seminal plasma. Later a bull semen extender, low in lipids, was developed (225) to examine the lipid changes in sperm associated with cryopreservation. Graham and Foote (350) reported that phoshatidylserine plus cholesterol could protect bull sperm during freezing and thawing equal to egg yolk. But the process of adding egg yolk was simpler and produced good results so the AI industry was not interested in pursuing this further.

The emphasis on lipids shifted to the changes in sperm lipids associated with capacitation and the acrosome reaction, which is discussed in the next section.

9.1 Capacitation, the Acrosome Reaction, and Hamster Oocyte Penetration

It was known that the sperm must undergo the acrosome reaction (AR) to penetrate the zona pellucida. Investigations were undertaken with bull sperm (1) to learn more about the induction of the AR, (2) develop procedures to control the rate of change to produce AR-sperm, (3) to compare the rates of change of sperm from individual bulls and relate this rate to their fertility, (4) to develop in vitro tests of fertility to compare with the AR and (5) to treat fractions of batches of commercially produced semen so that prescribed portions of sperm in each semen collection would theoretically be available to fertilize the oocytes in cows ovulating at variable times relative to insemination.

Liposomes made with phosphatidylcholine and varying fatty acid acyl chain lengths were tested. The 12-carbon chain (PC12) induced the AR rapidly without depressing sperm motility (347). Graham tested various liposome preparations with sperm from other species, and each species responded differently (347), to the AR and hamster oocyte penetration test (356). Fresh semen (351) and frozen semen (352) were treated with varying concentrations of PC12, and the percentage of motile sperm and acrosome-reacted sperm were compared to the number of sperm penetrating zona-free hamster eggs. Combinations of these tests and fertility were highly correlated. When a combination of percentages of motile and AR-sperm were compared with the nonreturn rates of bulls used extensively in AI, $R^2 \geq 0.97$ (354). This encouraging result prompted studies by Davis (362) on optimizing the hamster oocyte penetration assay to test the fertility of sperm treated with PC12 and make available to the AI industry multiple criteria of predicting fertility of bulls before use for breeding. Results of heterospermic in vitro hamster oocyte penetration assays with competing fluorochrome-labeled sperm were correlated with the fertility of the same bulls used for AI ($r \geq 0.86$).

The potential value of hamster oocyte penetration for testing the fertilizing ability of sperm from several species was promising. However, maintenance of a hamster colony to provide oocytes whenever they were needed was expensive. Tobback et al. (406) developed a procedure for successful cryopreservation that permitted a large bank of oocytes to be collected and banked. This was done before Lu and Gordon published work indicating that oocytes from slaughtered cattle could be used (see Gordon in the Appendix).

Major lipid classes of the plasma membrane and outer acrosomal membrane were characterized by Parks et al. (366). Then unilamellar liposomes made from various phospholipids and cholesterol were used to alter the composition of the sperm membranes (373). When treated with lysophosphatidylcholine, more AR-sperm were produced in the group of sperm with reduced cholesterol. Similarly treated sperm also penetrated more hamster ova (374), again suggesting that cholesterol removal plays a role in capacitation of bull sperm. This finding is consistent with studies (389) of oviductal fluid, in which high-density lipoprotein, a potential acceptor of cholesterol, is elevated during estrus and could result in cholesterol efflux from sperm in vivo.

Another method of rapidly capacitating sperm is exposure to calcium inophore A23187. Used in our laboratory primarily for in vitro fertilization (434), this procedure was simple, consisting briefly of exposing portions of sperm in each semen collection differently so as to produce a mixture of sperm programmed to be available to fertilize oocytes at different intervals following insemination. This concept is supported by the fact that fertility of male rabbits has been demonstrated to be related to timing of the insemination and the proportion of AR-sperm (338, 346, 386).

Further attempts were made to characterize biochemical changes in bull sperm when incubated with bovine oviductal epithelial cell cultures. This procedure was assumed to resemble physiological conditions. No change in cAMP or ATP associated with capacitation was detected (421).

10.0 Evaluation of Sperm in Fresh Semen

In a previous section on management of bulls at the time of semen collection mention was made of evaluating the semen quality and fertility of bulls (60, 61, 76, 166). Also, overviews of many studies on semen evaluation worldwide are included in review articles (149, 169, 294, 306, 369).

Before the development of AI, studies on semen often were focused on infections with leukocytes, and other seminal contaminants such as blood, urine,

and debris. Sperm morphology also was given high priority. Other experiments included research on bacteriology and asepsis of the semen collection procedure, asepsis during preparation of media for sperm preservation, and effects of bacteria on other tests of semen quality (9, 14), particularly high-temperature rapid tests of livability and metabolism (12, 14, 17). Morphology of sperm in successive ejaculates was found to be highly repeatable, provided that care was taken to prepare semen smears so as to minimize the introduction of artifacts (9, 10, 29). With the development of differential interference contrast microscopy and electron microscopy many details of sperm structure and changes with treatment could be evaluated (330, 423, 464, 484).

Sperm concentration is the characteristic in semen that is most variable. Therefore, errors in measurement can contribute substantially to errors in estimating the total sperm available in a semen sample. The hemacytometric procedure for estimating sperm concentration, adapted from counting red blood cells, was tedious and gave variable results. Following work in Minnesota, Salisbury et al. (11) found that estimates of sperm concentration by optical density, hemacytometer, and opacity standards gave similar results. The optical density method was adopted by most commercial AI organizations. Our laboratory calibrated many spectrophotometers for this group, using both the hemacytometer and Coulter Counter to obtain sperm counts. Repeated requests for help were partly answered by detailed presentations at technical conferences for AI personnel, including a demonstration of latex particles each organization could use for quality control (139, 202, 273).

The first intensive multiple comparison at Cornell of the value of most tests of semen quality that had been proposed by the early 1950s was published by Bratton et al. (66), correlating 12 tests of semen quality with fertility of semen diluted 1:100 and 1:300. Most correlations were statistically significant. The largest correlation was 0.39, between fertility and the number of motile sperm inseminated.

A Coulter Counter, Model A, which our lab could not afford to buy, was loaned to us in the 1950s. This became an interesting sophisticated "toy" to measure volume of blood cells and sperm of different species (Foote, unpublished). During these explorations considerable shifts in sperm cell volume were noted. Simultaneously it was observed that packed cell volume (74) changed correspondingly (Foote, unpublished). This led to electronic counter determinations and the less precisely measured packed cell volume to study changes in sperm volume associated with aging and death and as a measure of the integrity of the sperm cell membrane (143, 146). This procedure was demonstrated to researchers in other laboratories and years later it was modified and became the hypo-osmotic swelling test frequently used for testing human sperm viability. It's origin was with bull sperm. Cell volume also offered an approach to examining sperm prepared for cryopreservation (193) and for metabolic studies previously discussed (187, 188, 209). Parks et al. (337) developed a procedure for counting sperm in fluids with potentially interfering particles, thus extending the counter's range of usefulness.

In AI circles rate of sperm movement had been assessed only subjectively for many years (10, 66), but many cell biologists used electronic equipment to measure objectively the swimming speeds of sperm. Our first published experience by Wall et al. (289) was a comparison of a motility index, obtained by a swimup procedure using a flow cell and spectrophotometer, with subjective estimates of sperm motility. These were highly correlated ($r = 0.87$).

Sperm, at least in natural mating, must swim through cervical mucus, so migration through cervical mucus of sperm treated in various ways was compared (301), but the correlation with fertility of the donor bulls was low (303). One of the sources of variability in the assay was the individual samples of cervical mucus. A synthetic substitute for cervical mucus developed by Lorton et al. (302) was easily prepared, uniform, stabile for months at 4°C, and free from viruses and other contaminants. When migration of sperm through the gel was compared with other tests and correlated with fertility (358), the percentage of motile sperm and swim-up count were more highly correlated with fertility than was the gel penetration.

To study sperm changes in an environment, possibly simulating conditions to which sperm are exposed following deposition in cows, sperm were cultured with bovine oviductal epithelial cells (BOEC). Changes in sperm motility and the acrosome reaction associated with sperm attachment were monitored by conventional microscopy and scanning electron microscopy (410, 464). Sperm

attached immediately, and some were capacitated with acrosomal changes evident within 3 hours. Subsequently, computer-assisted-sperm-analysis (CASA) became available and motion characteristics of unattached sperm were included, along with SEM studies. Frozen sperm underwent the AR and motility declined more rapidly than in fresh sperm when incubated with bovine oviductal epithelial cells (BOEC) or oviductal explants. This provides support for the belief that frozen-thawed sperm do not survive as long as fresh sperm in the female tract.

The development of CASA made it possible to obtain statistically valid, rapid analysis of many sperm motion characteristics, including hyperactivity (462, 471, 474, 476, 478, 485, 488). When several of these characteristics were combined in a multiple regression equation, predicted fertility was highly related to fertility ($r^2 \geq .85$) of bulls used extensively in AI (488). The CASA of bull sperm is simple and can provide new meaningful results. The evaluation can include sperm suspended in whole milk or other media with particulate matter. This is made possible by the addition of ultraviolet light capabilities suitable for detecting DNA in sperm stained with Hoechst 33342 (476, 489).

Tests of semen quality in our laboratory associated with the AR in bull sperm were described in the previous section. Also, some of these tests were applied successfully to boar, ram, stallion, and rabbit sperm (see those sections). For exciting excursions into research worldwide the reader may wish to consult sources listed in the appendix as well as references in the papers abstracted here. One of our exciting times with CASA (also known as an exasperating time) was when a computer crashed before the data were backed up and a major experiment was lost.

11.0 Evaluation of Sperm in Extended Semen

This section deals with semen that has been preserved for use in AI. Bratton (45) coined the word "extender" to replace the word "diluter" "in view of the improved livability of spermatozoa in present day media." Of course, semen samples usually are diluted with simple compatible media to examine sperm initially (471), but the sperm used in AI has generally been preserved in egg yolk or milk (see reference to Salisbury in Appendix).

Most of the tests with fresh semen are also applicable to extended semen. A few differences are important, however. First, sperm in extended semen have been extended to some prescribed number, so concentration of sperm per milliliter and tests based on concentrated sperm no longer indicate semen quality. However, it is important to know the exact number of sperm packaged per breeding unit for insemination. Second, it is important to measure quality of sperm at different stages of the processing procedure, that is, soon after collection, after extension and cooling, and after freezing and thawing (222). Finally, AI organizations should retain a few breeding units (straws) from each batch of frozen semen for a period of time for quality control.

The percentage of motile sperm, AR, and oxygen uptake can be measured in some of the media used for freezing bull sperm, such as egg yolk-citrate (222), but for many tests, the extending medium used for storing or freezing sperm must be removed, usually by centrifugation. An example is a study of the AR in fresh semen (351) versus in frozen semen (352).

Also, particles in semen and the extending medium can result in errors when using CASA. Some of these can be overcome by judicious use of the various instrument settings when performing the assays (474). When there are massive amounts of interfering material, one option is to wash the sperm by centrifugation and resuspension, but this takes time and the separation procedure may alter the sperm. To overcome this problem, our laboratory assisted in the development of instrumentation using a strobed ultraviolet light source. Haploid sperm were stained with Hoechst 33342 DNA dye to distinguish sperm from non-DNA material or diploid cells (476, 478). Because of the short exposure of the sperm to the ultraviolet light with the strobe light source, there was no change in motility of the exposed sperm stained with useful concentrations of the dye. Therefore, this procedure can be applied rapidly to living sperm without introducing possible artifacts that can occur when using a separative procedure.

12.0 Extenders for Liquid Semen

Throughout AI development great importance was rightfully placed on the preparation and use of media that would best preserve fertility of the sperm.

The semen processing laboratory has complete control over this aspect. A detailed discussion of the published literature and some unpublished results were presented by Foote (272, 326).

The early work at Cornell by Salisbury and coworkers focused on yolk-citrate (YC) extender and modifications, compared with the yolk-phosphate extender developed by Philips at Wisconsin (3, 7, 10). Egg yolk made up 50% (v/v) of the YC and yolk-phosphate extenders. Concentration of the sodium citrate in YC, pH, cooling to 5°C, and warming rates of YC were evaluated in terms of the percentage of motile sperm. Fertility obtained with the use of the YC and yolk-phosphate was similar, but sperm assessment in the field by microscopic examination was vastly improved with the optical clarity of the citrate-buffered egg yolk. This extender was widely used in AI.

The addition of sulfanilamide improved fertility of YC by about 5% (31). Bacterial control and livability of spermatozoa were improved by adding a variety of antibacterial agents (36, 37), but *Pseudomonas pyocyaneus* was resistant to these. Then I obtained experimental polymyxins not yet on the market. A combination of antibiotics and sulfanilamide provided excellent bacterial control (49). The fertility of bull sperm used in AI was greatly increased (50). Almquist, and others elsewhere also reported that antibiotics had beneficial effects.

These antibiotics, without sulfanilamide (omitted from frozen semen because of toxicity during freezing), formed the basis for worldwide use to control infectious agents in bull semen. There was a dramatic decrease in delayed returns to cows requiring reinsemination (54, 55, 56), and the Diagnostic Laboratory at the New York State College of Veterinary Medicine reported (personal communication from Dr. McEntee) that vibrios is due to *Campylobacter fetus venerealis* had disappeared from herds using only AI. *This improvement in reproductive efficiency was worth billions of dollars to the AI organizations and the cattle industry they served.* It was a gift, as we did not wish to benefit, although, in view of the minimal research support through the years, millions of dollars foregone in royalties could have been invested wisely in accelerated research. These antibiotic combinations made it feasible to using liquid semen at ambient temperature without bacterial growth (52).

Simultaneously, other research was designed to improve the YC extender by the addition of catalase (42), which protected sperm against oxygen damage, but failed to improve fertility (51). The YC-sulfanilamide extender resulted in about a 5% increase in fertility (nonreturn rate) over a similar extender without sulfanilamide, and fertility results were satisfactory when 5 to 10 million motile sperm were inseminated (39).

In preparing egg yolk for YC extender the largest volume of the egg, the egg white, was discarded. The question raised was, Why? Was it toxic? Whole egg, without separating the egg white, was simple to prepare, so it was tested with a different sulfa drug to adjust the pH. Fertility was equivalent to that obtained with YC-sulfanilamide (47, 48, 57). Yet this good extender was not widely used, perhaps because the AI staff was pleased to have the egg whites for angel food cakes and other baked "goodies."

Workers at Cornell (83, 84, 85) and elsewhere reported that sperm survival was equal or better when the formerly used 50% egg yolk was reduced to 20% egg yolk (v/v). The concentration of egg yolk could be reduced to 5% for effective sperm preservation at 5°C and to 1% (v/v) for sperm preservation at 25°C (272). Food colors also were tested successfully (94) to color semen from different breeds of bulls to reduce possible errors in semen identification.

Interest in ambient temperature preservation of sperm led to our testing more balanced-buffer solutions, including self-generating CO_2 systems (83). These extenders included glycerol as potential all-purpose extenders for both fresh and frozen sperm. These investigations resulted in two extenders by Foote, CU-16 and CUE. These extenders preserved the proportion of motile sperm remarkably well for more than one week (83). Fertility trials with large numbers of inseminations resulted in 60-90-day nonreturn rates of 76.0% for the CU-16 extender and 76.6% for the CUE extender (84). Routine use of CUE by the New York Artificial Breeders Cooperative, Inc., for inseminating hundreds of thousands of cows resulted in a 60-90-day nonreturn rate of about 75%. The cooperative noted that this was worth hundreds of thousands of dollars to members annually. This fertility rate is believed to be the highest sustained in AI anywhere. A high level of fertility was maintained until frozen semen

was adopted. These reports came to the attention of New Zealand researchers, who used liquid semen for their AI program. The Cornell extenders were modified and became the Caprogen widely used in New Zealand (272).

Many modifications were tested in an attempt to improve these extenders for use at 5°C and 25°C by changing buffer composition and adding catalase and the inhibitors promazine and chlorpromazine (83, 84, 85, 87, 90, 137). Despite some improvement in preserving the proportion of motile sperm during storage, no beneficial effect on fertility was found in trials in which tens of thousands of cows were inseminated. This lack of a beneficial effect on fertility may have resulted from the use of excessive numbers of sperm for insemination.

These CU-16 and CUE extenders preserved sperm motility much better than did heated skim milk. However, additional studies were conducted with milk in an effort to improve upon the detoxification of milk with cysteine instead of by heating (198). Also, we investigated the interaction of a variety of components of milk with osmolality of the medium (136) and the use of various buffers (208). An important finding was that the harmful effect of anisotonic media was greatly reduced when the macromolecular content was increased by adding skim milk components (198).

Further studies with the CU-16 and CUE extenders demonstrated in fertility trials with 64,930 cows (90) that insemination with 5×10^6 motile sperm resulted in only about 1% reduction in fertility ($P > 0.05$) from the 76% obtained with 10×10^6 motile sperm per milliliter of extended semen. Control of pH was important, especially because CO_2 could be lost in the partially filled test tubes of extended liquid semen. Citric acid was added successfully in storage studies, comparing CUE with the Illinois Variable Temperature Extender. In two large field trials (109), fertility of the semen was 0.7% higher (not significant) when processed in CUE at a pH of 6.50 compared with a pH of 6.80. Thus the pH of an extender can be considerably below the neutral point. *Further studies with CUE revealed that sperm in sealed ampules with an inert gas or with catalase remained motile for 30 days at 5 °C (126).*

An interesting biological experiment was conducted with a group of researchers in the plant sciences (92) who used coconut milk fractions for culturing plant cells. They were the first to demonstrate full development of carrots through controlled culture (cloning) of individual somatic cells, not unlike the 1997 publication by Wilmut and associates in the production of the lamb Dolly. A new egg yolk-tris (EYT) extender developed at Cornell (93) also was tested in these experiments.

The EYT medium was a promising semen extender, particularly because it contained glycerol (93) and was highly compatible with sperm (97) at 5°C and at -79°C (the temperature of solid carbon dioxide used before liquid N_2 was available). Somewhat surprisingly, the EYT with glycerol was superior to EYT without glycerol. In field trials, with 135,579 inseminations, fertility of liquid semen in EYT plus glycerol was as high as in CUE (181). The fertility obtained with 4×10^6 motile sperm in EYT per insemination was 72.7% versus 73.7% with 8×10^6 motile sperm. The excellent results with EYT encouraged us to test the extender with other species (310) and to explore the effectiveness in semen of other species of new antibiotics on bacteria resistant to penicillin and streptomycin.

Our wondering if bull sperm in the cow tract were affected by the steroid hormones progesterone and estrogen opened another area that was briefly investigated. A "shot in the dark" experiment (81) revealed no detectable effect on fertility, but sperm were kept at 5°C. Later studies (205, 210), and especially current research in other laboratories on the influence of steroid hormones on sperm capacitation, suggest that detailed in vitro incubation studies might lead to devising a practical method of preparing sperm for AI controlled to fertilize oocytes at different times.

Finally, it is often overlooked that water is the chief ingredient (106) in any medium used to suspend sperm or other living organisms. Purity of water and other ingredients in synthetic media used to evaluate sperm in fresh semen (471) is vital.

12.1 Extenders for Frozen Semen

The extenders used for frozen semen were derived from those used for unfrozen semen (270, 272, 308). The major change in composition of egg yolk and milk extenders was the addition of glycerol (discovered by Polge and associates) to protect sperm during freezing and thawing. Many other additives have been tested to enhance the protection

afforded by these extenders against thermal and osmotic shock. These additives have been combined with a variety of freezing procedures.

Our first field studies with frozen sperm (62) demonstrated that freezing sperm to -79°C and packaging it carefully in insulated containers with Dry Ice® (solid carbon dioxide) can result in high fertility, but similarly frozen sperm cannot be warmed quickly and distributed as fresh semen to be used the next day without a substantial loss in fertility (62). A challenging sidelight of these Dry Ice experiments was the daily muscle-building experience of restocking the huge storage chest (67) with blocks of Dry Ice and grinding these blocks up for packaging sperm going to the field for breeding with frozen semen.

Semen was frozen in the 1950s in a homemade alcohol bath chilled with Dry Ice. We added the extended sperm, packaged in ampules, directly into the bath at various subzero temperatures. Transferring the ampules from +5°C to the bath at -20 to -40°C gave superior sperm survival and was a rapid, simple procedure (82).

Because various commercial organizations were interested in using EYT with glycerol, many experiments were done to determine the optimal concentrations of Tris, tri(hydroxymethyl)-aminomethane, and glycerol, the effect of changes in pH, addition of catalase, removal of oxygen from the ampules, and varying equilibration times on sperm survival following freezing and thawing. Small improvements were made (110, 112, 121), but the largest variable was bulls. Also, the optimal procedure at one stage of freezing often was altered by changes at other stages.

Other studies were designed to replace part of the Tris molecule with various combinations of Na^+, K^+ and Ca^{2+} (124), as well as to add different concentrations of macromolecules (130), but the original EYT with glucose and glycerol was usually the best extender. During cooling much Na^+ and K^+ were fluxed out of the sperm, but intracellular K^+ was maintained at three times the extracellular concentration (127). The critical temperature during freezing was found to depend on the ionic concentration of the extender (125).

We found that glycerol in EYT was not harmful to sperm (181). However, reports in the literature suggested that glycerol was harmful to sperm after considerable exposure. We developed a technique (153) to freeze the sperm in 10 seconds or less after exposure to glycerol. This procedure resulted in a higher proportion of motile cells before and after freezing than longer exposure. The question remains whether glycerol entered the sperm at 5°C in ≤ 10 seconds or whether glycerol protection was extracellular. In a similar extender with glycerol omitted, all sperm were killed by freezing, demonstrating the importance of glycerol.

Another question concerned the possible effect of light on inducing photo-oxidation in sperm. Because of the potential damage of the shorter wavelengths of visible light, frozen semen should be processed under reduced intensities of visible light (129, 260).

When sperm are carefully stored in liquid nitrogen at -196°C, (obviously in the dark), there was no loss of fertility over a period of at least 18 months (201, 262), although small biochemical changes can take place (Wall and Foote, unpublished). Other reports confirmed that semen kept constantly at -196°C maintained fertility for many years.

Control of bacteria in semen was of continued concern to the AI industry and resulted in an evaluation of many combinations and concentrations of antibiotics in EYT, whole milk, and skim milk extenders (245, 251, 335, 340, 357, 361, 363). These were primarily "insurance" studies because it was not clear if antibiotics were required since the large AI organizations basically had specific pathogen-free bulls. Antibiotics were useful as a precautionary measure should there be contamination with bacterial or mycoplasma during semen collection or processing; however, their addition did not enhance fertility (361, 363).

In several experiments (340, 357, 361, 365), the addition of low concentrations of the detergent triethanolamine lauryl sulfate improved sperm motility following cryopreservation. It is postulated that the detergent rendered sperm membranes more permeable to various molecules and reduced osmotic damage. Fertility of sperm frozen in EYT with the detergent tended to be improved (340). Scores of other experiments were completed as a part of thesis research (Parrish, Wall and others), but only appeared in annual reports.

Since the late 1930s, it was known that lipids were important in sperm preservation, initially to protect against cold shock (45). A lipid-deficient

extender was developed to examine loss of sperm lipids during cryopreservation. Although this extender (225) permitted us to measure changes in sperm (not possible to detect when adding massive amounts of egg lipids), we could not be sure that the same lipid changes occurred in extenders with 20% egg yolk. Both phosphatidylserine and cholesterol were important in protecting sperm during freezing in defined media (350).

These changes in phospholipids also have been linked with capacitation and the acrosome reaction. There were reports that the enzymes β-amylase and β-glucuronidase could capacitate sperm and increase fertility. We (249) were unable to confirm the fertility results reported elsewhere.

The development of the embryo transfer industry provided solid evidence that damage to embryos was less when they were seeded to form ice crystals before extensive supercooling occurred. Seeding also was reported to improve cryopreservation of human sperm. What could seeding do for bull sperm? Seeding mechanically or with silver iodide reduced supercooling, but it did not significantly alter sperm motility (436). The complex factorial experiments did demonstrate that freezing and thawing rates significantly affected the percentage of surviving sperm, and the optimal procedures differed between whole milk and EYT extenders.

Multiple laboratory experiments (436) and field trials (442) were conducted with whole milk and EYT containing antioxidants (hypotaurine, taurine) and nonpermeable sugars (sucrose and trehalose). These antioxidants have been reported to benefit cultured embryos and the sugars reported to protect many cells during freezing, but they did not enhance the fertility of sperm when tested in whole milk (441).

In summary, the most notable achievement in semen preservation was the work by Davis et al. (93) with EYT. This extender with glycerol is one of the most widely used media in the world for cryopreservation of bull sperm. This contrasts with our total failure (Foote, unpublished) to preserve bull sperm in 1947 with ethylene glycol in yolk-citrate at -10°C and to successfully freeze-dry sperm in the 1950's. The helium system we used was a good one made available by Dr. David Lee (1997 Nobel laureate in physics), but sperm could not survive the shock of freeze-drying in 6 seconds.

13.0 Extending and Processing Liquid Semen

Extending media used to preserve liquid semen was discussed in a previous section. Two additional critical components in the laboratory phase of processing liquid semen are the cooling system employed to reduce the semen temperature to about 5°C and the extension rate to produce a prescribed number of total or motile sperm for insemination. The glass equipment available then (and plastic equipment later) for inseminating the cow were designed to hold 1 ml conveniently. Sperm numbers for each insemination usually translated as the number of sperm/ml with 1 ml of extended semen used for insemination.

The storage temperature for liquid semen of approximately 5°C was established partly because of the convenience of the household refrigerator, which was run at about 5°C, and the ease of maintaining a temperature slightly above 0°C with an ice pack during distribution. We used a balloon filled with water, frozen, and then brought to the thawing point before wrapping the glass tubes and later plastic tubes of semen around the balloon. "Jiffy" bags inside heavy cardboard boxes were used for shipping until Styrofoam was introduced. Shippers then were fabricated in Ithaca. The semen was collected at 5 A.M., extended, cooled, packaged, and taken to the post office in a station wagon, which then was used to pick up office workers before 8 A.M. The local radio disk jockey (at that time accomplished organist, Jack Deal) used to look out the broadcast studio window and say, "There go the girls from the artificial breeders." They were not pleased by this notoriety.

A third consideration in processing semen was the possibility that removing nonmotile sperm would improve the quality of the semen distributed (324) in either a liquid or frozen semen program. Unpublished studies at Cornell and elsewhere indicated that potential gain by various separative procedures would not offset the work involved and the possible damage to live sperm caused by the separation procedures.

Cooling semen from body temperature has been extensively studied. The optimal rate likely is different for different extenders, but generally a slow cooling rate from body temperature to 5°C is recommended. Willett and Salisbury (10) recommended a

rate of 5°/20 minutes when cooling from 37°C to 5°C. If storage was to be at 1°C, cooling 5°C per 2 hours was recommended. This slow cooling and low temperature were not practical for commercial use.

Although these studies (10) and other published research indicated that sperm should be protected during cooling, the local AI organization, for convenience in cooling small samples, cooled the neat semen slowly and then added the larger volume of yolk-citrate-sulfanilamide extender. Foote and Bratton (45) presented further evidence from laboratory studies that adding some extender before cooling (called pre-X), and the remainder at 5°C, was clearly superior to adding all of the extender after cooling (called post-X). A split-ejaculate study with 8,518 services clearly revealed the advantage of the pre-X procedure (59.3% nonreturns) over the post-X procedure (52.8% nonreturns). Thereafter, semen was never cooled without first adding protective extender. Note the low nonreturn rates here were in the 1940s.

13.1 Sperm Numbers and Fertility of Liquid Semen

The early studies on reducing sperm numbers per insemination by adding substantial volumes of extender proceeded cautiously (10), in response to comments that "diluting sperm was like watering the milk," obviously not to be done. However, the extension of semen with extender moved onward from 1:4 (10) to 1:16 and 1:32 (13), and 1:50 (18), without reducing fertility. The fertility was maintained at a 1:100 extension of semen (25), and the number of sperm per milliliter averaged about 13×10^6. In some studies, higher dilutions of semen resulted in higher fertility, although the differences were not statistically significant. This positive result may be the fortunate effect of diluting the *Vibrio fetus abortus* (Campylobacter) organisms to a subinfective dose before reducing sperm numbers to a subfertile dose. In fact, later studies by Dr. McEntee in the New York State College of Veterinary Medicine reported that high dilutions could reduce the concentration of organisms to a noninfective dose. This organism was ubiquitous when AI started.

In a small study (39), a 400-fold and 800-fold extension of semen resulted in 37% and 44% nonreturn rates, compared with 61% with the 100-fold control extension. A more extensive fertility trial also produced a downward trend in fertility when comparing 100-fold with 400-fold extensions of semen. In another large trial (66) with yolk-citrate-sulfanilamide extender, semen extended 1:100 versus 1:300 resulted in 52.8 and 56.6% 60- to 90-day nonreturns rates, respectively.

When 5, 10 and 15 million motile sperm/ml of yolk-citrate-sulfanilamide extender were compared, the 60- to 90-day nonreturns rates were 66.7, 70.9, and 70.5%, respectively (59). In this report Bratton and Foote (59) diagrammed a predicted relationship between numbers of motile sperm inseminated and fertility. This concept was later expanded in the book by Salisbury et al. (see Appendix).

With the development of improved extenders for semen, such as CUE and EYT (83, 90, 93, 181), little difference was found when semen was extended to 5×10^6 or 10×10^6 motile sperm/ml, but conservatively the 10×10^6 dose of motile sperm was used routinely (61). Antibiotics were now included in the extender in these studies. Several of these studies were summarized recently (453). With approximately 10,000 inseminations per treatment, 4×10^6 and 8×10^6 motile sperm/ml of CUE resulted in 72.7 and 73.4% 60- to 90-day nonreturn rates, respectively. Corresponding values with EYT were 72.7 and 74.1%. The actual number of sperm inseminated was probably about 60 to 70% of the sperm number packaged per milliliter because of sperm loss in the catheters (453).

These were great days of inseminating cattle, with two excellent extenders containing antibiotics and a happy clientele of dairy farmers. With the high extension rate of semen, sperm numbers obtainable from genetically superior sires were sufficient to inseminate 200,000 cows per sire annually, if there was the demand.

Clearly, the better semen extenders with antibiotics (56) played a crucial role in raising the nonreturn rate nearly 20% over a period of a few years. Minimizing the exposure to light and packaging semen in full tubes to avoid agitation during shipment (128) may have been beneficial. Paufler and Foote (129) found that light and cysteamine had striking effects on DNA, with swelling of sperm heads, presumably caused by disruption of disulfide bonds. This finding, published in the *Journal of Dairy Science*, was generally unnoticed by the biological community and later rediscovered by

others. The AI organizations changed from the short wavelength blue lights in their labs to light with longer wavelengths and increased their quality control measures (138, 430), all of which helped to sustain AI at a high level of efficiency.

14.0 Extending and Processing Frozen Semen

Anyone who had processed liquid semen for use in AI had mastered the initial steps in processing semen for freezing. The chief differences between the two types of semen processing are that the extenders described for liquid semen in a previous section are now prepared with a cryoprotectant (glycerol usually), extended semen is packaged as individual insemination doses in straws, and the straws are frozen and stored in liquid N_2 at -196°C. One well-remembered, nontechnical difference is that the normal semen processing day, which started at 5 A.M. with liquid semen, shifted to 8 A.M. with frozen semen. Fewer "all-nighters" were required to prepare ingredients for research thereafter.

In the early studies with frozen semen we used an egg yolk-citrate extender with a final concentration of 7% glycerol (v/v). After the semen was cooled to 5°C and made up with the YC to one-half the planned final volume, an equal volume of YC with 14% glycerol (v/v) was added in small increments over a period of at least 1 hour (62, 67). Equilibration with glycerol was thought to be important, and several hours of equilibration at 5°C was allowed before freezing was initiated. During this time semen was dispensed into small plastic test tubes and tightly corked or into glass ampules and tip-sealed. Later, the more effective pull-seal glass ampules were introduced with curved shoulders and bottoms that didn't break off during freezing or thawing. This innovation reduced the number of exploding small glass "bombs" with nitrogen leaks in the old tip-seal ampules, and square bottoms that gently cracked off, wasting millions of sperm from each ampule.

Freezing was done slowly in an ethanol bath by adding chips of dry ice to lower the temperature to -79°C over a period of about 40 minutes. Excellent control of temperature around each tube or ampule was maintained by circulating the liquid with two motorized stirrers in the bath. Later the cooling and freezing time was shortened by a direct transfer of the extended semen from +5°C to temperatures from -20°C to -40°C (82). Eventually ethanol was replaced with methanol or isopropyl alcohol, as some questions were raised about the alcohol consumption at the frozen semen laboratory. Dr. Bratton, the author, and the small staff were "teetotalers."

In an early trial (67), semen was frozen with enough sperm initially so that there would be about 10×10^6 motile sperm following freezing and thawing, equivalent to the number of sperm used in the liquid semen control. Excellent fertility was achieved with the control fresh semen (71.0%), and frozen semen used within 1 week (73.2%) or at 17 weeks (69.8%), based on 60- to 90-day nonreturns.

During the subsequent years, many unpublished experiments (Parrish and Foote and others), appearing only in the annual research reports to granting agencies (approximately 5,000 pages over 40 years), dealt with glycerol concentrations in milk and egg yolk extenders, equilibration times, methods of adding glycerol, freezing in ampules, pellets and straws, freezing equipment and procedures, and thawing rates. Simultaneously, other laboratories were reporting on some of these subjects.

One of our more interesting studies (Yasseen and Foote, unpublished, 1965) dealt with equilibration of extended semen at 5°C. Cryobiologists indicated that equilibration of sperm with glycerol was important. While our studies clearly confirmed the benefit of a period of cold equilibration in producing sperm with maximal survival following freezing and thawing, glycerol equilibration time appeared to be unimportant. Later, Berndtson and Foote (194) used the pellet freezing technique and homemade equipment to freeze sperm in 10 seconds or less after adding glycerol. Various extenders were tested, including a nonpermeating egg yolk-lactose extender that Nagase and Graham had developed. These experiments (194) conclusively demonstrated that long-term exposure to glycerol was harmful and that exposure to glycerol for 10 seconds or less gave maximal protection. This finding revised the concept that "glycerol equilibration" was required at 5°C. A more correct understanding was that equilibration of sperm at 5°C was useful, but freezing could commence as soon as glycerol was added to the cooled extended sperm and they were packaged for freezing.

With the development of the egg yolk-tris extender, which contains glycerol initially, a study

was conducted comparing the EYT with the standard egg yolk-citrate. Glycerol was added to EYC slowly or in one step either immediately after cooling to 5°C or just before filling ampules and freezing them (180). Four field trials provided data demonstrating that there was no difference in fertility among different sperm treatments, again supporting the view that the time at which glycerol was added was not critical. For routine processing of semen the AI organization added extender with glycerol at 5°C by the drop method after part of the extended semen was cooled to 5°C.

Various commercial freezers were used in the different experiments. For easy access to straws frozen differently, however, a large open-top liquid nitrogen tank with controlled release of N_2 vapor was developed (Simkin, Foote, and Kaproth, unpublished) and used by Coulter and Foote (222). Large numbers of straws were frozen simply and cheaply, using wide-mouth liquid N_2 tanks already available and surplus N_2 vapor escaping from the N_2 storage tank. Incorporating suitable temperature sensors reduced batch variability (222), and sperm in millions of straws with whole milk-glycerol extender have been frozen successfully this way by the local AI station.

Because of the relatively low cost of processing frozen semen, the possibility of banking thousands of straws from highly selected young bulls and then slaughtering them was studied extensively (219). Banking the semen from young bulls, when the quality of sperm and the libido of bulls is high, offers a potential fertility advantage. Injuries to older bulls would be avoided. Housing, handling, feeding, and manure disposal costs would be reduced. The system has economic advantages for bulls representing breeds with a low population of cows. One objection to this system was that farmers liked to come and see the bulls. This objection is no longer valid, as restrictions relative to disease prohibit visitors from visiting the bulls.

14.1 Sperm Numbers and Fertility of Frozen Semen

Most of the unfrozen (liquid) semen studies were based on a planned number of motile sperm to inseminate. The percentage of motile sperm was estimated subjectively, however, and with frozen semen the percentage of motile sperm was reduced by freezing and thawing. Eventually, the frozen semen program was based on providing a standard total number of sperm per breeding unit. With good quality control procedures (234, 369), this system has consistently provided frozen semen with a high fertility potential. The policy of Eastern AI Cooperative, Inc. (now Genex Cooperative, Inc.) has been to provide at least 20×10^6 total sperm per straw. This number should provide extra sperm. Our studies had indicated that 12×10^6 motile sperm before freezing gave satisfactory results (180), and research by Pace and others at American Breeders Service demonstrated little decrease in fertility until total sperm numbers per breeding unit were reduced below about 10×10^6, yielding about 4×10^6 motile sperm after freezing. In a recent study with 88,486 first services, Foote and Kaproth (486) reported no change in fertility when total sperm numbers per breeding unit decreased from 40×10^6 to 20×10^6, and little change when using 10×10^6 total sperm. The 60- to 90-day percentages of nonreturns for 20×10^6, 16×10^6, 13×10^6, and 10×10^6 total sperm were 71.5, 73.1, 72.2, and 70.5, respectively, not significantly different.

It is clear that the possible beneficial effects of extender modifications and semen processing are not likely to be detected when field trials are conducted with 20×10^6 sperm (441). Each dose contains more than enough fertile sperm. Few procedural improvements have been reported for many years. It is possible, however, that improved methods of partially dehydrating sperm before freezing (Liu and Foote, unpublished) will lead to methods for preserving more viable sperm. The fertility tests would need to be done with fewer than 10×10^6 total sperm to detect an effect. Currently, judicious reduction of sperm numbers per breeding with the semen from genetically superior and highly fertile sires can continue to increase the genetic merit of the next generation of dairy cattle.

14.2 The Bull and Fertility

With the elimination of low fertility bulls for many generations in AI, few low-fertility bulls exist in the lines of cattle selected to produce new sires. Nevertheless, differences in fertility among bulls exist, although heritability of fertility is low (144, 177). In the field trials conducted to test various extenders and other factors affecting fertility (76,

166, 180, 323, 486), variance among bulls was always a significant component. It was very large before the time of antibiotic-treated semen, when certain bulls were infected with organisms causing venereal diseases (54, 55, 56, 63, 265). In addition to the bacterial contaminants controlled by antibiotics, mycoplasma (307) also is controlled by the antibiotic treatment of semen. Furthermore, bulls in the large AI organizations are specific-pathogen-free of many viruses capable of transmission through semen (388).

In the early days of AI, Blom in Denmark, Hancock in the UK, and others identified several types of abnormal sperm associated with reduced fertility. We had observed an unusual case of a proven bull in the 1950s in which about 50% of the sperm displayed severely bent or broken tails. Fertility was normal. When the bull was slaughtered it was discovered that the pathology occurred only in one epididymis. Either these sperm were also fertile or the number of sperm inseminated was more than double the total sperm needed for normal fertility.

We rarely observe serious morphological defects in sperm in the bulls housed in Ithaca, New York for use in AI. However, a stump tail defect (330, 427) was found in two related Ayrshire bulls that was so severe the bulls were not used for AI. Semen from one bull was used in the original owner's herd on a limited basis, and fertility was zero.

A Brown Swiss bull in Wisconsin had complex chromosomal translocations (404, 433) that resulted in much lower fertility than was normal for the breed. This condition was found to be present in a limited number of progeny, demonstrating that the condition could be transmitted.

14.3 Insemination Procedures and Fertility

There was minor concern, but little evidence that the egg yolk in semen extenders could cause an immune response and affect fertility. Coulter et al. (250) obtained evidence that there was a higher incidence of repeat breeder cows with detectable titers to egg yolk in their blood serum compared with fertile cows. However fertility of rabbits was not impaired by immunization with egg yolk.

The time of insemination relative to ovulation has been extensively studied in many places. The Trimberger AM and PM rule (145) refers to the recommendation that cows first detected in estrus in the morning should be inseminated the same day in the afternoon. Cows first detected in estrus in the afternoon should be inseminated the following morning. This practical recommendation was borne out in a large-scale study by Foote (265, 281) involving 44,707 cows and heifers. When the dairy farmers do their own inseminations, some perform multiple inseminations with problem cows but with limited success (344). No precise best time for insemination has been established. The range in insemination times without markedly affecting conception rates appears to result from the range in time from the onset of estrus until estrus is first detected, the variation in ovulation time of individual cows, and the length of time sperm remain fertile in the female reproductive system (226).

Many recent studies, particularly at Virginia Polytechnic Institute and State University, confirm that even with the use of various aids for detecting estrus and regular observation of cows to detect onset of estrus, insemination time can differ without marked differences in conception rates. More information on detection of estrus is included in the section on cow management.

A variety of studies have been conducted around the world on depth of insemination beyond the cervix and insemination in both uterine horns or only on the side ipsilateral with ovulation. Studies by Bratton et al. (unpublished, Cornell University) on depth of insemination and volume of semen inseminated did not produce definitive results, perhaps because of the superfluous number of sperm used.

In another experiment, Cooper et al. (328) found that clitoral massage caused uterine contraction (327, 328), so it was hypothesized that the massage and the associated uterine contraction might enhance sperm transport. However, clitoral massage had no beneficial effect on fertility. Other manipulations of the reproductive organs had no effect on uterine contractions. Oxytocin did initiate uterine contractions in cows in estrus, but the dose required was higher than that needed to stimulate milk ejection. Obviously, injection of this dose at random

insemination times relative to milking was not practical to test as a way to affect fertility.

14.4 Microinjection of Sperm and Fertilization

Microinjection of sperm probably has little practical application in commercial AI of dairy cattle, but it is a useful experimental tool (426, 432), and could be used with sexed sperm and IVF. Frozen sperm from the bulls with either a severe sperm morphologic defect (330, 427) or a chromosomal anomaly (404, 433) were available to compare with sperm from two bulls differing in fertility and used routinely in AI (432). Sperm from the two bulls used routinely ranked the same in the in vitro fertilization (IVF) procedure as in their normal AI procedure, and the sperm with abnormalities resulted in 1 and 2% fertilization. With microinjection the fertilization rates of the four bulls were much more similar, demonstrating the importance of normal sperm morphology in oocyte penetration in vivo as well as in the usual IVF procedure.

15.0 Sperm Manipulation and Sex Ratios

Theories and procedures to alter the sex ratio have been proposed for many years. Many theories have been tested and when tested properly (314) usually have yielded negative results (191, 261, 265, 311, 313, 336). Gledhill and associates at Lawrence Livermore Laboratory, however, using sensitive cell-sorting equipment, were able to detect differences in the DNA content of sperm containing an X versus a Y chromosome (see reference 313). Procedures were modified at the USDA, and Johnson and associates demonstrated that the sex ratio could be altered by using sperm separated by this procedure. Because the separation of sperm was slow, considering the millions of sperm needed for AI of cattle, its application has been limited to IVF or microinjection techniques.

We have researched gravimetric procedures (311) further and found that the water component of the sperm had a substantial effect on its density. Therefore, if any such procedure holds promise for separating sperm on density gradients (Foote et al., unpublished), sperm should be shrunken to minimal size (minimal water content) and velocity of movement through harmless density gradients used to isolate sperm with different densities.

16.0 Nutrition of Holstein Heifers

One component of a longtime regional project on reproductive failures in dairy cattle dealt with nutrition (68). The nutrition of bull calves was discussed in Section 5.0. Similar studies were carried out by Sorensen et al. (75) on heifers. Four calves per group in five groups were replicated across planned intakes of 60,100 and 140% of the Morrison total digestible nutrient (TDN) standards for dairy heifers (4 ×5 × 3). The five groups were calves slaughtered at 16, 32, 48, 64 and 80 weeks of age. An additional four calves were slaughtered at birth. Detailed measurements of body growth on the live animals and weights and histologic appearance of the reproductive tract and endocrine organs at slaughter were recorded. Extensive summaries and photographs are included in the bulletin (75). High energy intake markedly accelerated age at sexual maturity. Other studies (not published) showed that the high level of feeding was associated with lower production in adult cattle. The effect of high energy diets on suppression of development of the lobular-alveolar system was shown in elegant studies elsewhere by Swanson at Tennessee, in studies with identical twins.

16.1 Growth and Sexual Behavior of Holstein Freemartins

In 1779 Hunter first observed that most female calves born co-twin with a male calf were reproductively poorly developed, were partial intersexes, and were sterile. Lillie and associates added much to our knowledge of the development of "freemartins" in the 1920s.

We were interested in detection of estrus in cows, the homosexual behavior of cattle, and the libido of bulls at the time our studies with these calves were initiated. We were aware that neural and sexual development were influenced before birth or very early in development (see research in rats by Gorski and others). Therefore, we postulated that freemartins would be a useful model to study sexual behavior without surgical intervention because nature had provided a genetic female that had been exposed to androgens before birth.

These investigations resulted in a series of studies of sex-chromosome ratios (254) and their relationship to sexual development, the mammary-gland response of freemartins administered steroid hormones (259), the release rate of steroid hormones from implants over an extended period of time (271), the behavioral characteristics of hormonally-treated freemartins (266), and finally, growth and sexual development of freemartins hormonally treated for 79 weeks (283). These studies were so different from most of our research that they were challenging, stimulating, highly instructive, and fun. The stories are told in the original articles. Of most interest to the application of improving detection of estrus in cattle submitted for AI was the similarity of responses found in our work with steroid hormone-treated steers and ovariectomized adult cattle (299, 317, 319). This finding led to the conclusion that neural tissue of both sexes associated with sexual behavior responded similarly to the same hormones in many respects.

17.0 Reproductive Management of Cows and Detection of Estrus

Most of the herd studies relate to cows housed and managed in the northeastern area of the United States (24, 32, 33, 232, 304, 342, 344), although experience and experimental data also were obtained with a herd of 8,000 cows in the hot and humid area of south central Florida (322, 331, 332, 333, 344) and in hot, dry Israel (372, 392, 414, 435). Great emphasis is placed on reproductive management in Israel to keep milk production high (392).

In the early studies efforts were made to describe the age of the cow populations being inseminated (24, 33) and to examine the effects of season and light (32, 33). Season had little effect on fertility in the Northeast (33), but the summer season had a disastrous effect on the conception rate of lactating cows in south Florida (333). The hot, dry summers of Israel had less effect in depressing conception rates, but differences in imposed management schemes affected production and reproduction (392, 414, 435).

As the average milk production in herds increased, more problems in detecting estrus and obtaining pregnancies were reported from the field. A study of DHI records (232) revealed a marked decline in conception rate as milk production increased. Butler at Cornell and others at many experiment stations subsequently have been researching the effects of negative energy balances on reproduction (473).

Obviously, managing a large herd of high producing cows is very complex. The original procedure of professional inseminators providing expert service with high-quality unfrozen semen has been replaced with probably less fertile frozen semen, often sold directly to the dairy unit. The busy dairy operator may or may not be highly skillful in using the semen on the farm to inseminate the cows expertly near the optimal time to achieve highest fertility (344).

These studies demonstrated that the most important aspect of management was to have an informed and dedicated manager. Techniques that facilitated reproductive management, especially in large herds, were to group the cows according to their reproductive status (322) and to employ a variety of techniques to assure that true estrus was detected properly (226, 236, 247, 279, 284, 286, 332, 342, 473).

A simple procedure that was effective in improving accuracy of detection of estrus and increasing conception rate and reducing the calving interval (Foote, unpublished and 226) was to train individuals to observe for estrus at specified times and to check off on simple forms the specific symptoms observed each time. This way true estrus (standing for mounting) was not confused with other signals. After this system was incorporated, calving interval in one large herd was reduced a full month the next year, with a 1000 pound increase in average milk production.

Animals with increased libido, such as ovariectomized females given estrogen (299) or cull females treated similarly, are easy to prepare. Treatment of males or females with estradiol stimulates mounting behavior (226, 247, 266, 299). An active mounter is valuable to detect animals that are in "standing" estrus. These animals also stimulate sexual activity in a herd. Bulls with a deviated penis or steers with hormone implants, along with Chin Balls or KaMar Heatmount detectors, can be very useful (226, 247). A good stripe with colored wax crayons on the rump of animals (tail head chalking) is smeared by mounters when cows in estrus stand to be mounted. This is an inexpensive, useful way to detect estrus. Pen-O-Blocks to prevent mating are not practical (247).

Activity meters on cows, as pedometers (Foote, unpublished) can be helpful. Many electronic devices measuring animal activity have been reported by other researchers. They help detect cows in estrus, but they may not be cost-effective.

An electrical conductivity probe was developed at Cornell (285), and various designs were tested to monitor changes in the electrical conductivity of vaginal mucus associated with estrus. Its use (312) and effectiveness in detecting cyclicity, estrus, and pregnancy in cows and heifers have been described in detail (243, 279, 332). Cows in commercial herds with no visually detected estrus but inseminated on the basis of electronic probe measurements indicating estrus (280) became pregnant at as high a rate (52%) as cows in the same herds inseminated on the basis of visually detected estrus (49%).

The electronic probe measurements were compared with milk progesterone changes (243, 279). The hormone patterns with the estrous cycle were more consistent than were the electronic probe values, but the electrical conductivity changes measured with the probe provided a basis for making a decision immediately. Cowside milk progesterone tests were not available then. In addition to measuring cyclical changes (236, 243, 279, 284), the milk progesterone test is useful to detect pregnancy, to monitor cows with reproductive problems and to evaluate herd reproductive status (284, 286, 291, 298, 332). Also, we found it effective in detecting suspected embryonic death (277), including early abortions induced by the misapplication of $PGF_2\alpha$ (331).

Data accumulated from these studies, much unpublished work, and the results published by others were used to model reproduction (287). Various strategies to improve detection of estrus and conception rates were cost-effective.

Another method for inseminating cows at the proper time is to control estrus rather than watch for it. Synchronization of estrus to inseminate at a specified time has been extensively investigated for the past five decades by Casida and co-workers at Wisconsin, Hansel at Cornell, and many others (274). Several procedures in synchronizing estrus have been developed (343) but have not been widely applied to dairy cattle AI. They have been more useful in breeding beef cattle and synchronizing donors and recipients for transfer of fresh embryos.

The advances in neuroendocrinology and hormone biochemistry led to the availability of gonadotrophic releasing hormone (GnRH) and analogs. Early work by Britt and others indicated that GnRH could induce early ovulation in the postpartum cow. Heuwieser et al. (457) obtained mixed results in a large study when GnRH was administered 28 to 35 days postpartum, and these authors concluded that routine use of GnRH was not economical (413). Another study (334), however, under more tropical conditions, indicated that cows that had been treated with a GnRH analog could be inseminated sooner postpartum. Selective treatment with GnRH of cows with slow uterine involution about 2 weeks after calving increased conception rates and reduced the postpartum interval to conception (Foote and Riek, unpublished, Cornell University).

18.0 Superovulation in Prepubertal Cattle

The initial studies on induced ovulation and superovulation in our laboratories were performed with rabbits (95, 116). The results with prepubertal rabbits were exceedingly promising. Calves have antral follicles (197), and Casida and co-workers had reported that induced ovulation in young cattle was possible. So superovulation of calves appeared to offer the possibility of reducing the generation interval and of progeny testing young dams to produce sires for use in AI (147). As a high energy intake was found to decrease the age of Holstein heifers at puberty (75), a group of calves were fed a high energy and protein diet from 8 to 16 weeks of age (152) in hopes of accelerating reproductive growth and function. Body weight was accelerated nearly 50%, but the average number of ovulations (37) at Week 17 was not different from that of the controls (34). Thus, follicular response was little affected by treatment.

Subsequent studies by Larson et al. (1990) demonstrated that there was marked proliferation of uterine glands and development of the reproductive organs between birth and 2 months of age with some precocious development of the uterus under the influence of progesterone following superovulation. However, the reproductive system still was relatively primitive at 2 months of age.

Many schedules, using several combinations and concentrations of PMSG-hCG or FSH-LH, were tested with calves, starting at about 9 weeks of age (147, 171, 182, 189). By 5 months of age 1,500 to 2,000 IU of PMSG resulted in an average of 77 ovulations per calf (189). There was considerable variation in response, and the fertilization and recovery rates often were low. Liquid semen produced higher fertilization rates than frozen semen, but we developed a procedure of nonsurgical intrauterine insemination that resulted in probably *the first report that fertilization with frozen sperm was possible in the calf* (171).

The infantile reproductive tract of calves (190) appeared to be hostile to embryos (184). More embryos reached the uterus 5 days after LH than 3 days after LH, but the percentage of abnormal and degenerating embryos increased (189). Progesterone concentrations rose to nearly 100 ng/ml of plasma by 8 to 10 days after the LH surge, and this could have impaired early development of the embryo. Cyclic patterns were not initiated in these calves. Progesterone was always detectable after superovulation of calves, in contrast to a return to cyclic behavior by postpubertal cattle (214).

There was an abundance of luteal tissue at 5, 10, 15 and 20 days after superovulation. In vitro production of progesterone was high at all intervals tested, but with more variation among corpora lutea at 20 days, some of which were undergoing regression, in studies reported by Spilman et al. (196).

Ova and embryos collected surgically from prepubertal cattle were cultured. Modified Ham's F10, TCM199, follicular fluid, bovine or rabbit sera, or ligated rabbit oviducts provided the culture environment (157, 184). No embryos cleaved past the 16-cell stage (157). Of course, no one at that time could culture embryos from adult cows beyond the 16-cell stage. It is wonderful how much has been learned about embryo culture in the past 30 years.

Several embryos obtained from a superovulated calf that were transferred to a synchronized recipient (Seidel et al., 184) resulted in birth of a 45 kg calf 275 days later. *This is believed to be the first calf produced from an embryo obtained from a 5-month-old mother.* But the efficiency was low, grant support diminished, and the project was terminated. We still feel the goals were worthy.

18.1 Superovulation in Postpubertal Cattle

At the same time as the calf work was in progress, we gained initial experience with superovulating adult cattle (214). Various preliminary, unpublished experiments in the late 1960s were conducted by Onuma, Hahn, and Larson, particularly to develop a nonsurgical procedure of embryo transfer, based on the work of Sugie in Japan (see 182).

Before long, interest in importing exotic beef breeds was increasing in North America. Embryo transfer (ET) was thought to be the best method to import the desired genetics. Interest in ET heightened. A few commercial centers were established in Canada and the United States to obtain and transfer embryos surgically. Through the research of many groups, especially Sugie in Japan and Rowson and co-workers at Cambridge, a nonsurgical technique of uterine flushes and transfer of embryos was developed. Research flowed from many laboratories (too many to list here, but see reviews 349, 355). Now superovulation, estrous cycle regulation, and embryo transfer could be added to AI as additional animal reproductive biotechnologies (274, 320, 321, 349, 355, 455). Commercial organizations contributed much to technical knowledge, and we were especially fortunate to gain knowledge and experience shared by Hasler and McCauley (318, 353) at Em Tran. Their extensive data sets offered much information on all aspects of ET.

Research at Cornell focused on the endocrine and ovulatory response of heifers and cows treated with different hormones or hormone agonists (214, 268, 349, 382, 383). Whereas, following superovulation some progesterone secretion continued and cycling was not initiated in prepubertal animals, postpubertal animals continued with estrous cycles following superovulation (214). Presumably prepubertal animals did not release luteolytic amounts of prostaglandin $F_2\alpha$ ($PGF_2\alpha$).

The availability of $PGF_2\alpha$ (268) began a new era of experimental destruction of the corpus luteum. Detailed endocrine changes (estradiol, progesterone, and LH) were measured following various treatments to induce superovulation. An interesting practical finding was the high correlation (0.76 to 0.99) between peak plasma progesterone concentration and the number of corpora lutea (214, 268).

This was before ultrasound equipment was widely available, and accurate determination by palpation of many corpora lutea following numerous ovulations, was not possible. As a potential indication of the number of ovulations and embryos to expect we measured milk progesterone. The correlation between milk progesterone and the number of embryos recovered was 0.86 (316). These collective studies indicated that plasma progesterone and its transfer to the mammary gland were related to the amount of luteal tissue. There was no feedback that set a limit on the pharmacological concentrations of progesterone circulating after extensive corpora lutea formation.

As gonadotropic releasing hormone became available, we tested an analog, Buserelin, in an attempt to regulate the time of LH release, increase the ovulatory response and improve fertilization rates (382, 383). Variability among cows in the time from onset of estrus to the spontaneous release of LH prevented any standardized injection schedule of Buserelin from improving ovulation rates or fertilization results. A rapid ELISA test for progesterone was confirmed as useful in identifying cows unsuitable for superovulation treatment (368), but this would not have solved the cow variability problem. Finally, a study was conducted to eliminate the requirement for multiple FSH injections by implanting capsules (459), but this did not reach a point where the capsules gave a repeatable release rate of FSH.

Data kept very carefully by Hasler at Em Tran (318, 353) have provided valuable insight into all factors affecting the success of an embryo transfer program. One question raised was whether superovulated oocytes were normal (367). The potential alteration in the follicular waves of the ovary possible as a result of elevated FSH concentrations following the usual FSH injection schedule could produce abnormalities. Yet the fertilization rate of oocytes in superovulated cows was similar to the rate in unstimulated cows. Some individual cows in commercial ET centers produced large numbers of oocytes with nearly 100% fertilization. This was similar to our results with extensive embryo transfer studies in rabbits (185). Therefore, the inescapable conclusion was that the superovulatory regimens tested had little effect on the developmental potential of ovulated and fertilized oocytes.

19.0 Embryo Evaluation and Direct Transfer

The early commercial ET procedures consisted essentially of embryo collection followed by evaluation and transfer as soon as possible to synchronized recipients. Rapid evaluation of embryo quality was important. Probably the most useful tests of the quality of embryos and their value in predicting the probability of a pregnancy have come from large embryo transfer facilities such as Em Tran (318, 353), Colorado State University, Alta Genetics, and TransOva Genetics, where thousands of transfers were made. Much valuable information has appeared each year in the January issue of the journal *Theriogenology*.

We conducted one large-scale study with Buserelin to increase progesterone secretion in recipient cows soon after transfer of fresh or frozen-thawed embryos (428). Results were negative. Buserelin did not elevate plasma progesterone. Also, there was no relationship of embryo quality to pregnancy rates.

Export of pathogen-free embryos is another important consideration. As other groups have reported, viruses can be transmitted to cows through semen and are present on the surface of embryos produced by infected cows (370, 387, 388). The importance of following the International Embryo Transfer Society recommendations for washing embryos is emphasized.

The ability to transfer embryos and achieve a high pregnancy rate has opened the gate for a host of biotechnologies to follow. These include embryos produced in vitro (IVF), cultured in vitro, sexed, genetically engineered, and cloned (320, 321, 455), all followed by embryo transfer to suitable recipients. This has stimulated many basic studies around the world on embryo development, particularly facilitated by improved methods of culture.

19.1 Embryo Culture and Transfer

Studies by Wright and co-workers, First and co-workers, Rexroad, and others indicated that the bovine zygotes encountered a block to further development in culture at the 8- to 16-cell stage and that coculture with somatic cells supplemented with serum could partially overcome this block. The

emphasis in our laboratory was to compare in vitro culture systems with in vivo culture systems and to develop the simplest culture system compatible with the development of zygotes into blastocysts, suitable for transfer (391, 395, 405). We had developed a successful protocol for recovering bovine zygotes (407). When zygotes were cocultured with monolayers of freshly prepared bovine oviductal epithelial cells (BOEC), the simple CZB medium without glucose during the first 48 hours of culture was equal or superior to coculture in more complex media with serum (391), but serum was needed for sustained culture of the somatic cells. In the next phase of research (395) it was demonstrated that bovine 1- to 2-cell embryos cocultured in CZB medium with the BOEC for 5 days developed as well as comparable embryos incubated for 5 days in ligated rabbit oviducts. Pregnancy rates were the same (57% for coculture and 58% with live rabbit oviduct incubation). Finally, our studies (405) demonstrated that development of zygotes to blastocysts in simple CZB-BOEC coculture was equivalent to in vivo development of embryos for 6 days in the donor cows.

It was remarkable that collectively research advanced technology so rapidly. The simple in vitro system mimicked in vivo conditions sufficiently that it could be used as a model to study in vitro requirements for embryo development and for transfer of cultured embryos.

One major drawback of these studies, based on procuring zygotes produced in vivo, was the cost. While this research was in progress in the late 1980s, Lu and Gordon in Ireland were conducting extensive culture studies with oocytes recovered from ovaries of slaughtered cattle (see Gordon's book listed in the Appendix). These oocytes were matured and fertilized in vitro. We used this technical advance, with some modification, in our embryo culture program thereafter.

19.2 Embryos Produced and Cultured in Vitro (IVF)

The simple CZB medium with the BOEC coculture provided excellent support for the development of bovine zygotes produced naturally (in vivo), and also provided a simple semidefined system for developing embryos that were produced in vitro (390). We did include serum in the oocyte maturation medium, as was commonly done with IVF studies in other laboratories.

Experiments on embryo culture continued with the addition of several growth factors to different media, including the replacement of serum with bovine serum albumin (445). This elimination of serum was part of our continued effort to develop a completely defined system of embryo culture that would allow rigorous study of the influence of different growth factors on early development. Several growth factors increased the proportion of embryos that developed into morulae and blastocysts (445).

We and other researchers had reported that the approximately 20% O_2 in a 5% CO_2 :95% air mixture was too high for optimal development of embryos from laboratory animals and that the O_2 requirement for the somatic cells of the coculture might be higher than required by the embryos. In a study by B. K. Yang et al. (458), a 20% O_2 gaseous environment was inferior to 5 or 10% O_2, and in another experiment with BOEC or buffalo rat liver cell cocultures, embryo development in 10% O_2 was superior to 5% O_2.

The most exciting results were associated with a simple KSOM medium developed by Biggers at Harvard for mouse embryos. This medium was equivalent or superior to the complex Menezo B_2 medium for culturing bovine embryos evaluated both by stage of development and cell number. It was further demonstrated (469) that KSOM was equal or superior to Menezo B_2 with or without the buffalo rat liver cell coculture, thus providing a defined system for studying bovine embryo requirements for normal development.

It was postulated that the superior results reported earlier for cultures with serum in the media were due in part to antioxidant components in serum, as well as to the inclusion of numerous growth promoters. A series of experiments by Liu et al. (466, 467 and 475) demonstrated that 5% O_2 was optimal in a modified simple KSOM culture system, and that addition of taurine nullified the detrimental effect of 20% O_2. Insulin also was beneficial. A concentration of 95 mM NaCl in KSOM was optimal. Increasing NaCl or total osmolality of the medium was harmful. Some commercial media, such as TCM 199, contain more NaCl than is optimal.

An experiment with a variety of amino acids and selective addition of α-amanitin (470, 480)

showed that amino acids and protein synthesis were vital throughout embryo development. An exogenous source of amino acids appeared to be especially critical at the blastocyst stage. Many individual amino acids and combinations were tested. No single amino acid was found to be especially beneficial, but the Eagle group of so-called nonessential amino acids was especially beneficial when added to KSOM, which contains glutamine but no amino acids.

20.0 Oocyte Maturation and IVF

The IVF system adopted initially for producing embryos in vitro (355) to study requirements for good embryo development was modified from various reports in the literature, although the different procedures reported from various laboratories had not been compared at one time and place. To choose which method worked best for us we compared various media, sera, and hormones in a large study (434). No major difference was found among methods in maturing oocytes. Treating sperm with 0.1 μM of calcium ionophore (A23187) for 1 minute induced more acrosome reactions than did either 10 or 100 μg/ml of heparin. The procedure with A23187 to induce the acrosome reaction was fast and simple to use and was adopted in further IVF studies.

Suzuki et al. (456) performed a detailed scanning electron microscopic study of bovine oocytes during in vitro maturation and after IVF. It was planned to have a parallel group of oocytes and embryos produced in vivo for comparison during Dr. Suzuki's fellowship, but the control animals failed to yield sufficient oocytes or embryos for study. A second group of potential donors was prepared from cows that previously had responded well to superovulatory treatment at a large successful commercial ET center. This group also failed to produce oocytes and embryos. This double failure was most unusual, but as many have experienced, in research, "the best laid plans" don't always work out.

20.1 Oocyte Maturation, Activation, and Cloning

Other studies with oocyte maturation and activation were designed to increase understanding of the activation phenomenon initiated by the fertilizing sperm cell (424). This was critical for successful cloning (424, 451). An activation procedure was developed consisting of exposing in vitro matured oocytes for 5 minutes to 7% ethanol, followed by incubation for 20 hours with 10 μg/ml of cycloheximide. This procedure, with minor modifications, was used by Yang and associates in many papers published from the laboratory but not included in the list here.

Our studies with cloning in rabbits and cattle (364, 376, 437) involved injecting isolated blastomeres into enucleated recipient oocytes. Studies by Koyama et al. (450) indicated that little polarity occurred in cattle embryos until after the 16-cell stage and in rabbits until after the 32-cell stage. Thus, these young embryos were used to obtain blastomeres for the production of cloned offspring (376, 437). The first cloned calf born in 1992 at Cornell was a high milk producer in the Cornell herd. Intensive efforts at the same time to develop lines of stem cells (424) were not sufficiently successful to publish.

Some of these advances over the past 50 years have been fun to incorporate as background for an applied genetics course at Cornell, ranging from AI in the 1940s to cloning prospects for the twenty-first century. Also, this long history of experience seems to have provided some credibility as one is asked to present workshops or seminars reflecting on developments in this field for students, science writers, lawyers, and government agencies. More importantly, it is exciting to see former students, as well as other excellent investigators, exploring innovative approaches to unlock the biological secrets of mammalian development with enormous potential benefits for human medicine and agriculture. What a bright future lies ahead! To you young researchers, especially, I say "think, plan and go for it."

Chapter III. Rabbits

21.0 Male Reproductive System

The male rabbit is a good, inexpensive model for studying many aspects of reproductive physiology and technology (169) in domestic animals. It is the smallest animal from which one can collect semen easily (108); yet the testes are large enough that testicular tissue can be obtained repeatedly by biopsy with minimal effects (111, 161). As described subsequently, the rabbit is useful for studying blood flow to the testes, formation and transport of sperm, seminal characteristics, and fertility following artificial insemination timed accurately relative to ovulation by treatment of females with luteinizing hormone (LH). Thus lessons learned with the male rabbit usually can be extrapolated in principle to other species (169, 381).

The extensive replication possible in rabbits provides reproductive data for estimating variances and planning the size of experiments with clues as to the most sensitive end points (375, 378, 425) to detect treatment effects. This information can be useful in planning initial experiments with a species lacking the necessary prior data.

The internal anatomy of the rabbit male reproductive system was described in detail by Holtz and Foote (275). Testis size can be measured externally in the live animal (148, 487). Size is an important indication of the gross normality of the testes and expected sperm output. The accessory sex glands are complex and they can serve as a bioassay of circulating concentrations of testosterone. In castrates (258) the size of these glands is decreased and fluid from accessory sex glands obtainable by ejaculation also is decreased. However, when only the spermatogenic elements are depleted and Leydig cell function is maintained (468), accessory sex gland weight and fluid secretion also are maintained.

21.1 Testicular Function: Spermatogenesis

A fundamental understanding of the quantitative dynamics of spermatogenesis was gained by making a detailed sequential analysis of the association of spermatogenic cell types (98). Males were given a tracer dose of ^{3}H-thymidine which had just become available (88, 115). The cell nuclei incorporated the radioactive tritium into DNA. By applying a liquid nuclear emulsion directly to the histological sections on slides, the advancement of cell types could be traced because the radioactive DNA reduced the silver grains in the emulsion. To improve the procedure, we designed special stainless steel spreaders that distributed a uniform thin layer of emulsion over the tissue. Also, to prevent fogging the emulsion the nuclei were stained after preparing the autoradiograph (ARG).

A microspectrophotometer worth $40,000, described later by Paufler and Foote (129), was built for $5,000 with the help of Leitz experts and odd parts from a warehouse in New York City. By taking microspectrophotometric measurements of the DNA in Feulgen-stained nuclei of various types of cells Swierstra was able to relate the ^{3}H-thymidine incorporation to the nuclear DNA content (115).

These studies, patterned particularly after those of Clermont and Leblond in Canada and Ortavant in France, increased the understanding of spermatogenesis to the extent that it was possible to predict sperm production (169, 209) from quantitative measurements of the testes. Also, this knowledge permitted pathological conditions of the testes observed on biopsy specimens (111, 161) to be evaluated more precisely (339, 378, 425, 468, 481).

Swierstra continued these testicular studies elsewhere in boars and stallions and collaborated on a study with dogs (199). *This research, begun in the 1950s, we believe was at the forefront of investigations at that time to unravel the events taking place along the seminiferous tubules.*

Earlier Asdell and Salisbury (5, 6) had studied the rate of degeneration and regeneration of spermatogenic elements when the testes were placed in the abdomen for various lengths of time. After specific stages of spermatogenesis had been described for the rabbit (98) the stage classification procedure was used by Igboeli and Foote (163) to study effects of cryptorchidism on spermatogenesis.

Also, we used ultrasonographic equipment assembled by Dr. Stouffer to evaluate testes of bulls. These extensive studies showed that tumors,

tissue necrosis, and other pathology could be detected by ultrasound (Hahn and Foote, 1968 unpublished). It was not known whether exposing testes to ultrasound would have any deleterious effect, so a study of the effects of ultrasound on rabbit testes was undertaken (145). One testis of 25 males was exposed and one was not exposed to different doses of ultrasound. Detailed examination indicated that exposure to ultrasound did not affect ejaculated sperm or stages of spermatogenesis.

21.2 Testicular Function: Steroid Hormones and Behavior

It was established in the 1930s that testosterone could maintain spermatogenesis in sexually mature rats and rabbits. Our interest in testosterone and other steroid hormones related particularly to libido. In various experiments we had collected hundreds of ejaculates of rabbit semen and noted a considerable difference among males in libido.

Sexually immature males were castrated and given testosterone implants immediately or after a delay of 26 days. Both groups of implanted males developed libido while on the implant regimen, and circulating testosterone concentrations were higher than in intact males of a comparable age. However, libido and seminal-fluid volume were higher in the intact controls (224), indicating that products of the testes in addition to testosterone were required to produce normal sexual development and activity. Further studies (258) with androgens and estrone indicated that both hormones played a role in maintaining libido and function of the accessory sex glands, thus indicating involvement of both central and peripheral areas of control. These results were similar to findings with hormone-treated steers (317).

Cryptorchidism did not reduce libido in sexually mature rabbits (163), a finding consistent with the established ability of Leydig cells to continue to function at body temperature. Furthermore, rabbits maintain libido and accessory sex gland function when exposed to agents that interfere with spermatogenesis, but not with Leydig cell function (see section 26.0).

Other studies were designed to ascertain factors controlling blood flow rate to the testes because volume of blood could be as important as concentration of hormones in the blood. No difference was found in blood flow rate to the testes of young and older males, other than what was accounted for by changes in testis size (221). Administration of FSH and LH to 12-week-old rabbits did not affect blood flow rate to the testes.

21.3 Epididymal Storage and Transport

Sperm undergo dynamic changes as they are both stored and transported in the epididymis. Labeling the progenitor cells of sperm with ^{3}H-thymidine during DNA synthesis permitted tracking sperm cells as they left the testis to be transported through the epididymis, and ejaculated (88, 115, 123). Results obtained by autoradiography and liquid scintillation counting were in agreement that epididymal transport of sperm required 8 to 10 days, depending on the male.

By placing ligatures unilaterally at various locations on the epididymis (165), it was determined that sperm could be stored for 12 weeks in the cauda epididymides with negligible resorption, but resorption could occur in the caput region. Sperm accumulation in males unilaterally vasectomized for 12 weeks was not substantially less than the number of sperm ejaculated (6 ejaculates per week) from the intact side, although prolonged blockage of the ductus deferens resulted in considerable resorption of sperm in the epididymis, with minimal formation of antibodies to sperm detected in blood serum (Foote and Simkin, unpublished, 1975, Cornell University).

Further studies (209, 218) comparing numbers of sperm collected from rabbits with a unilaterally cannulated ductus deferens to sperm ejaculated from the intact side, and counting sperm voided in the urine, indicated that there was little resorption of sperm by the paired epididymides in sexually active males. When males were sexually rested, substantial numbers of sperm produced were unaccounted for and presumed to have been resorbed by the epididymides.

Unilateral ligation of the epididymis at different locations permitted comparisons of sperm morphology, motility and fertility during changes over time in the same space on the ligated side, versus changes with time and space on the control side (142, 151). Sperm retained in the caput region of

the epididymis increased in motility but did not become fertile, although transport through the epididymis for a similar period resulted in achievement of fertilizing ability. Sperm in the caudal region retain fertilizing ability for at least 35 days, a period much longer than anyone has achieved storing sperm in vitro at the same temperature. What are the animal's secrets?

Ligation of the ductus deferens (142, 154) for 12 weeks had little effect on changes in sperm characteristics (except sperm accumulation) or on spermatogenesis. Thus the vasectomy did not appear to suppress spermatogenesis. Occlusion of the ductus deferens for longer periods (Foote and Simkin, unpublished) however, reduced spermatogenesis with no effect on libido. Vasectomized rabbits were used for years to collect seminal fluids and for induction of ovulation in mated females without initiating pregnancy. *These results were especially relevant to the controversy at the time surrounding potential complications following vasectomy in humans.*

The fact that epididymal temperature is lower than body temperature appears to be of great importance for preserving the fertilizing life of sperm cells, as pointed out by Bedford in discussing evolution of the descent of the testis-epididymal complex. Whereas anchoring the testis-epididymal complex in the abdomen for several days (6, 163) interrupted spermatogenesis after 24 hours, sperm in the cauda epididymides retained fertilizing ability for several days. These sperm appeared to remain uncapacitated and survived much longer than capacitated sperm at the same temperature in the female reproductive tract.

22.0 Semen Collection

Our initial semen collection studies in rabbits were done with the Cambridge-model artificial vagina (AV) described by Walton. The Cambridge type of AV was effective, but it was cumbersome to assemble and use with the small Dutch-belted rabbit. A simple, smaller AV was designed (108) that was effective in stimulating males and could be cleaned rapidly. Semen usually was ejaculated directly into the semen collection tube with little loss. The new AV saved an enormous amount of time over the years in collecting thousands of samples of semen.

We collected many samples, partly because frequent semen collection is essential to obtain a reliable estimate of sperm output. Rabbits were ejaculated either daily for 43 weeks or once weekly in the control group (71). Daily ejaculation resulted in the harvest of four times as many sperm harvested from the 7X/week group as from the 1X/week group without loss of libido or fertility.

Another method we used for collecting semen was to cannulate the ductus deferens (218) to measure sperm output without potential sperm resorption in the epididymis or voiding of sperm in the urine (209). These studies indicated that it was necessary to ejaculate rabbits 4 to 6 times weekly to harvest most of the sperm produced, a finding similar to results obtained by Amann, then at Pennsylvania State University. Two ejaculates can be collected daily (Foote, unpublished), but our routine was two collections every other day (264).

23.0 Semen Composition and Evaluation

Composition. Our early interest in preservation of liquid semen from several species of farm animals and the beneficial effects of catalase on survival of bull sperm led to a study of the endogenous catalase content of semen (91). Rabbit semen contained much more catalase (275 μg/ml) than semen from other species. It was associated with the seminal plasma, whereas in the ram, two-thirds of the catalase was associated with the sperm. Sireson values in rabbits were highly correlated, indicating that catalase content of semen was partially an inherited characteristic. It was not due to bacterial contamination.

A detailed study of rabbit semen and the origin of various components was reported by Holtz and Foote (264). Fructose was found mainly in the prostate gland, citric acid in the vesicular gland and the ampulla of the ductus deferens, and glycerylphosphorylcholine was contributed by the epididymis. Fructose and citric acid were lower in second ejaculates than in first ejaculates, suggesting that much of the secretions from the glands producing these compounds was removed with the first ejaculate.

Evaluation. The need for standardizing conditions for evaluating semen quality with high repeatability and minimal artefacts has increased as more rabbits are being used for reproductive toxicology studies. Many andrology laboratories and toxicology laboratories use computer assisted sperm analysis (CASA), so CASA was tested for evaluating rabbit semen.

First we compared media used in our laboratory to other media available and found that a Tyrode's albumin-lactate-pyruvate (TALP) solution was much superior to phosphate-buffered saline (PBS; 471). Little change in the motility of sperm occurred during 2 hours of storage at 30°C in TALP.

Some of the granules in rabbit semen will be counted as static sperm unless the settings on the CASA instrument is set to screen them out, primarily on the basis of size, shape, and brightness (474). Errors of this nature would bias estimates of sperm concentration and motility. With proper settings both concentration and motion characteristics of sperm can be estimated objectively. Under these conditions repeatability of the measurements is high when adequate numbers of sperm are sampled (462). With 12 fields examined and about 20 to 25 sperm per field, repeatability of most sperm characteristics approached 1.0 in our CASA studies.

Hamilton Thorne CASA equipment now includes an optional ultraviolet light source capable of monitoring sperm stained with the DNA-specific stain Hoechst 33342. This system makes it possible to analyze rabbit sperm motion, excluding all potentially interfering granules in rabbit semen because they do not contain DNA (478). The CASA analysis has been adopted by many as an important component of sperm analysis (472).

24.0 Semen Processing and Insemination

The number of sperm inseminated is critical in any study in which evaluation of treatment effect includes measurement of fertility. Studies with inseminates of 25,000 to 2,000,000 sperm demonstrated that some fertilization occurs when as few as 25,000 sperm are inseminated (448). Although 2,800 oocytes or embryos were used in these studies, the numbers per subclass were not large enough to exclude most of the variation caused by differences among females and other sampling errors. The correlation between semen quality and fertility was $r = 0.53$. In another study (468), 400,000 sperm were required for maximal fertility. So, even with low sperm numbers, many replicates are needed to detect treatment effects.

Competitive fertilization. Competitive fertilization occurs when sperm from two males are marked to identify the source of sperm, then mixed and inseminated. This technique has been demonstrated by researchers elsewhere (Beatty, Dziuk, Saacke, and others) to be a highly efficient method for ranking fertility of males or semen treatment.

We developed a colony containing Dutch rabbits homozygous for the Dutch pattern plus an albino strain of Dutch. These strains are useful for many studies, including competitive fertilization. The coat color serves as an excellent fetal and postpartum marker. TEPA also can serve as a label for sperm, but fertility was reduced (329). Subsequently, Parrish and Foote (338, 346) used the fluorochrome markers, fluorescein isothiocyanate (FITC) and tetramethylrhodamine B isothiocyanate (TRITC), to color-mark competing sperm. The dye concentrations were effective in marking oocytes with red or green dye from the sperm, and the dye had no effect on fertilization. This method facilitated studies on cooling sperm, on frozen-thawed sperm, and on capacitation as these affected sperm transport and fertilization rates (346). Freezing sperm affected all of these criteria, particularly causing reduced sperm transport. Moreover, paired bucks that had been ranked according to the proportion of fertilized oocytes with the dye method (328) had the same ranking when used to produce color-marked young (Vallet and Foote, unpublished). Concentration of acrosomal enzymes also was evaluated in these studies, but they were not highly correlated with fertilization rates.

Capacitation. Experiments to capacitate sperm in vitro indicated that the acrosome reaction (AR) could be induced with lysophosphatidylcholine (LPC). Individual males differed in the percentage of AR-sperm initially and in the response to treatment with LPC (379). Chen et al. (380) noted that freezing of rabbit sperm increased the number of AR-sperm, but no difference in capacitation time was detected. However, differences among males

in fertility associated with time for capacitation of their sperm was detected by timed insemination studies (386). Thus fertility of different males was affected by the time of insemination relative to ovulation.

Freezing sperm. We spent many years developing our colony of colored as well as albino Dutch rabbits. Experiments with frozen semen were pursued partly to develop methods to preserve sperm from some of the most fertile males in the two strains. A series of studies was conducted to test different freezing conditions (380, 454). The most beneficial treatment was initiation of ice crystal formation (seeding) at -6°C, instead of allowing the semen extender to supercool and seed spontaneously at about -14°C, followed by rapid ice crystal formation and a rise in temperature. Seeding at -6°C resulted in a post-thaw motility of more than 60% (454). However, this technique did not improve the post-thaw quality of semen from males whose semen froze poorly using other methods (Foote, unpublished).

25.0 Sperm Antigens and Fertility

For many years we noted that sperm from some males occasionally had many clumps of agglutinated sperm that, nevertheless, resulted in fertilized eggs when used for insemination. In the early 1970s there were many conflicting reports as to whether antibodies to spermatozoa in humans were a substantial cause of infertility. Some of the difference in reports likely was due to the different techniques used to measure the antibodies. We explored this controversy and tested procedures that led to temporary infertility.

Kummerfeld et al. (240) immunized 48 female rabbits with preparations of washed epididymal, washed ejaculated and β-amylase-treated rabbit sperm. All of these antigenic preparations produced high titers in the serum. All immunizations rendered the does sterile, but fertility gradually returned. The titers obtained with the Shulman assay procedure were more highly correlated with the suppression of fertility than were titers from the Kibrick procedure. These differences may explain some of the conflicting published reports based on different assay procedures. These results were confirmed in additional experiments (241) that included embryo transfers. These tests demonstrated that the infertility caused by immunization was primarily due to a block to fertilization and not to embryo mortality. Blockage of fertilization is a valuable attribute for a contraceptive, but the procedure for immunization was not practical for humans and the block to fertility was transient.

26.0 Reproductive Toxicology in Males

When atomic energy became available for commercial use the politicians and public became highly sensitive to potential genetic damage and induction of cancer from radiation and other environmental pollutants. Effects on reproduction, which could be detected in a population sooner than genetic effects and cancer, tended to be neglected. This was unfortunate, in my opinion, as appropriation and distribution of federal research funds did not match real national needs. However, when reproductive failure of groups of men, such as those exposed to dibromochloropropane (DBCP), received national publicity, the Environmental Protection Agency (EPA) became interested in developing a small animal model for risk assessment of agents that might interfere with reproduction in the human male (315). Rats had been used, but it was not possible to collect semen from rats, and semen was the chief material used in the laboratory for evaluating reproductive potential of men. The EPA sponsored a meeting to discuss this and it was clear that the rabbit provided a nonrodent model for comparison, and the male could be easily trained to ejaculate into an artificial vagina.

We had collected thousands of semen samples this way from male rabbits, and had extensive experience evaluating spermatogenesis. Dr. Eric Clegg, from the EPA, asked us to develop the rabbit model and the agency provided some funding. We picked DBCP because while there were many epidemiologic studies of people consuming DBCP in drinking water due to its long biological life after being widely applied to crops, there were no comparable controlled data for animals. Fortunately, the series of doses we chose to test covered the range from no effects to major disruption of spermatogenesis.

We used a combination of tests based on serial collection of semen samples and detailed evaluation of the vital organs and the reproductive system at the end of treatment with DBCP (339, 341). At the highest DBCP dose sperm numbers were decreased, the percentage of abnormal sperm increased, spermatogenesis was disrupted, and FSH increased, but LH, testosterone, libido, and accessory sex gland weights were unaffected (339, 341).

The papers provided fundamental data used in multimillion-dollar lawsuits in California and were described as "classic." Fortunately, these data demonstrated that even if humans were much more sensitive than rabbits, they would have to consume thousands of liters of California groundwater every day to have even the slightest subclinical effect, with no clinical manifestations of a reproductive problem. Original papers need to be consulted for the many details of the research. The author learned much about legal procedures which are so different from teaching. Never explain anything you don't have to in court. Keep the opposition guessing, and they may entrap themselves.

Later studies were equally successful in establishing the dose-response relationship between consumption of ethylene glycol monomethyl ether (EGME) and reproduction (468, 481). Again, the highest dose caused disruption of spermatogenesis and oligospermia, but as long as some sperm were produced, fertility surprisingly was maintained. Animals on all doses were clinically normal and otherwise healthy. These studies provided much longitudinal data on the same animals that could not be obtained with rats. Furthermore, rabbit testes were at least 10 times as sensitive to EGME as were the testes of rats and mice.

One reviewer of the papers we submitted for publication appeared to be a rat-oriented toxicologist. The reviewer tried to block publication on the basis that a second animal model was not needed for reproductive toxicology. The rat was "it." In the earlier DBCP work another reviewer stated that the paper was better suited for a journal on reproduction because toxicologists were not interested in reproduction. Fortunately the editor agreed with me. What a change in the past one or two decades! Toxicology programs now contain major sections dealing with reproduction.

These papers (339, 341, 468, 481) provided the opportunity to compare the sensitivity of end points for toxicology studies and experimentation. It was possible to suggest the size of experiments needed to accomplish particular objectives with different end points considering both sensitivity and costs (see details in 378 and 425).

In the review by Foote and Berndtson (425), work done earlier on triethylenemelamine (TEM) was included for comparison. The TEM study also included cadmium chloride (159), because at that time we had just received a shipment of new cages dipped in $CdCl_2$ instead of zinc. This was before the days of all stainless steel cages. Another feature of the TEM experiment was that it was planned to continue collection of data long enough after treatment ceased so that potential recovery of spermatogenesis and sperm output could be examined. Suppression of spermatogenesis was dose-response-related. Recovery followed cessation of treatment. Testicular biopsies facilitated making the simultaneous comparisons of testis characteristics (98) with semen quality.

Rabbits like to chew on cages, and this raised the question whether metallic cadmium would be absorbed and possibly interfere with our reproductive studies. No ill effects were found in control rabbits, but treating rabbits with cadmium chloride caused extreme testicular damage with epididymal hemorrhaging (159).

The sensitivity of quantitative and qualitative analyses of testicular histology was noted on another occasion (104). We had been using amphotericin B to control some difficult fungal contaminants in bull semen, and had seen a paper indicating that this antibiotic had been tested systemically in dogs with no apparent effects. We had observed an effect on spermatogenesis in treated rabbits (104). However, the testes of the dogs had not been examined, and we were able to secure blocks of testicular tissue stored from the dog experiment. Examination of histological sections revealed a retention of some sperm after Stage 8, as we had observed in testes of rabbits treated with the antibiotic (104).

During the course of these studies it become apparent that the familiarity we had gained with the male reproductive system of the rabbit was

extremely valuable in detecting dysfunction. *These studies on normal rabbits and those exposed to toxic insults helped to establish the rabbit as a useful animal for risk assessment of reproduction in the male.*

27.0 Timing Oogenesis and the Aging Ovary

As a graduate student I learned in courses in histology and embryology about some of the known features of spermatogenesis and oogenesis. The evidence for the association between onset of spermatogenesis and sexual maturity was irrefutable, but the evidence was challenged from time to time that oocytogenesis essentially was completed neonatally and no new germ cells were formed in the adult. When I taught this subject, students would occasionally ask, "How do you know this to be true?" I had to answer that, as with many things, I was not sure, or I did not know. The world is full of mysteries. I decided that we should find the answer if possible.

The development of radioisotopes, especially ^{3}H-thymidine, and autoradiography, enabled us to provide a more definitive answer to the above question. We were using these procedures in our laboratory to explore spermatogenesis. Two crucial bits of evidence were necessary to support or reject the hypothesis that the process of oocytogenesis did not occur in the adult. Kennelly designed a Ph.D. thesis in 1961 to obtain this evidence. The first evidence was based on permanently tagging reproductive cells neonatally with ^{3}H-thymidine and determining if essentially the same population of reproductive cells was labeled in the adult. Did all the germ cells in the adult female represent those formed neonatally? The second bit of evidence was sought by attempting to tag reproductive cells postnatally with ^{3}H-thymidine and to compare labeling of female germ cell nuclei with nuclei of cells in tissues undergoing rapid DNA synthesis and cell division. This would indicate whether any new germ cells were formed postnatally.

The evidence for the rabbit, at least, was clear-cut (118). When newborn females were injected neonatally, about 90% of the oocytes in ovaries removed unilaterally contained DNA labeled with ^{3}H-thymidine. This percentage of labeled oocytes continued as the remaining ovaries were removed at various intervals for 40 weeks. No oocytes were labeled in animals injected at 20 weeks of age and no meiotic figures were observed postnatally. This research (118) represented a huge effort in tissue processing, grain counting and statistical analysis on the mechanical calculators available at that time, but it was worth it! *The evidence was overwhelming. The dynamic process of oogenesis stops at birth in the rabbit, and probably in all mammals, and only the completion of meiosis occurs in the adult.*

The timing of oogonial mitosis and the prophase I stages of meioses up to dictyotene was determined (183). Oocytes remained "in storage" with meiotic activity arrested at the dictyate stage until the oocyte reposed in a follicle selected to be the dominant follicle. Mechanisms regulating maturation of oocytes and events leading to fertilization or to the successful use of enucleated oocytes as "synchronized" recipients of transferred genetic material (cloning) are currently topics of great interest. This research (118, 183) provided strong evidence of what really happens and when it happens; today, new research is revealing how this biologic machinery functions.

The foregoing studies pointed to the stability of DNA. Current fingerprinting of pieces of DNA from prehistoric organisms supports this concept on a very long-term basis. The biological stability of the DNA was not substantiated at that time, but the stability found in the oocytes provided an opportunity to explore another intriguing question of reproduction: What are the components of the reproductive system that contribute to the well-established reduction in fertility and embryo survival as females age? To what extent is this associated with the aging oocytes, the aging ovary (endocrine component), the aging pituitary, and the aging uterus? Obviously, the DNA of the oocytes was as old as the mother. This topic is covered in section 33.0, and the work of Maurer and Foote (185, 229) also is relevant here.

28.0 Ovulation and Superovulation

Ovulation. One of the advantages of the rabbit model over the spontaneously ovulating farm animals was that the rabbit is an induced ovulator. This made it possible to conduct experiments in

which the interval from insemination to ovulation and the age of oocytes and embryos following ovulation could be predicted rather precisely.

In our early experiments we relied on breeding males or vasectomized males to induce ovulation, but some sexually mature females failed to copulate, or at least to copulate with specified males. This problem prevented us from completing a balanced experimental design. In addition, much time was spent in attempting to make the planned matings.

The solution to this problem was to develop a reliable method of inducing ovulation by the injection of luteinizing hormone (LH) combined with artificial insemination (95). As a result of injecting 2.5 mg of Armour PLH into 57 Dutch rabbits at the time of insemination, 91% of the does kindled and delivered 307 young. Obviously, this simple, rapid procedure was effective.

With the advances in endocrine chemistry, gonadotropin-releasing hormone (GnRH) and several analogs became available. A simple intramuscular injection of 2.5 μg of GnRH or 1.2 μg of Buserelin ovulated Dutch rabbits (444). Moreover, there was no antihormonal effect with repeated injection of GnRH or its analogs.

Superovulation. The value of the rabbit model for reproductive studies would be enhanced experimentally and economically if a large number of oocytes and embryos could be obtained repeatably. There was evidence from Adams at Cambridge, Chang at the Worcester Foundation, and others that the rabbit could be superovulated, but various pituitary extracts, not commercially available, were used. We had tried pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with only moderate success.

Armour sold standardized batches of follicle-stimulating hormone (FSH) and LH, which were the early NIH standards. These were used in an extensive study (252 females) comparing different superovulatory regimens (116). The FSH-LH treatment was superior to PMSG-hCG. Six injections over 3 days of 0.5 mg of FSH followed by LH into Dutch rabbits resulted in an average of 54 ovulations per doe. This treatment administered to thousands of does continued to give an average of about 45 to 50 ovulations per doe with high fertilization rates. *It was one of the most useful animal procedures fine-tuned in our laboratory.*

Another objective of the early studies was to establish a dose-response rate of LH to induce multiple ovulations following FSH injections and to study the stage of embryo development associated with timed recovery of embryos following the LH injections (122). The dose of LH was important to ensure that it was enough for an optimal ovulatory response without wasting hormone or giving a high antigenic stimulus that could result in inhibition of LH in the future. The establishment of precise timing of early embryonic development was important in planning experiments for which oocytes or embryos had to be collected at a specific stage of development. An injection of 1.0 mg of LH per kg of body weight into Dutch rabbits weighing 2 to 2.5 kg gave high ovulation rates. There was some variation in the time of first cleavage of the embryos collected, probably because of the time required for a large number of follicles to ovulate.

The developmental potential of embryos produced from superovulated oocytes also was important. Maurer et al. (135) superovulated does with FSH-LH or PMSG-hCG combinations versus the controls ovulated with LH. They collected and transferred 538 embryos to 66 recipients that resulted, respectively, in pregnancy rates of 92%, 71%, and 87%. Young born from all groups were normal. Obviously embryos produced from superovulated oocytes were normal.

Later Maurer and Foote (185) transferred more than 3300 embryos obtained from superovulated does that resulted in large numbers of normal young being born. More recently Carney and Foote (399) found that embryos remaining in superovulated donors for 84 hours did not develop as well as those removed 19 hours after LH and transferred to synchronized recipients. This difference could be caused by crowding and/or hormonal effects of superovulation on the uteri of these donors.

The dose of LH given to induce superovulation is much greater than the endogenous release of LH by either the mated or GnRH-treated rabbit. The release of LH by the female rabbit following varying doses of GnRH or analogs (444) will not

cause superovulation. It is logical that evolutionary pressure for survival has favored animals capable of producing large litters, by producing enough embryos to fill the uteri without overcrowding and causing embryo mortality.

29.0 Oocyte Maturation and Micromanipulation

Oocyte maturation. In the early 1970s culture of rabbit zygotes to blastocysts had become routine in our laboratory. Information was beginning to appear on culture of mouse oocytes. Nothing was known about maturation of rabbit oocytes, and we were curious to know how the culture requirements for these oocytes compared with rabbit embryos and mouse for oocytes. Bae conducted a series of experiments, utilizing oocytes from follicles of different sizes (228), on the role of glutamine and carbohydrates as a source of energy and protein (230), on ammonia production (233), and on the effect of osmolality of a defined medium on oocyte maturation (290).

The basic medium used was Biggers, Whitten and Wittingham's (BWW) medium, adjusted to alter osmolality (290). The optimal osmolality was about 270 mOsm, similar to the requirements for rabbit embryos (168). Pyruvate and glutamine promoted oocyte maturation (228). Progesterone stimulated oocytes from large follicles especially to develop more rapidly in culture. LH had no effect. Some cumulus cells were still attached to the oocytes because complete removal of cumulus cells was damaging to the oocyte. Consequently, the action of progesterone may have been on the somatic cells or directly on the oocyte, or on both pathways.

All three carbohydrate sources tested, pyruvate, lactate, and glucose were beneficial for maturation of oocytes, but they were not essential if the medium contained glutamine (233). Glutamine was utilized as a substrate for energy and protein synthesis (230) by both the oocyte and the cumulus cells. This resulted in ammonia production, but oocyte maturation was not inhibited.

These basic studies initiated 25 years ago contributed to understanding of the requirements of the oocyte for maturation, but the in vitro culture results were not phenomenal. There was no compelling need to develop the methodology at the time, and this topic did not have priority with NIH, so these studies were discontinued. Remarkable advances on oocyte maturation have been made in recent years by Eppig and others in studying the mouse.

Micromanipulation. In recent times, with human IVF, maturation and micromanipulation of oocytes aspirated from the ovary has again become very important. The success of microinjection of sperm (394, 426, 432) and nuclear or stem-cell transfer (402) depends substantially on use of properly nourished and matured oocytes. Yang et al. (394, 402) have shown that microinjection is facilitated by brief exposure of the oocytes to hypertonic solutions.

30.0 Embryo Culture and Evaluation

Early development of the embryo is a fascinating subject. First, the embryo undergoes several cleavage divisions in which there is no increase in size or mass, but a host of internal changes take place as the embryonic genome relieves the maternal genome of most responsibilities. Then, as the blastocyst forms, the mass of the embryo increases daily at the highest rate achieved during development when considering the gain as a proportion of pre-existing mass.

Culture. *The ability to culture embryos successfully has great practical as well as research value. All of the procedures involving embryo injection, IVF, sexing, and other embryo biotechnologies involve holding the embryos in culture for variable periods of time.* The success of biotechnological procedures, including simple embryo transfer, depends partly on providing the needs of the embryo so that it is not compromised while in vitro. For these reasons our laboratory has devoted a major effort to culturing embryos since the late 1960s.

When we initiated the studies on rabbit embryo culture, no one had been able to culture 1-cell or 2-cell rabbit embryos beyond the morula stage. Onuma et al. (140) tested heated rabbit serum and heated bovine serum with or without the addition of 1% glucose because use of glucose by the embryo was known to increase at the blastocyst stage. In addition, after 52 to 60 hours of culture,

1% pronase was added for 5 minutes to weaken the zona pellucida. Blastocysts were produced in all treatments. Glucose did not increase the proportion of blastocysts formed. Pronase promoted hatching, with 83% of the blastocysts hatching or hatched within 24 hours of pronase treatment. Embryos in similar media (175) were transferred to recipients after culture for 0, 24, 36, 48, 62, 72, 88 and 97 hours, with good survival rate up to 62 hours of culture.

Kane undertook the task of determining the components in serum that promoted embryonic development (174, 176) and used this information to develop an excellent serum-free medium, but it contained BSA (172, 186). *The report by Kane and Foote (172) was the first to demonstrate that 2-cell rabbit embryos could be cultured to the blastocyst stage without adding serum or uterine fluids.* This paper was first sent to *Science*. Despite photographs and irrefutable statistical evidence, the paper was rejected because the reviewer, supported by the editor, said it was not possible to achieve this. This was a clear case of biased censorship. We resubmitted the paper elsewhere (172) after a cooling-off period, and it was accepted.

Serum was fractionated, and addition of various extracts to a simple synthetic culture medium resulted in some blastocyst formation. The dialysate fraction was the most effective, and Kane and Foote (174) concluded, that "these experiments indicate that it should be possible to construct a synthetic medium containing low molecular weight constituents and BSA that will promote the development of rabbit ova to the blastocyst stage." Serum was further fractionated and the amino acid composition of the fractions determined (176). A total of 35 treatments were tested for promotion of embryo development. Amino acids were implicated as being especially important. Various combinations of amino acid were added to a synthetic medium that Brinster had used for mouse embryos. In the best treatment, 73% of the 2- and 4-cell embryos developed into blastocysts. Omission of hypoxanthine and thymidine improved embryo survival, an unexpected effect. These studies resulted in the BSM II medium containing 1.5% BSA as the only macromolecule (172). Many components are the same as in Ham's F10. Also, we were the first to introduce Dow Corning 360 Medical Fluid to replace the paraffin oil to cover the cultures because different batches of oil behaved differently.

Naglee et al. (168) designed a study to determine the optimal osmolality of this medium. Some embryos developed in culture over a wide range of osmolalities. The optimal osmolality was 270 milliosmols, 88% of the embryos developing into blastocysts. We referred to the medium as chemically defined. However, we were mistaken as the highly purified BSA included was not chemically defined.

The BSM II medium was used successfully by many people for culturing rabbit embryos. Drawbacks were that it was not commercially available and it took much time to prepare. As time passed, information on growth factors emerged and exploded. A medium free of any unknowns (different lots of BSA vary and contain many unknowns) was needed to study these and other specific factors required by the embryo at different stages. Furthermore, even BSM II medium was not as good for embryo development as the natural environment, so there was ample room for improvement.

Carney reinvestigated culture systems for rabbit embryos (397, 399, 403, 408). He eventually was able to modify commercially available media so that good blastocyst formation was promoted with no unknowns or protein in the medium. Several commercial media were tested with and without rabbit oviductal epithelial cell coculture (397, 403). It was hypothesized that coculture would provide a system equal to the in vivo environment. The coculture system promoted more rapid cellular proliferation than did the medium alone. However, one interpretation could be that the coculture protected the embryos against certain undesirable components or toxic concentrations of components in commercial media. It should be recognized that commercially available media were designed to culture somatic cells, not embryos.

Eventually (408), a protein-free medium was developed that sustained development of 1-cell embryos to the hatching blastocyst stage. *This was the first report of such an event in rabbits.* The medium was a 1:1 mixture of RPMI-1640 and

Dulbecco's minimal essential medium (MEM) plus polyvinyl alcohol (PVA). The medium was equal or superior to the BSM II medium that Kane had developed 20 years previously.

While these studies on media were in progress, the gaseous environment was investigated. This component of a culture system is simple to regulate. With a synthetic medium and no coculture, the optimal gas concentrations might differ from systems using serum and coculture. Work by Bavister's group at Wisconsin indicated that a CO_2 concentration of 10% was superior to 5% for culturing hamster embryos. In our studies, oxygen concentrations varying from 1% to 20% and CO_2 concentrations of 5 and 10% also were compared (431, 460). We concluded that 5% O_2, similar to the concentration of O_2 in the oviduct, was superior to 1% or 20% O_2 and that 10% CO_2 was superior to 5% CO_2.

Many laboratories used a 5%CO_2:95% humidified air culture system which provided nearly 20% O_2. In this system, the addition of taurine or superoxide dismutase, but not catalase, increased the proportion of zygotes developing into blastocysts after 72 hours of culture, and this rapid development resulted in more cells per blastocyst (439).

Several preliminary observations indicated that glucose concentration was higher than optimal for newly formed embryos. So Li and Foote (438) reevaluated the range of O_2 concentrations from 1 to 20% in the presence of 5% CO_2 to support development of embryos cultured with a lower glucose concentration (408). Under these conditions of lowered glucose, both 1% and 5% O_2 promoted production of a high proportion of hatching embryos (67 to 72%), and cell counts for embryos cultured for only 84 hours were relatively high.

Some commercial media, such as TCM 199, contain higher NaCl concentrations than we had found optimal in preliminary studies. This may be one reason why coculture improved embryo development when combined with TCM 199 (399). One series of experiments was undertaken to establish the optimal concentration of NaCl and the effect of the osmolytes, glycine, and inositol in anisotonic media (461). In another series of experiments osmolality was altered by varying NaCl and sorbitol in the protein-free, low-glucose medium used to culture zygotes and 2-cell embryos (461, 479). These studies collectively showed conclusively that optimal embryo development is highly dependent on using media with an optimal NaCl concentration and an appropriate total osmolality. Both glycine and inositol assisted the embryos in overcoming many of the adverse effects of moderately hypertonic media. One-cell embryos were more sensitive than 2-cell embryos to effects of media that were not optimized for NaCl concentration and total osmolality. These studies resulted in a protein-free medium with optimal conditions for culturing rabbit zygotes into blastocysts.

A novel antioxidant, tempol, that has theoretical advantages over superoxide dismutase (482), failed to protect rabbit embryos in experiments, again demonstrating the positive effects of added superoxide dismutase or taurine in the presence of 20% O_2. Embryos reaching the blastocyst stage in 48 hours in this culture system resulted in 50% live young compared with 58% resulting from transfer of uncultured control embryos. But embryos developing slowly resulted in only 12% young following embryo transfer.

More recently, studies with growth factors have been initiated using this completely defined medium (483). With the high rate of development of embryos into blastocysts in the controls neither platelet-derived growth factor nor mouse nor human leukemia inhibitory factor improved embryo development. Adding 10% fetal bovine serum was beneficial, however, suggesting that a sequential combination of factors likely is needed to approach conditions found in vivo.

Recent studies with KSOM, developed for mouse embryos by Biggers at Harvard, indicated that KSOM can be modified to provide an excellent medium for culturing rabbit embryos. The modified KSOM, with Eagle's essential and nonessential amino acids, resulted in 100% of the zygotes and 2-cell embryos becoming blastocysts after 3 days of culture (482). Rabbit embryos developed into 100% morulae, but no blastocysts formed without the amino acids, indicating that the initial oocyte had a limited reserve of substrate for protein synthesis. However, experiments by Liu and Foote (477) with amino acids and α-amanitin, an inhibitor of RNA polymerase, clearly demonstrated the absolute requirement for protein

synthesis for the embryos to be transformed into blastocysts. Kane and Foote (176) had reported that amino acids were important even when the medium contained substantial amounts of BSA.

Evaluation. Throughout the embryo culture studies various methods of evaluation have been employed. These include morphology of embryos, rate of cell division, development into various advanced stages, number of inner cell mass cells, number of trophectoderm cells and total cells, and development following embryo transfer. In addition, it was important initially to establish that embryos from superovulated donors developed normally. This was established early on by Maurer at el. (148, 185). More recently Carney and Foote (399) reported that embryos retained for 84 hours after LH before recovery from superovulated does were slightly compromised compared with those removed 19 hours after LH injection and transferred to recipients. Although development of embryos into young following culture is the most critical test, embryo transfer is not always feasible. Rate of embryo development in culture and differential counts of cell types also are important, but cell counting is laborious. Giles and Foote (463) reported that the ratio of ICM:TE cells increased during development, occurring at about the same rate in vitro and in vivo when embryos were 3.5, 4.0, and 4.5 days old.

Koyama et al. (450) examined rabbit embryos with the scanning electron microscope at different stages of development in vivo but not after culture. The objective was to establish changes in polarity during development that could affect totipotency of individual blastomeres used for cloning.

31.0 Embryo Storage and Metabolism

In 1970, when these studies were initiated, we believed that there would be interest in developing a cattle embryo transfer industry. Little was known about storing embryos (the first paper on freezing mouse embryos was published in 1972), but it occurred to us that if embryo transfer were to occur successfully over any substantial distance or time, the embryos collected from superovulated donors would have to be held in a dormant state until they could be transferred to multiple recipient animals in the same stage of the estrous cycle. Furthermore, if sufficient embryos could be harvested to expose them to a variety of treatments, much could be learned by comparing enzyme kinetics and embryo development of control and stored embryos. The rabbit was chosen as a model, for it was possible to obtain large numbers of embryos, culture and store them in various ways, analyze their energy metabolism and protein synthesis, and finally transfer them to recipients. Anderson designed a series of experiments to do all of the above (220, 227, 231, 235). All experiments were highly replicated, as were hundreds of experiments with rabbits.

A storage temperature of 10°C was chosen as one that would inhibit embryo development, yet not cause the many unknown deleterious effects of freezing and thawing. Storing 2-cell rabbit embryos at 10°C for 24 hours greatly reduced pyruvate metabolism (220), but after embryos were rewarmed to 37°C, pyruvate and glucose metabolism were not different from uncooled controls. Embryos collected at the 2-cell stage were transferred to recipients as controls or after cooling and then were recovered from the recipients as blastocysts. Again cool storage did not impair embryo functions, as blastocysts developed well from both control and treated 2-cell embryos, and did not differ in the functions tested.

In another experiment 2-cell embryos cooled to 10°C and then cultured in vitro or transferred to recipients were compared as blastocysts with controls (227). The cool storage had no effect on incorporation of ^{3}H-uridine into RNA or ^{14}C-leucine into protein, but blastocysts grown in vivo synthesized more RNA and protein than those grown in vitro.

Other studies (231, 235) revealed that extended storage at 10°C decreased embryo survival and development upon rewarming. After storage, development to blastocysts in vivo was superior to development in vitro, reflecting the fact that the "good" culture system was inferior to the natural environment. Following embryo transfer, the losses associated with stored embryos were found to occur before Day 12 of gestation. Subsequent prenatal and postnatal changes in control and treated embryos were equivalent.

The storage media used to hold the embryos at 10°C included 4% Promine-D, a soybean protein

we had used successfully in media for freezing bull sperm (225). This protein also protected the embryos at 10°C. In addition, these cooling studies were steps toward potential freezing of embryos. However, many subsequent studies with 8-cell to morula-stage rabbit embryos frozen with a variety of cryoprotectants yielded only a modest proportion of viable embryos after freezing and thawing (Foote, unpublished). Many researchers know well that some carefully planned experiments don't yield positive results.

32.0 Embryo Manipulation and Cloning

Our early work was planned to test and improve micromanipulation procedures applied to rabbit embryos. Half embryos developed into blastocysts at a rate equal to controls, and homologous and heterologous zona pellucidae receiving half embryos gave equal results. Twin progeny were produced (364). In another study Yang and Foote (376) combined morulae to produce chimeric rabbits representing four parents (376). Adjusting times of embryo transfer to recipients temporally asynchronous by 0, 12, and 24 hours did not improve pregnancy rate (396). Successful nuclear transfer experiments followed in 1992 (429).

We used coat-color markers to identify well-developed chimeras, but these markers were not useful for detection of young chimeric embryos. As possible markers for any chimeric tissue, Giles et al. (447) identified several specific enzyme markers for lines of rabbits in our colony that were homozygous for different alleles. ICM cells transferred directly or after culture into morulae or blastocysts contributed to embryonic development. At midgestation, eye pigmentation was a more sensitive marker than the enzyme assay.

These studies formed the basis for micromanipulation of sperm into oocytes for IVF (394) and the disaggregation of embryos to insert single blastomeres into shrunken enucleated oocytes (401) for cloning (402). Blastomeres from 32- to 64-cell embryos were isolated, inserted into enucleated oocytes, fused, cultured, and transferred to recipients to produce young. Results were positive, but efficiency was low.

Finally, rabbit embryonic stemlike cells were passaged. These cells were microinjected into enucleated oocytes (465) and developed into morulae and blastocysts (23%) compared to 34% for embryos cloned from blastomere injections into enucleated oocytes. At this point the team went different directions. The planned sequential embryo transfer experiment with stem cells was not done.

33.0 Aging Female Rabbits and Reproductive Failure

We had noted a decline in fertility of the females in our colony as they grew older, similar to farm animals. However, in large farm animals it was prohibitively expensive to study experimentally the multitude of factors associated with aging. The rabbit had many attributes that made this species an excellent model for studying causes of reproductive failure associated with aging. We had clearly established that the oocytes were as old as the female (118, 229). The rabbit was large enough that we could obtain blood samples as often as needed to monitor changes in reproductive hormones. Because the rabbit is an induced ovulator, ovulation and pregnancy could be programmed to follow experimental protocols. The life span was such that in a few years we could monitor the changes during the reproductive life of the female. Most of the techniques needed to isolate the components in the pituitary-ovarian-uterine axis responsible for reproductive failure were relatively routine in our rabbit colony and laboratory. Finally, and very importantly was the fact that over a period of years the author had multiplied an albino gene in the colony by interbreeding animals with various coat colors to get maximal genetic diversity, while saving albinos. The heterozygous animals for coat color were used in various experiments. We were able to use the albino and Dutch effectively by transferring embryos from color-marked young and old donors to the opposite uterine horns of young recipients. The reverse procedure was followed using old recipients.

We tested several null hypotheses over a period of years. These were as follows:

1. Age has no effect on ovulation rate, fertilization, cleavage, implantation, or young born.
2. Age has no effect on uterine collagen, collagenase, or organ weights.
3. Age has no effect on circulating concentrations of LH, progesterone, or estradiol.
4. Age has no effect on blood flow to the uterus or on hormone binding to the uterus.

The reciprocal transfers of embryos between young and old animals separated out the effects of aging oocytes versus extraovarian conditions. Papers providing convincing evidence to accept or reject the several hypotheses were published by several excellent young scientists (134, 162, 185, 200, 203, 204, 206, 207 and 213).

The animals programmed to be ovulated several times over a period of years would have to receive multiple injections of gonadotropins. Maurer et al. (134) established that when four injections were administered 16 weeks apart, ovulatory response declined by the third or fourth injection. Evidence of antihormone production was obtained.

The plan in most experiments was to administer LH at the time of insemination, because LH is released at the time of natural mating. However, to be sure superovulated animals responded the same as controls not receiving FSH, experiments were conducted to study insemination times arranged to age sperm or to age ovulated oocytes before fertilization (162). Results were affected by the timing treatments, as expected, but superovulated and control animals responded similarly.

The largest experiment we conducted with rabbits involved over 3,300 embryos transferred by Maurer at 6-month intervals for 3 years to sort out ovarian aging of oocytes versus extraovarian changes with age (185). A natural pregnancy was scheduled to intervene between each superovulation to reduce possible hormone antibody problems and as a control test of reproductive fitness. Despite repeated surgeries and aging, we lost few animals. During 3 years the embryos obtained from young and aging donors had similar developmental potential, but aging donors showed an impairment in potential to produce young from transferred embryos. Aging donors had a higher preimplantation embryo loss of 45% compared with 33% in young donors. Ovulation rate also was much reduced in old donors, but fertilization rate was not reduced. Following transfer, embryos from 18 does 229 weeks old produced only 26% young following transfer versus 45% with embryos from young donors.

Detailed studies of circulating concentrations of plasma progestins and progesterone at various stages of pregnancy in relation to reproductive efficiency in young and old rabbits were conducted by Spilman et al. (203). The loss of fetuses appeared to be responsible for a decline in progestins, rather than the reverse. Thus uterine dysfunction, as animals aged, appeared to be one cause of reproductive failure.

Maurer and Foote (200) had shown that the uteri of young does contained more collagenase and less collagen than uteri of old does. Larson and Foote (204) reported that uterine blood flow per 100 g tissue per minute in young does was double the blood flow to the uteri of old does. This could signify a potential shortage of many components needed for continued development of fetuses in old does.

A study of progestagens and LH in young and old rabbits gave equivocal evidence (206). The pituitary LH content and progesterone synthesis in vitro were similar between the age groups. Progesterone was higher in ovarian venous blood of young rabbits than in blood of old rabbits.

A study by Larson et al. (207) of the binding of progesterone and estradiol by the uteri of young and old rabbits provided another important piece in the puzzle of causes of reproductive failure. There was a two- to four-fold greater uptake of both steroid hormones by the uteri of young animals than by the uteri of old animals. The lower binding of hormones by old animals was interpreted to be one of the factors limiting their ability to maintain pregnancy. This was before the era of receptor biochemistry. Further studies with supplemental progesterone and estradiol administered to pregnant young and old does gave negative results (213), probably because the binding sites (receptors) were already saturated.

Chromosomal analysis (213) of 14 fetuses indicated that 11 were completely normal, but most of the embryo loss in old recipients occurred earlier in pregnancy. From these collective studies the following was concluded (213): *"Most of the embryonic wastage could not be attributed to ovulation rate, fertilization rate, ovum potential, CL function, circulating levels of progesterone and estrogen, or to chromosomal anomalies of the fetuses. It was concluded that uterine factors apparently limit reproductive performance in aged rabbits."*

34.0 Pregnancy, Embryo Mortality, and Contraception

Many of the studies in previous sections described various aspects of pregnancy as a part of the physiological measurements taken. A few experiments summarized here were planned specifically to study factors inhibiting pregnancy. Studies by Chang had indicated that progesterone (P_4) inhibited capacitation of rabbit sperm. We observed occasionally that superovulated does had a few 3- or 4-day-old corpora lutea at the time we flushed 1- or 2-celled embryos. Unfortunately, when this occurred we always harvested unfertilized oocytes, presumably because enough P_4 was produced by the time of insemination to prevent fertilization. This could have been due to an effect on sperm, but also oocytes ovulated at the time of P_4 secretion could have been less fertile or their transport accelerated. Rapid transport would result in asynchronous arrival of eggs into the uterus. Incidentally, the FSH preparations contained some LH and when we reduced the dose of the first FSH injection, premature ovulation and P_4 secretion were essentially eliminated.

We were interested in obtaining a fuller understanding of P_4 interference with pregnancy, as it was being touted as a potential contraceptive. What would happen if a person took what was a subcontraceptive dose of P_4 for that person? We used the rabbit as a model.

Several experiments were designed to explore the effects of subcontraceptive doses of P_4 administered slightly before the time of ovulation. This would induce an asynchrony relative to the normal pattern of P_4 secretion starting a few days after ovulation (212, 253, 276, 360). Allen and Foote (212) were successful in administering subcontraceptive doses of P_4 starting either 2 days before or 1 day before ovulation. Fertilization was not blocked, but the proportion of embryos reaching the blastocyst stage in the P_4-treated groups was reduced. Transfer of blastocysts from the treated groups to synchronized recipients resulted in fewer implants on Day 12 than in the control group. Single and double transfer experiments demonstrated that exposure to the environment of a P_4-treated mother for 4 days caused embryonic death between 4 and 12 days post-conception, but those fetuses surviving past 12 days had as high a chance of developing into young as those in the control group. So exposure to the environment of P_4-treated does during the first 4 days of pregnancy caused latent effects on the embryos. Other data collected suggested that part of this detrimental effect may have been caused by accelerated transport to the uterus. Uterine secretions under the influence of exogenous P_4 also could be produced asynchronously.

The next study by McCarthy et al. (253) was designed to expand knowledge gained in the previous study (212) and to relate embryo mortality to altered secretion of uterine luminal proteins. Treatment of does 2 days before ovulation resulted in normal survival of embryos retained for up to 3 days (embryos reside in the oviduct most of that time). By Day 4, embryo mortality had occurred, and the reduced ability of these embryos to survive when transferred to control recipients was reduced. Uterine protein secretion was increased by the injection of progesterone, and uteroglobin secretion was advanced. Evidence that asynchronous secretion of protein was embryotoxic was obtained by in vitro studies. Placement of normal embryos in media containing uterine fluids from temporally asynchronous control does suppressed embryo development.

These studies continued (276). Normal 2-day embryos transferred to the oviducts of P_4-treated rabbits for 24 hours developed normally when retransferred to control does. Day 3 and 4 embryos exposed to the uteri of P_4-treated does for 24 hours were severely compromised following retransfer to normal recipients. Exposing normal embryos to uteri of asynchronous untreated recipients did not

result in toxicity. Thus the administration of P_4 before ovulation produced toxic effects, presumably because the abnormal protein pattern was harmful to the embryos.

Further studies on progesterone were directed at protein concentrations in blood and in the uterus and the effect on pregnancy maintenance of the proximity of corpora lutea to the pregnant horn of the uterus (360). Does were unilaterally ovariectomized. Hemiovariectomy did not produce any gradients in P_4, and embryos transferred to the ipsilateral uterine horn versus the horn contralateral to the ovariectomy developed at the same rate. In contrast to cattle, proximity of the embryos to the corpora lutea was not related to embryo survival.

One other series of experiments relating to pregnancy dealt directly with contraception (119). The rabbit was chosen as a possible model to test intrauterine devices (IUDs). Various sizes of IUDs were fabricated and inserted unilaterally or bilaterally into the uteri of mature does 1 week before they were ovulated and inseminated. Laparotomies were performed on Days 11-13. The IUDs reduced the number of implants but did not prevent implantation. Normal young were born, indicating that the rabbit was not useful as a species for testing mechanical IUDs. However, based upon our P_4 studies, devices releasing P_4 likely would be contraceptive.

Conclusion. These many studies with rabbits provided a wealth of information, much of which was applicable to other species, including humans. In addition, the many fine graduate students and undergraduate honor students obtained experience in preparing designs for and statistical analysis of large experiments, experience with handling animals, surgical experience and laboratory experience (yes, even washing dishes very carefully). Dozens of others gained experience in the proper feeding, care and handling of animals. Many have gone on to outstanding careers in physiology, veterinary medicine, human medicine, industry and agriculture.

Chapter IV. Horses, Swine, Sheep, Goats, Dogs, Ferrets, and Humans

35.0 Horses

We conducted research with different classes of farm animals to gain some direct experience, particularly important in teaching comparative reproductive physiology. Often this provided an opportunity for undergraduate students to obtain useful experience with animals, gain an understanding of animal behavior, and become involved in research projects.

The first step in both teaching and research was to review what was known about each species. An opportunity to share thoughts relative to spermatogenesis and semen quality was provided as part of a symposium on animal reproduction (278). This brief review (278) sketched out genetic and environmental factors that affect spermatogenesis from conception to maturity and those that affect semen quality at the time semen is collected. Attention was called to the fact that sperm formation and transport take many weeks. Therefore, changes in quality of semen collected may reflect conditions that affected the testis (such as a hot summer or illness) many weeks earlier.

35.1 Equine Hypothyroidism

Lowe et al. (217) thyroidectomized 6 yearling horses (3 males and 3 females) and compared them to controls. Detailed studies were conducted on growth, behavior and blood characteristics. Growth, metabolism, and appearance were affected. Feeding thyroprotein dramatically reversed the signs of hypothyroidism. When the thyroprotein was discontinued, the thyroxine levels returned to nondetectable levels, but other hypothyroid conditions were not as severe as those found previous to the thyroprotein supplementation.

Weekly semen collections were made when the stallions were 25 to 39 months of age (238). We first attempted to train them for semen collection in their exercise yard. This effort was completely unsuccessful because this was their playground. Then we took each stallion to a nearby indoor area (judging pavilion), which was new to them. The stallions soon mounted a mare in estrus and were trained for semen collection. Thereafter they were conditioned for this event when taken to the judging pavilion. Libido was low in the thyroidectomized stallions. It was improved by thyroprotein supplementation. There was no effect of thyroidectomy on testicular histology or semen quality, a fact previously unknown in horses.

The thyroidectomized mares were studied during two breeding seasons (359). Their reproductive behavior was somewhat suppressed, but the duration of their estrous cycles, blood progesterone, and peak LH concentrations did not differ from those of controls.

Of special interest was the pregnancy of a thyroidectomized female following insemination with semen from a thyroidectomized male. Thyroxine was detectable in the blood of this mare during the last two-thirds of pregnancy and returned to nondetectable levels at parturition. Although only one case, it was a clear signal that the fetal thyroid glands were producing thyroxine, and that this hormone passed through the placenta from the fetus to the mother. *This was the first report we could find documenting thyroxine passing the placenta. Also, noteworthy was the normal pregnancy produced by thyroidectomized parents.*

35.2 Milk Progesterone in Mares

Possible use of milk progesterone to monitor postpartum reproductive activity and early pregnancy was evaluated in 13 mares (269). Milk and blood samples collected three times per week for 5 months were analyzed. The progesterone pattern was similar in milk and blood, and milk progesterone could be used as a part of a breeding management program.

35.3 Stallion Semen Evaluation and Preservation

All other studies with horses dealt with semen collection, evaluation, and processing (310, 356, 377, 384, 398, 400, 409, 412, 419, and 422). This fitted our research program and was consistent with the increasing interest in artificial insemination of horses. A study by Arriola and Foote (310) was designed to examine the use of amikacin in

stallion semen to control organisms that were associated with infections in mares, and were not controlled by commonly used antibiotics. Sperm motility was not depressed by concentrations of amikacin up to 2,500 μg/ml. This concentration was much higher than the dose required to control various pathogenic organisms in bacteriologic screening tests.

In another study Graham et al. (356) investigated methods of inducing the acrosome reaction of stallion sperm in vitro. This could be useful in the laboratory to develop in vitro tests of stallion semen quality, and possibly to enhance fertility of sperm from some stallions when used for AI. Treatment with liposomes to affect the acrosome reaction (AR) was examined by staining sperm and testing the potential for fertilization by penetration of hamster eggs. Under the optimal conditions tested, 54% of the eggs exposed to treated stallion sperm were penetrated.

Padilla and Foote (412, 419) followed these studies with experiments to improve the quality of stallion semen processed for potential use in artificial insemination. There was a major interaction between stallions and the effect of centrifugation on survival of extended semen. These studies resulted in a modification of the Kenney extender by adding a high potassium Tyrode's medium, which greatly improved survival of stallion sperm at 5°C.

Further studies (419) utilized the technique developed earlier (356) to induce the AR. The hamster egg penetration test was employed to evaluate potential fertility of extended semen. These studies indicated that stallion sperm can be stored for at least 24 hours at 4°C without a decrease in potential fertility. The hamster egg penetration test was useful, but it was difficult to have a fresh supply of hamster oocytes available as needed. Fortunately, it was possible to cryopreserve hamster oocytes and maintain a bank of oocytes (406). With the improved extender (412), longer storage of liquid stallion semen for artificial insemination seemed to be possible.

The other published papers on stallion semen and fertility were a series by Jasko et al. (377, 384, 398, 400, 409 and 422). The first study was an examination of the sources and magnitude of the variances in measuring sperm motion characteristics by computer-assisted sperm analysis (CASA). This study was based on CellSoft equipment. Later this equipment was compared with the HTM-2000 Motion Analyzer (400). At that time, software and instrument settings needed improvement. Many of the sperm measurements of semen quality differed considerably, but the estimated mean percentages of motile sperm with the two instruments agreed more closely than did the CASA values with subjective estimates made by a technician viewing sperm on a TV screen. The CASA procedure also was used to evaluate a swimup procedure for selecting the more motile sperm in the ejaculate (384).

These studies included performance of stallions on many breeding farms. A low but significant correlation was found between the morphology of the sperm and the fertility achieved by the stallions (398). Another study of stallion fertility (422) compared the relationship between CASA and fertility during 99 breeding seasons on Thoroughbred and Standardbred farms. A total of 7,017 estrous cycles was included. Many of the measurements were correlated with fertility, but the correlations were low. The highest correlation was between the percentage of progressively motile sperm and fertility ($r = 0.34$).

Because of the high repeatability of the CASA measurements, a few ejaculates per stallion may be sufficient per stallion to evaluate fertility potential of the stallion by CASA. Seasonal effects were found (409). Generally, semen quality was lower in the winter months.

Other potentially useful studies were conducted on handling liquid and frozen stallion semen. However, these ended up in a master's thesis (Smith, unpublished, 1987, Cornell University).

36.0 Swine

Limited studies were started with swine early in my career because of the interest in developing AI in swine and because of the need to obtain practical experience with swine for more effective teaching. Also, because of the tremendous number of sperm collected from boars compared to other farm animals, spermatogenesis in this species was of special interest (105, 169). With this background, an opportunity was provided at a meeting

on comparative animal production to discuss various attributes of boar sperm and their production (278). One reason that boars produce so many sperm is that spermatogenesis takes less time to complete in the boar than in bulls, rams, and stallions (278).

36.1 Boar Testes and Sperm Production

In 1958 Kennelly initiated studies (105) on sampling boar testes and quantifying the various elements composing the testes to predict sperm production as part of an M.S. thesis project. Little was known about the details of spermatogenesis in the boar, so the first study involved sampling four areas of the testis to examine uniformity. Some differences were found, but samples from the left and right testes provided concordant data. The tubular, intertubular, and supporting components of the testes were quantified. *From these data total sperm production per day was calculated from a newly proposed formula (105).* Actual sperm collected per day was 63% of the predicted production. The difference of 37% presumably reflected degeneration and resorption of spermatogenic elements in vivo and losses of sperm in the urine. Boars differed in the number of sperm collected and in calculated sperm production.

36.2 Boar Semen Collection, Evaluation, Preservation, and Use

The main objectives of developing an artificial insemination program for swine were to make genetically superior boars more widely available and to reduce disease. To make rapid progress in boar progeny testing, it is important to sample boars at an early age. Our first studies (73, 79, 80) were designed to train boars to mount a dummy at as early an age as possible. Boars were trained for semen collection at 4 months of age, and different methods of semen collection were tested. It was found that boars maintained high libido when semen was collected every second day. By the time a boar was one year old as many as 67×10^9 sperm could be harvested after giving the boar a 2-day rest. Ten-month-old boars yielded 68 billion sperm in a 3-day period when ejaculated daily. To facilitate rapid estimation of the sperm concentration in an ejaculate, a procedure was developed to calibrate a photoelectric colorimeter (80). This was patterned after procedures used with bull sperm.

Many types of semen extenders were compared as well as packaging units (79). Sealed storage containers were superior to corked tubes. Storage of sperm at 15°C was superior to 5° or 25°C. Because of the apparent sensitivity of boar sperm to oxygen, the catalase content of boar semen was investigated (91) and found to be almost nondetectable (0.2 μg/ml); it was lower than in other farm animals.

An extender containing 20% egg yolk (v/v), 0.33% sodium citrate dihydrate, 0.33% glycine, 1% glucose, and 1,000 units of penicillin plus 1,000 μg of streptomycin per ml was tested on a farm in the 1950s (Foote and Young, unpublished, Cornell University) where semen was collected from boars two or three times per week and extended 1:4 or 1:8. The semen was stored at 15°C and used both on the day of collection and for 1 or 2 more days. Pregnancy rate following a single insemination by the herdsman was 65% (53/81). *The farm planned to have a sale advertised as the first one in the United States in which all pigs were sired through AI. However, the breed society would not support this idea and the sale proceeded with no mention of AI.*

With the limited opportunity to inseminate large groups of gilts or sows to test various treatment effects, an attempt was made to treat boar sperm in vitro (356) and test fertility of boar sperm using the hamster egg penetration test. Modifications of the procedure used successfully for other farm animals failed to work with boar sperm.

36.3 Estrous Cycle Activity and Fertility in Gilts and Sows

Extensive research at other stations (see 197 for references) has demonstrated that feeding and hormone treatment can advance puberty. One of the problems with gilts was to detect estrus conveniently and precisely enough to use AI successfully. We measured several biochemical changes in the vagina, but test results were erratic (Foote, unpublished). Then we modified an electronic probe used successfully for cattle (279, 280, 285). This probe, modified for swine, was tested on

sows still in farrowing crates. When timing of AI was based on changes in electrical conductivity with the probe, pregnancy rates were as high as when AI was based on detection of estrus by a boar (Foote, unpublished). However, the swine farm manager considered the use of a boar to be the simplest procedure for detecting estrus.

Earlier studies (117) indicated that estrus could be synchronized in gilts by including progesterone or progesterone and estrogen derivatives in the feed. Natural mating was used at the synchronized estrus, and several treated gilts refused to mate with the boar, yet conception rates and litter sizes of treated gilts were equivalent to those of the controls. Variability was the most pronounced characteristic found in gilts, which is consistent with results published by others.

Minimizing embryonic mortality, with the consequent increase in litter size, obviously is important in the success of a swine enterprise. Pope et al. (348) studied the sensitivity of embryos to different concentrations of estradiol on Days 9 to 13 of gestation because the literature revealed that estrogen secretion by pig blastocysts increased greatly from Day 9 to 12. Results of the study by Pope et al. (348) demonstrated that the uterine environment of the embryos in pregnant gilts was more sensitive to shifts in estradiol on Days 9 and 10 than on Days 12 and 13.

37.0 Sheep

Sheep were included in our research program for reasons similar to those discussed with other farm animals. Also, sheep are of great economic importance worldwide, and some of the students came from areas of the world where sheep were much more important than dairy cattle. It is amazing how much one learns by examining the similarities and differences among species and by appreciating how they have become adapted to exist in various ecological niches. Some of this information, gained partly from others, was included in the symposium paper on semen quality in farm animals (278). It is fascinating that sheep respond to decreasing daylight so that with fall breeding lambs are born in the spring following a gestation of nearly 5 months. We investigated the role of lighting and hormones on male reproduction in reverse lighting experiments (El-Alamy et al., unpublished, Cornell University). In contrast, the horse responds to increasing daylength. With a longer gestation, and after breeding in the spring, the young are born the following spring. This is common knowledge, but reflect on the mechanisms that evolved over eons of time to make this differential response to daylength.

37.1 Ram Semen and Sperm Preservation

Because of the potential benefit of catalase added to semen, catalase content of ram semen was compared with that of other farm animals. Ram semen catalase was higher than in some farm animals, and 66% of the catalase in ram semen was associated with the sperm.

In other studies, Jones (136) and Jones and Foote (198) examined the effects of low molecular and nondialyzable components of skim milk on the preservation of ram and bull semen. Ram sperm survived better at 37°C in the low molecular weight (dialyzable) component. Heating the nondialyzable portion improved sperm survival. Raw milk and pasteurized milk contain a substance toxic to spermatozoa. This toxicity was eliminated by heating milk to at least 92°C or by adding 0.5 mg of cysteine·HCl/ml.

Perhaps the most important finding with ram semen was that the toxic effects of anisotonic buffer solutions on sperm survival were greatly decreased by increasing the high molecular weight (nondialyzable) component. This protective effect of the large molecular weight substances was useful in designing better extenders for freezing ram sperm. Extensive studies on varying concentrations of skim milk and egg yolk for cryopreservation of ram semen were conducted by Lathrop for an M.S. thesis (Lathrop, unpublished, 1986, Cornell University).

Testing the fertility of ram semen with adequate numbers of inseminations was not possible in our laboratory. Therefore, we pursued with ram sperm the possibility of inducing the acrosome reaction with liposomes (356) and testing fertility based on hamster egg penetration. The initial tests failed, but when sperm concentration was increased to 20×10^6/ml, 52% egg penetration was achieved. With bull sperm, 92% of the eggs

exposed to 6×10^6 cells/ml were penetrated. The reason for the difference between species is not known, but it appears that ram sperm were more resistant to induction of the acrosome reaction in our system. To facilitate further work with hamster egg penetration, a successful technique for cryopreservation was developed by Tobback et al. (406), and a large bank of cryopreserved embryos was prepared.

Young male sheep also were used for toxicologic studies associated with fertilizing land with municipal sludge (325). The cadmium content of tissues of sheep grazing on grass-legume forage growing on soil treated with the sludge was slightly higher than tissue cadmium in the controls. Although cadmium is a potent toxin (159), the exposure concentration was low, and no effects were found on the testes, epididymides, or progressively motile sperm of the rams.

37.2 Techniques for Monitoring Reproductive Phenomena in the Ewe

Lambs, like other prepubertal farm animals, have oocytes in ovarian follicles capable of responding to gonadotropic hormone (197). However, our studies with female sheep were limited to reproductive cycles in mature ewes.

In a group of 18 rams used for detecting estrus, nine had their penises deviated, three were vasectomized and six were intact controls (267). Penis-deviated rams were raddled. They proved to be as useful for detecting estrus in ewes as were the typically used vasectomized rams. Two major advantages of the rams with deviated penises were that 1) that their breeding potential was preserved and 2) it was easier to collect semen from rams with deviated penises. All semen characteristics in the surgically altered rams were equivalent to those of control rams.

Nine ewes had specially molded silastic cannulae installed in their oviducts. They were designed so that it was possible both to inject spermatozoa into the oviduct and to collect fluid and oocytes or embryos from the oviduct. Diagrams of the design and placement of the cannulae are in the paper by Ball et al. (288). Cannulation appeared to reduce superovulatory response to gonadotropins. Blood plasma LH and progesterone and estrous cycle length were normal. Cannulae were still functioning at the conclusion of the study 6 months after installation. Subsequently, functional cannulae were retained in ewes for a year and fluids were collected throughout the breeding and nonbreeding seasons (Ball and Foote, unpublished, 1976, Cornell University).

38.0 Goats

Mellado et al. published a series of papers (415, 416, 417, and 418) on nutrition, reproduction and lactation in goats. The original plan for the thesis was to study sperm transport and fertilization under different conditions of insemination in cattle. This was to be part of international collaboration. After doing all the preliminary planning and research at Cornell, there was a change in the administration at Mellado's home cooperating institution. The program abroad was canceled so Mellado ingeniously put together a goat project.

The first paper (415) is a detailed report of the botanical composition of the diets selected by goats at different seasons of the year in northern Mexico. The goats' performance was remarkable despite the nutritional inadequacy of the diet.

Another study (417) dealt with mortality of goats related to age of the goats and seasons of the year. With semiconfinement the annual mortality was 21.5%, and the highest mortality rate was among young animals. The data obtained provided a basis for recommending changes in management to reduce losses.

Nubian goats under intensive management, with uniform feeding conditions, produced young throughout the year, although there were seasonal effects (416). Rainfall and temperature influenced sexual activity more than length of daylight, and the conception rate was lowest in the winter.

The fourth paper (418) involved evaluation of lactation and reproduction in crossbred goats receiving no concentrate supplement to their range feeding. Relationships between parity, body weight, litter size and lactation were analyzed. The larger goats with good body condition were the highest milk producers and had larger litters. International programs are very important, but complications arise when a cooperating institution

fails to meet a commitment, as happened in this instance.

39.0 Dogs

Many of the undergraduate students in the reproductive physiology course were interested in dogs. Some students came from families who bred dogs commercially. The students related various problems about bitches sent to them for breeding, such as mating incompatibility or infection. They had simple questions such as "How often should a stud dog be used?" There seemed to be little information in the literature about spermatogenesis, sperm production, and AI in dogs. Fortunately, Cornell had the McCay Dog Farm which Dr. McCay had used meticulously to study nutritional requirements for dogs. This colony of beagle dogs was kindly made available for reproductive studies at essentially no cost for the dogs.

39.1 Spermatogenesis in Dogs

Little was known about the kinetics of sperm formation in the dog, so availability of male dogs provided an opportunity to produce new information on this important organ. Also, toxicologic studies with male dogs in the past had not included reproduction, and a standard description of spermatogenesis was essential if the testis was to be included in toxicologic investigations. Evaluation of the dog testis was initiated in the 1960s and finally was published by Foote et al. (199). Spermatogenesis in the dog was similar enough to several other mammals studied so, with a few modifications, the eight-stage system we used for the rabbit (98) was modified to fit the dog. Maintaining the eight-stage classification system facilitated comparison with other species. Details of the classification system used and photographs typical of the histological appearance of testis cross sections for each of the eight stages were included in the publication. The duration of one cycle of the seminiferous epithelium was 13.6 days compared to 10.9 days for the rabbit (98). *This publication (199) provided the first detailed description of spermatogenesis in the dog.*

Also, we tested testicular biopsy, combining this procedure with regular semen collections to obtain comparable data longitudinally. When done properly, the biopsy is a relatively harmless procedure (161, 487) that has minor effects on sperm production. In toxicologic studies, testicular changes during treatment and during the recovery phase following treatment can be monitored by biopsy in the same animals. Recently, hundreds of human males have had testicular biopsies performed to obtain sperm for intracytoplasmic sperm injection (ICSI). Often little attention was given to the procedure used or possible follow-up. Few experimental data had been published in this field. So these studies (161, 487) provide both precautions and objective evaluation, bridging the gap that currently had existed to much earlier literature on testicular biopsy with which most clinicians are not familiar.

39.2 Semen Collection and Evaluation in Dogs

Training dogs to serve an artificial vagina was relatively simple and provided an opportunity to study quantitatively the effect of frequency of ejaculation on sperm output per ejaculate and to obtain an estimate of daily sperm production (70). Different methods of semen collection were tested, resulting in use of a simple clear collection funnel and tube that allowed visualization of the ejaculation process and resulted in deposition of the sperm into the graduated semen collection tube without loss or damage that occurred on the rubber lining of the conventional artificial vagina (70, 133, 216). An optical-density method of determining sperm concentration was developed (70, 107). Thus sperm concentration, ejaculate volume, and the percentage of motile sperm could be determined rapidly. Frequency of semen collections, ranging from twice per week up to twice per day, produced sperm output data indicating that stud dogs could be ejaculated every 2 days on a regular basis and even daily on a short-term basis without depleting the number of sperm per ejaculate. The standards determined in this study proved to be useful guidelines in establishing procedures for subsequent studies (100, 101, 102, 103, 179, and 472). With the clarity of the ejaculate in dogs, computer-assisted semen analysis (CASA) has been added as

a procedure for evaluating semen where this instrumentation is available.

Tranquilizers were sometimes administered to easily excitable dogs to facilitate handling or transport. Foote and Gray (99) administered chlorpromazine to males with no effect on libido or semen quality. Promazine or chlorpromazine (200 μg/ml) added directly to semen did not depress sperm motility.

39.3 Semen Preservation in Dogs

Our first series of experiments (100, 101 and 102) were designed to improve the storage life of liquid semen at 5°C. Modifications of semen extenders used with bull sperm preserved the motility of dog sperm at high levels for at least 4 days at 5°C. Egg yolk-based extenders were superior to heated milk (100). Glycerol could be included in a yolk-citrate-glycine-glucose extender with a slight reduction in sperm motility.

During the many semen collections, we had noted that collection of the last fraction of the ejaculate containing a large volume of prostatic fluid was not only time-consuming, but sometimes decreased the percentage of motile sperm in the final ejaculate. So the next series of experiments (101) was designed to study the effect of ejaculate fractionation and frequency of ejaculation on quality of semen stored for up to 16 days at 5°C. There was little difference in survival whether the third fraction of semen was included or not, and frequency of ejaculation had little effect. What was important was to extend the semen enough that the sperm concentration was about 20×10^6/ml and to include glucose as a source of energy for continuous storage at 5°C (101, 102). Dog semen contains 5 μg/ml of catalase, and addition of catalase to extended semen did not improve sperm survival. One of the simple lessons we learned from these complex experiments was not to ignore the basics. These were the lessons: a basic source of energy in the extender and the right numbers of sperm are important. Use enough sperm to perform the required task, but do not crowd them.

If artificial insemination in dogs were to become practical, especially where the principals in special matings were far apart, frozen semen would be a big help. We had frozen semen from many bulls, so our next step was to freeze dog semen (103). Best survival of sperm was in a 20% yolk-tris extender containing glucose and 8% glycerol. The postthaw motility of sperm was 41%. This extender was used for freezing sperm (179) that were inseminated. Fertility and whelping rates following insemination with extended semen held for 0 or 24 hours at 5°C were similar to control results (natural mating), but results with frozen semen were negative. We had planned to repeat these experiments, using concentrated sperm following freezing, removal of most of the extender in the washing process and insemination of sperm into the uterus. However, egg yolk-tris was used successfully in Norway with dogs and foxes, with an intrauterine insemination technique. About 67% pregnancies and normal litter size resulted from use of frozen dog semen (Farstad and Berg, J. Reprod. Fertil. Suppl. 39:289-292. 1989).

40.0 Ferret Nonsurgical Artificial Insemination and Embryo Transfer

For the past several years interest in using noninvasive (nonsurgical) techniques in animal experimentation has increased greatly. Also, with endangered species, use of surgical techniques to assist reproduction can lead to tissue damage, adhesions, and other postsurgical trauma. Studies were instigated to develop several nonsurgical techniques to assist reproduction.

The black-footed ferret is an endangered species. The largest supplier in the world of a related ferret is located near Cornell. This made possible studies by Kidder et al. (papers and Ph.D. thesis in preparation, 1998) on AI, embryo collection, and embryo transfer in ferrets. Special equipment and techniques were developed by Kidder to pass the cervix. This was the critical step in being able to produce pregnancies by nonsurgical artificial insemination, *a step never achieved before in ferrets. Likewise, embryos were collected, examined, and transferred non-surgically, with normal kits resulting, also another first.* Early embryo development also was studied.

41.0 Human Semen Analysis

Probably the most important problem facing the world today is the increasing human population, outpacing our ability to provide food, eliminate wastes, protect the environment, and conserve our diminishing natural resources. We have depleted more natural resources in the past 60 years then in all previous history since human beings emerged. The world population is increasing at a catastrophic rate. Meanwhile, billions of dollars are spent annually on reproductive problems in humans. With the difficulty of obtaining experimental data in humans, many procedures are based on experience and biases without solid supporting evidence. Our experimental experience with semen from several species facilitated performing various analyses of human semen.

As sperm normally must pass through cervical mucus, the medical field logically sought to develop a test of sperm fertilizing ability based on penetration of cervical mucus. Where could one obtain an adequate supply of cervical mucus? The cow was chosen by others as a commercial source. Our experience with cattle indicated that there was great variability in cervical mucus among and within cows, depending on the exact day of collection relative to estrus (279, 280, 285). This could complicate any systematic testing of sperm penetration. Companies producing mucus test kits for sale failed to appreciate this fact. So we developed a synthetic mucus made from polyacrylamide gel (301, 302 and 305) and applied this to human sperm (309). The gel was stable for a long time and different batches gave uniform results. The gel worked well, but the gel penetration test did not add much information to what could be obtained by analyzing semen samples for the percentage of motile and normal sperm.

The development of computer-assisted semen analysis (CASA) offered an objective and rapid method of evaluating many motion characteristics of a statistically valid number of sperm. When this research was started more information was needed on the assay procedures. Were live and dead sperm detected accurately? Were estimates of sperm concentration accurate? Were assays repeatable? We had studied these questions previously with semen from several species and applied the same analysis to human sperm.

With human semen it was found that different machines gave the same results when analyzing the same videotapes used to record suspensions of sperm (462). Another factor studied was the way to maintain sperm quality from the time of ejaculation until they are evaluated. Media were tested and procedures were established to accomplish this (471, 478).

When there was much debris in the semen, or extenders were added, that obscured the sperm (such as whole milk) the CASA was less accurate or could not be used. We assisted Hamilton Thorn Research in testing a unit with an ultraviolet strobe light that was equipped to detect sperm stained with the specific DNA stain, Hoechst 33342 (476, 478). This unit was validated against phase contrast illumination on samples suitable for both types of illumination. Selected variables were highly correlated with fertility of bull semen (488). So careful CASA of human sperm can be used to evaluate quality of human semen, and this may provide an estimate of the fertilizing ability of human sperm.

Chapter V. Designs

Just as "beauty is in the eye of the beholder," the word "design" will convey different meanings to different people, depending upon the context in which it is placed by individuals. For the artist, the design may be some spatial arrangement of colors of different sizes and shapes. The architect will include definite quantitative relationships in an architectural design or plan. Others may contrive plans that are even selfish or harmful. So design, by itself denotes neither good nor bad.

The researcher must consider various experimental designs, all having important quantitative components. Different designs are needed to fit different situations to yield credible results.

42.0 Experimental Design and Modeling

The preceding chapters cover an array of hypotheses that have been tested. In research, choice of the experimental design is the next step after deciding which hypothesis to test. The investigator must supply answers to the following questions. What constitutes an appropriate control? How many animals or subjects are needed? How many replicates within animals or subjects are needed? Answers to these questions will depend on the end points selected, the magnitude of the differences one wishes to detect and the probability chosen to detect Type I and Type II errors. What are the most important end points biologically? What is the sensitivity, repeatability and cost of obtaining each end point?

We have considered several end points for evaluating bull semen (72, 169, 211) and for rats and rabbits in toxicologic studies (378, 425, 481). Based on the variances obtained one can calculate the size of the experiment needed to accomplish the desired objective.

Similar data are equally valuable in deciding what management strategies will be most effective to accomplish certain goals (292, 293, 305). These can be research management strategies or strategies to improve management in a business.

Success in life, as in experiments, can be measured in many ways. Certainly it is important to make decisions as objectively as possible and to stack the probabilities in one's favor. Also, as we pursue this important urge to explore, learn, and understand, it is the author's hope that in this quest we will work together to understand the needs of each other, so important for the fulfillment of the need to be understood.

43.0 Animal Biotechnology and Ethics

Many miracles in reproductive technology have been performed many places through the ingenuity and persistent efforts of people in academia, industry, and government. These miracles have revolutionized animal breeding programs and the ability of the medical profession to assist in completing the reproductive process in human beings. I have been privileged to observe these changes and to have been a part of selected aspects from *AI to cloning* during the past half-century. This involvement is chronicled in the previous sections. These 50 years also have provided an opportunity for reflection. A few of these reflections have appeared in print, such as found in Chapter I and in the following: sex control (191); artificial insemination and cryopreservation of sperm (306, 308); embryo transfer (320); IVF and embryo transfer in animals and humans (355); embryo cryopreservation and depositing patented animals (385); and various combinations of animal biotechnologies from AI through transgenic animals and cloning, including ethics (393, 411, 420, 424 and 443). Researchers working with animals in the future will be increasingly challenged on the ethics of their research. When planning an experiment, researchers always should answer the question thoughtfully "Why should this research be done?" Other ethical factors to consider were summarized by Foote (420).

One memorable event, not cited in this book, is the series of meetings organized by The National Institutes of Health (NIH) that led to an Ethics Advisory Report included in the HEW report "Support of Research Involving Human In Vitro Fertilization and Embryo Transfer," 1979. Dr. Biggers and I were technical consultants. The author had been a prime reviewer on a solid grant proposal to NIH by Dr. Soupart to investigate chromosomal characteristics in human embryos in vitro. This stirred a never-ending controversy, and NIH was prohibited from investing funds in this

research and in related areas dealing with human material. So the Soupart proposal was not funded.

We predicted then that clinics would be established in response to couples' demands, and that there would be a lack of knowledge and expertise initially to provide effective assistance in many clinics. This is precisely what happened. Millions of dollars were spent by couples who remained childless, partly because the procedures and needed skills had not been developed through research. Through trial and error and the introduction of ingenious procedures by IVF staffers, progress was made. Also, many embryologists with excellent ability and technical skill, honed by research with animal embryos, helped to fill the need by accepting employment in human IVF clinics. Today there are many excellent IVF clinics.

44.0 Cloning

Previous sections referred to our specific studies related to cloning (364, 376, 396, 401, 402, 424, 429, 443, 447, 465). Other laboratories have made major advances in cloning, and Granada Genetics, Inc. initiated a major program of cloning cattle by nuclear (blastomere) transfer (see the chapter by Massey in the book listed in the Appendix, edited by W. Hansel and B. J. Weir). This program eventually ceased for a variety of reasons, one of which was that it produced some giant calves. The mechanism responsible for producing abnormally large calves is not yet understood.

In 1997, a bombshell article on cloning was published by Wilmut and associates (Nature, 385:810. 1997). The editors of *Science* called this accomplishment the "Breakthrough of the Year (*Science*, 19 December 1997). The lamb Dolly was produced from a cell derived from mammary-gland tissue of an adult (6-year-old) ewe. Birth of the lamb from this mammary cell indicated that no detectable modification of key genetic information occurred during development that was not reversible by the procedures used. Furthermore, Dolly grew normally and was fertile. Production of cloned cattle followed, although donor cells were from fetuses. In 1981, McKinnell (see his chapter in the book listed in the Appendix, edited by Brackett, Seidel and Seidel) concluded his chapter with the statement, "Whether or not cloning of adult nuclei becomes possible depends upon increased understanding of the differentiative process."There is still much to be learned about the differentiative process.

The discovery that dedifferentiation is possible opened up a new world of hope and opportunity for investigating important mechanisms in developmental genetics and physiology. An increased understanding of cell function and malfunction will help in discovering procedures for early detection or prevention of diseases such as cancer. Valuable medicinal products, such as blood clotting factor and other blood proteins, or even spare parts, will be produced by genetically engineered cloned animals. *The magnitude of the opportunity to advance knowledge in the twenty-first century is unprecedented.*

A great furor has arisen over the concern about cloning humans. Multiple sources of viewpoints on ethics, morality, legality, technical feasibility and costs can be found in the electronic network. If cloning can be done from adult somatic cells in human beings, why would anyone want to do it? Presently, it would be very expensive, unlikely to succeed, and possibly produce abnormal babies. There are much more efficient, medically proven ways of helping most infertile couples to produce offspring.

If the reason for producing a clone is the desire to have an offspring that will be an exact copy of an adult, that desire is based on an absolutely false assumption. *An exact copy will not happen using nuclear transfer!* In the process of cloning one will start with an aged cell. Whether the process of aging is completely reversed by the cloning procedure is unknown. Furthermore, will the cell or cells chosen to be cloned have undergone a mutation or mutations during development and aging? What effect will the small amount of mitochondrial DNA in the cytoplasm of the recipient oocyte have on development? Will there be any environmental influences during in vitro manipulation in culture that will impinge negatively on some genetic component? What will the environment be like that will surround the clone during pregnancy? Most important of all, what kind of an environment will a clone grow up in? How will the likely stress and

expectations during this critical period of development affect the behavior of the clone? Would you like to be born as a clone?

There are many unanswered questions that need to be researched. One of the intriguing scientific questions being studied intensively is the relative "amount" of donor and recipient mitochondrial DNA replicated, and the impact that this has on future development. True clonal lines of cells currently are produced and genetically engineered to perform many valuable functions. Some day true clones of animals from bone marrow stem cells or other sources likely will be produced without the necessity of incorporating mitochondrial DNA, as occurs with current nuclear transfer techniques.

· Any attempt to clone human beings certainly is premature, and, from my standpoint, represents a major financial effort for questionable benefits. Education of the public of the great potential value of cloning research is a responsibility of all knowledgeable people. No legislation should be enacted that will interfere with research on cloning human cells for many purposes, including possible homologous batches of cells for organ repair.

Ethics. Researchers have a great responsibility to act ethically in this and all areas of science. We can and should serve as role models. In Cornell Focus [6(3):4-9, 1998] Foote was quoted as follows: "In the quest for knowledge and understanding, judicious use of animals has benefited humans and animals. In contemplating research, the first question to ask is *why* is it important to try to solve a particular problem? Then *why* is followed by *what* will the probable impact of a solution be upon society? And finally, *how* should one proceed to employ the best method available to achieve that goal? Cloning of medicinal products, cells, tissues, and animals offers extraordinary possibilities for increasing our knowledge and ability to improve the social and economic well-being of people and the health of animals. As long as we continuously combine high ethical, moral, and research standards in the pursuit of truth, great benefits can result." It is important that we keep the public and our leaders informed of our purpose and progress, and that mass media report our findings responsibly.

There are many great needs in society such as family planning and helping those born under impoverished circumstances to develop according to their abilities. Let us use our time and talents to help others in really meaningful ways through research and teaching and through applying our knowledge and resources to make the future of our home (planet earth) the best place for all who dwell therein.

ABSTRACTS

1. **Some Problems in Bull Semen Storage.** G.W. Salisbury, E.L. Willett and I.C. Gunsalus

This paper is part of a small conference on artificial insemination (A.I.) in which many of the pioneers in A.I. reported on early work on preservation of semen obtained from bulls, stallions, rams and turkeys. The paper by Salisbury et al. discussed the need to control temperature, and the development of the Cornell artificial vagina to prevent coldshock during semen collection was described. Also, use of sterile equipment and minimizing bacterial contamination was discussed.

2. **An Artificial Vagina for Controlled-Temperature Studies of Bull Semen.** G.W. Salisbury and E.L. Willett

An artificial vagina having several advantages over the one in common use is described. While somewhat bulkier than the conventional-type artificial vagina, it is especially useful when samples are being collected in cold weather, for it maintains proper temperature for use longer and prevents chilling of the spermatozoa. A diagram of the artificial vagina is included and shows the warm water casing extended to enclose the semen collection tube.

3. **Preservation of Bovine Spermatozoa in Yolk-Phosphate Diluent and Field Results from its Use.** E.L. Willett, H.K. Fuller and G.W. Salisbury

Data are presented which show that the yolk-phosphate-buffer diluent maintains the viability of spermatozoa at a higher level during storage than semen stored without dilution. The odds that this is true are significant during each of the periods of comparison. Data on over 1500 inseminations in an artificial breeding association indicate that, when proper precautions are taken in handling the semen and diluent, semen may be diluted and stored up to four days with results as satisfactory as with semen used on the day of collection.

4. **Recent Research Developments in the Preservation and Handling of Bovine Semen.** G.W. Salisbury

An overview of the technical aspects of development of artificial insemination of dairy cattle in the U.S., particularly in New York State is given. The use of the artificial vagina and careful evaluation of semen for volume of the ejaculate, pH of semen, sperm concentration and the percentage of motile sperm are discussed. Semen should be cooled slowly to 5°C. An egg yolk-citrate diluter was developed in which sperm are visible has an advantage over the egg yolk-phosphate diluter published by Lardy and Phillips. Similar fertility results were obtained with both diluents. Bacterial contamination of semen was observed and proper washing of the sheath of the bull effectively reduced bacterial contamination.

5. **The Rate at Which Spermatogenesis Occurs in the Rabbit.** S.A. Asdell and G.W. Salisbury

Testes which had been placed in the abdomen for various times were returned to the scrotum. The degree of degeneration and the rate of regeneration were observed. A stay of 1 day in the abdomen produces considerable damage which is not fully apparent at the end of the 24 hours, as it continues after the testes are returned to the scrotum. A stay in the abdomen longer than 1 day produces somewhat more damage. The rate of recovery of the testis after its return to the scrotum is roughly proportional to the length of its stay in the abdomen. Approximately 1 week is needed for the establishment of each successive layer in the seminiferous tubules, and 2 weeks for the spermatids to grow their tails.

6. **The Viability of Spermatozoa in the Abdominal Epididymis and the Failure of Motile Sperms to Fertilize Ova.** S.A. Asdell and G.W. Salisbury

The testes of adult male rabbits were anchored in the abdomen for varying periods. Spermatogenesis ceased within 24 hours. The extreme fertile life of sperms was 8 days for 2 males in 6 tested at this

time. At 9 days none in 10 was fertile. No effect was observed on litter size at 8 days. Motility ceased at 14 days. Motile but non-fertile sperm failed to reach the oviduct or to survive to ovulation time after mating the bucks.

7. **Preservation of Bovine Spermatozoa in Yolk-Citrate Diluent and Field Results from its Use.** G.W. Salisbury, H.K. Fuller and E.L. Willett

An M/15 solution of sodium citrate mixed in equal amounts with fresh egg yolk produced a diluent which dispersed the fat globules and other material in the yolk so that when semen was diluted with it the individual spermatozoa could be readily seen upon microscopic examination. The yolk-citrate diluent and the yolk-phosphate diluent were apparently equal as preservatives of the motility of spermatozoa which were stored in them under standard conditions for two and four days. The yolk-citrate diluent was superior to the yolk-phosphate diluent for the preservation of motility when semen was stored six days or more. In actual insemination tests with semen stored up to 5 days no significant difference in fertility was found between the semen stored in the yolk-citrate and the yolk-phosphate diluents. The results of the fertility studies tend to bear out the results of laboratory storage studies on the maintenance of motility under standard conditions.

8. **The Bacteriology of Bull Semen.** I.C. Gunsalus, G.W. Salisbury and E.L. Willett

The bacterial count on 43 ejaculates collected from 19 bulls by means of an artificial vagina ranged from 1,000 to 22,000,000 per cc. It was found that by douching the sheath and washing the underline, if the bull was dirty, the number of bacteria in semen could be markedly reduced. Almost sterile yolk-phosphate diluent was consistently produced when fresh eggs from healthy hens were used and when aseptic methods were employed in the preparation of the diluent. Under other conditions the diluent may be responsible for the addition of large numbers of bacteria to semen samples. Bacterial growth during storage was held at a minimum by storing at 5°C or lower.

9. **The Effect of the Method of Making Semen Smears Upon the Number of Morphologically Abnormal Spermatozoa.** G.W. Salisbury, E.L. Willett and J. Seligman

Considering all factors involved in this study it would appear that the most satisfactory method to be used in the preparation of semen smears is that in which a drop of diluted semen is spread over a clear slide by simply placing another clean slide face down on it and pulling the two apart lengthways. Apparently the film of liquid between the two slides is sufficiently thick to protect the individual spermatozoa if undue pressure is not applied. This study emphasizes the importance of a standard procedure in the handling of semen and in the preparation and staining of slides when two or more males are to be compared with the view to assessing their relative fertility. It also shows that while tailless heads and spermatozoa with coiled tails, at least in some cases, may be considered to be artifacts, as the proportion of these types of abnormalities may be influenced by the method of treatment, the semen of individual males varies with respect to its resistance to those treatments. Finally, this study emphasizes the necessity of examining more than one sample of semen from a male when the proportion of morphologically abnormal spermatozoa alone is used as the criterion of fertility.

10. **The Effect of Various Diluters, Cooling Rate, Temperature Storage, and Some Other Factors, on the Livability of Spermatozoa in Stored Samples of Bull Semen.** E.L. Willett and G.W. Salisbury

This memoir includes data on semen collection, different types of diluters, cooling, storing and warming semen. Considerable attention is devoted to pH. The egg yolk-citrate diluent is recommended and a pH range of diluter from 6.5 to 7.0 did not differentially affect sperm survival. Cooling at a rate of 5°C per 20 minutes is recommended. Covering the semen with a layer of mineral oil prolonged sperm survival. Very rapid warming of semen (30°C/minute) was not harmful. Buffer coefficient

and neutralization curves are presented for semen from bull, man, stallion and dog.

11. **Rapid Methods for Estimating the Number of Spermatozoa in Bull Semen**. G.W. Salisbury, G.H. Beck, I. Elliott and E.L. Willett

The number of spermatozoa in bull semen may be estimated by means of the hemocytometer, the photoelectric colorimeter, and the visual comparison with opacity standards. The hemocytometer served as the basis of comparison for the other methods. It was shown that the concentration was determined in the colorimeter with an accuracy equal to that obtained with the hemocytometer. The visual comparison of diluted semen with opacity standards results in an estimate of concentration only slightly less accurate than that obtained with the other two methods. Directions are given for preparing opacity standards and for calibrating the standard tubes.

12. **Rapid Methods for Estimating the Quality of Bull Semen**. G.H. Beck and G.W. Salisbury

The decrease in motility of the spermatozoa in bull semen samples, diluted with yolk-citrate and stored 10 days at 5 °C after having been brought to storage temperature in steps of a 5 °C drop each 10 minutes, was positively and significantly correlated with the decrease in motility for similar samples stored in water baths for one hour at 46.5 °C, for 45 minutes at 47.0 °C, and for 30 minutes at 47.5 °C. The correlation coefficients were 0.9088, 0.8979, and 0.6731, respectively. Such an incubation test was suggested for use in determining relative livability of the spermatozoa in semen samples under the same and different handling techniques. A test for quality of semen in which the rate at which semen diluted with yolk-citrate diluent will reduce a dilute solution of methylene blue was suggested for use, especially by operators of artificial breeding circuits. The quantitative relationships between this test and several factors affecting it were presented. Under standard conditions the test was shown to be largely dependent upon the concentration of spermatozoa, the motility of the spermatozoa, and the concentration of ascorbic acid in the semen. The two tests may be combined for each semen sample. Thus information may be rapidly obtained on initial motility, duration of motility, and relative metabolic rate as influenced mainly by spermatozoan concentration and motility, and ascorbic acid content of the semen. All of these factors now appear to be important as indications of the potential fertilizing capacity of semen.

13. **The Effect of Dilution Rate on the Livability and the Fertility of Bull Spermatozoa Used for Artificial Insemination**. G.W. Salisbury, G.H. Beck, P.T. Cupps and I. Elliott

Bull spermatozoa diluted with yolk-citrate diluent showed a decreased ability to maintain their motility during storage at 5 °C with each increase in rate of dilution. Dilution rates of from 1 part of semen to 1 part of diluter, up to 1 part of semen to 32 parts of diluter were used. Semen possessing higher numbers of spermatozoa, a large percentage of which were actively motile on initial examination, survived storage at the higher dilutions better than did semen of lower spermatozoa count and lower initial motility. In field studies, when the semen was diluted at a predetermined rate based on the spermatozoa count and the percentage of motile spermatozoa in each semen sample, no differences were found in fertility between semen diluted at the rates of 1 part of semen to 2 parts of diluter up to 1 part of semen to 14 parts of diluter. Over 3900 inseminations were involved. In an investigation designed to eliminate arbitrary selection of the rate of dilution to be used for any satisfactory semen sample, no difference was found in fertility for semen diluted at the rates of 1 part of semen to 4, 6, 8, 10, 12 or 16 parts of diluter. Semen at all dilution rates was used for as long as 6 to 8 days after collection. No observable differences were noted in the maintenance of fertility for the several dilution rates during the first 4 days of storage. For diluted semen used longer than this period, the number of inseminations was too few on which to base conclusions.

14. **The Bacteriology of Bull Semen. II. The Effect of Bacteria Upon Rapid Tests for Semen Quality.**
I.C. Gunsalus, J.J.R. Campbell, G.H. Beck and G.W. Salisbury

When sanitary precautions are observed, the number of bacteria found in freshly drawn semen or freshly prepared yolk-citrate diluent is not sufficient to interfere with the methylene blue reduction test for semen quality. The short-time-high-temperature incubation test for semen quality may kill up to 50 percent of the bacteria present when the test is run at 45°C or above in the presence of methylene blue. A temperature of 46.5°C is recommended. The methylene blue reduction test is not recommended as a criterion of the quality of semen stored more than 2 days. The short-time-high-temperature incubation test is recommended as a criterion of continued livability of the spermatozoa in stored semen samples. Though the bacterial population of stored samples can be controlled by proper precautions, factors other than the number of bacteria, and the activity and concentration of the spermatozoa are involved in the methylene blue reduction rate of stored samples.

15. **A Controlled Experiment in Feeding Wheat Germ Oil as a Supplement to the Normal Ration of Bulls Used for Artificial Insemination.** G.W. Salisbury

Two comparable groups of 10 bulls each were selected. Both were fed a practical ration made up of common, natural feeds which supplied plenty of vitamin E for normal reproduction of rats. To determine whether or not additional vitamin E in the form of solvent process wheat germ oil would benefit the reproductive performance of bulls used extensively for artificial insemination, each of the bulls in one group received one ounce daily of the wheat germ oil during an experimental period of one year. Over 1,250 semen samples were collected from the 20 bulls, and over 8,200 cows were artificially inseminated during the experiment. A comparison of the results from the two groups warrants the following statements: The feeding of one ounce daily of solvent process wheat germ oil in addition to the normal ration did not: 1) Increase the volume of semen produced by the bulls; 2) Increase the spermatozoa concentration in the semen; 3) Improve the motility of the spermatozoa; 4) Shorten the time required for service; 5) Decrease the number of semen ejaculates which were discarded as being of too poor quality for use in artificial insemination; 6) Improve the fertility of the bulls to which it was fed; 7) Nor prevent two bulls from decreasing in fertility to such low levels as to force their withdrawal from use in artificial insemination. A study of the seasonal effects showed a highly significant decrease in percentage of motile spermatozoa during the early spring months and a highly significant difference between months in spermatozoa count. The lowest average count was found in August, but there was no significant difference in fertility of the bulls from month to month.

16. **The Concentration of Some B Vitamins in Bull Semen.**
N.L. VanDemark and G.W. Salisbury

The thiamine, riboflavin, pantothenic acid, and niacin content of fresh bull semen were found to be 0.89, 2.09, 3.71, and 3.63 γ per cc., respectively. The spermatozoan count was correlated with the concentration of all four vitamins. Initial sperm motility was correlated with the concentrations of thiamine, riboflavin, and niacin.

17. **The Methylene-Blue Reduction Test and its Relation to Other Measures of Quality in Bull Semen.**
N.L. VanDemark, E. Mercier and G.W. Salisbury

The relationship of the methylene-blue test to other criteria of measuring semen quality is shown by correlation coefficients. In the samples of this study the methylene-blue test was not significantly correlated with the proportion of morphologically abnormal spermatozoa, nor with ascorbic acid content or the initial glucose level of fresh semen, nor with the glucose loss in diluted semen after 10 days' storage at 5°C. Statistically highly significant correlations were shown between the methylene-blue reduction time and volume of the ejaculate, spermatozoa count, initial spermatozoa motility, initial pH, and the initial lactic acid level of fresh semen.

Equally significant associations were shown in diluted semen with the methylene-blue reduction time by glucose loss, by lactic acid gain, and by livability after an hour's incubation at 46.5°C, and by lactic acid gain and by livability after 10 days' storage at 5°C. The methylene-blue reduction test, along with spermatozoa count and initial motility estimates, is recommended for routine prediction of semen quality.

18. **Further Studies of the Effect of Dilution Rate on the Fertility of Bull Semen Used for Artificial Insemination.** G.W. Salisbury, I. Elliott and N.L. VanDemark

In a study involving 3,296 inseminations, dilution rates of 1 part of semen to 8, 12, 16, 24 and 50 parts of yolk-citrate diluter were compared. The experiment was designed so as to eliminate all arbitrary selection of the semen to be used at any dilution rate. On the average, the following number of spermatozoa were contained in the 1 ml of diluted semen used for each insemination: 150, 104, 80, 54 and 26 millions. No statistically significant differences were found in fertility of the semen for the 5 dilution rates compared. The semen diluted at the highest rate was not used as efficiently as the semen diluted at the lower rates, but, on the average, seemed to maintain its fertility as well for 4 days after collection.

19. **The Reliability of Estimates of the Proportion of Morphologically Abnormal Spermatozoa in Bull Semen.** G.W. Salisbury and E. Mercier

An experiment was conducted to determine how many spermatozoa must be examined on a single bull semen smear to obtain reliable data on the proportion of morphologically abnormal spermatozoa found on that smear. Five hundred spermatozoa on each smear were examined and classified as to structure. The data were recorded in units of 100 spermatozoa. The results showed that the proportion of abnormally-formed spermatozoa calculated on the basis of the examination of a single unit of 100 spermatozoa was as reliable as the ability of a single investigator to make two slides from the same ejaculate of semen and to examine 500 cells on each one. Also, the data showed that improvement in the reliability of making the morphological classification of spermatozoa was rapid as an investigator gained experience.

20. **Stimulation of Livability and Glycolysis by Additions of Glucose to the Egg Yolk-Citrate Diluent for Ejaculated Bovine Spermatozoa.** G.W. Salisbury and N.L. VanDemark

In an investigation to determine the effect of adding from 58 to 116 mgm of glucose per 100 ml to bovine semen diluted at the rate of 1 part of semen to 4 parts of the yolk-citrate diluent and incubated for 1 hour at 46.5°C or stored for 10 days at 5°C, it was found that: 1) The added glucose promoted increased livability and lactic acid production during incubation for 1 hour at 46.5°C and during storage for 10 days at 5°C. 2) The stimulation of motility duration and lactic acid production occurred in spite of the fact that the initial glucose stores of the diluted semen samples were not depleted. 3) The glucose loss during low-temperature storage in the three separate experiments proceeded at similar rates regardless of the quality of semen used. 4) Glucose loss was not directly related to the livability of the spermatozoa unless that sugar was glycolyzed to lactic acid. 5) The proportion of glucose loss recovered as lactic acid was dependent upon storage interval and upon the quality of the semen used in the separate experiments and varied from less than 25 per cent to complete recovery.

21. **Livability and Glycolysis of Bovine Spermatozoa in Yolk-Citrate, Incubated Eggs or Chick-Embryo Diluters.** G.W. Salisbury, J.A. Zelaya and N.L. VanDemark

Two different experiments were conducted to determine the value of a chick-embryo diluter as compared to the yolk-citrate. The chick-embryo diluent (C.E. I) made from whole-fertile-eggs incubated 9 to 11 days was only slightly superior in maintaining livability of the spermatozoa during low-temperature storage to the yolk-citrate. The difference was observed largely with the semen

samples of poorest original quality, which ordinarily would not be used in commercial artificial insemination. In a limited experiment with semen of better average quality no difference was noted in spermatozoan livability during storage between yolk-citrate, chick-embryo made from whole-fertile-incubated eggs, and a diluter made from the embryos only. The semen samples in the check-embryo I diluent produced slightly more lactic acid during storage than did the same semen samples in the yolk-citrate. Also, a larger proportion of the glucose lost on storage was recovered as lactic acid in the chick-embryo diluents. This fact suggests that the chick-embryo material aided in the proportion of glycolysis by the spermatozoa.

22. The Estimation of the Weight of Bulls from Heart Girth Measurements.

C. Branton and G.W. Salisbury

A study was made of the relationship between heart girth measurement and the body weight of 25 Holstein and 25 Guernsey bulls ranging in weight from 852 to 2351 pounds. The coefficients of correlation between heart girth measurement and body weight were 0.976 for all of the 50 bulls, 0.954 for the Holstein bulls, and 0.958 for the Guernsey bulls; all were highly significant statistically. A calculation of the regression of body weight on heart girth measurements indicated a straight line relationship. The error of estimate of body weight based on heart girth measurement was 83.8 pounds or 5.05 per cent. Estimates of the body weights of bulls were, therefore, very accurate. It was also of interest to note that the correlation coefficient between body weight and semen volume was 0.2079, which was not mathematically significant.

23. The Effect of Sulfanilamide Upon the Livability and Metabolism of Bovine Spermatozoa.

C.B. Knodt and G.W. Salisbury

The addition of 300 mg of sulfanilamide per 100 ml of yolk-citrate diluent, gave a significant improvement in the livability of ejaculated bull spermatozoa over a 20-days-storage period and prevented bacterial growth. Sulfanilamide depressed glucose and oxygen utilization at all concentrations studied. The accumulation of lactic acid was increased both absolutely and in relation to the glucose utilized in the presence of sulfanilamide.

24. The Influence of Age on Breeding Efficiency of Dairy Cattle in Artificial Insemination.

T. Tanabe and G.W. Salisbury

Since the beginning of the New York Artificial Breeders' Cooperative in June, 1940, until June 30, 1944, a total of 12,621 complete, recorded services to registered Holstein-Friesian cows, involving 41 bulls, have accumulated. This number is 22.4 per cent of all matings to Holstein-Friesian bulls. The average number of services required per conception when based on all females, infertile cows included, was 2.07, or a breeding efficiency of 48.2 percent. The influence of the age of the cow on breeding efficiency reveals a steady increase in the conception rate up to four years of age. Between the ages of five to seven years, inclusive, cows maintain a uniformly high breeding efficiency, which gradually declines with advancing age. Although the full effect of the age of the sire could not be obtained with a group of bulls selected for fertility, young bulls between the ages of one to three years, inclusive, have shown the highest breeding efficiency of all age groups. Within this group the peak in conception rates was found for the two-year-old bulls.

25. Fertility of Bull Semen Diluted at 1:100. G.W. Salisbury

In an investigation involving 5,765 inseminations no difference in fertility was found between dilution rates of 1:40, 1:60, 1:80, or 1 part of bull semen to 100 parts of the egg yolk-citrate diluent. At the highest rate of dilution on the average 12,836,000 spermatozoa were introduced in each 1.0 ml of diluted semen used for insemination.

26. The Effects of Season on the Spermatogenic Activity and Fertility of Dairy Bulls Used in Artificial Insemination.

E. Mercier and G.W. Salisbury

Five Holstein-Friesian and five Guernsey bulls were used for one year in artificial insemination.

Eight different semen characteristics were studied for most ejaculates. The variation among the bulls in these semen characteristics was marked. Also, important month-to-month differences were noted for most semen characteristics. A total of 328 ejaculates were used for 20,689 inseminations, of which 58.2 per cent were apparently successful. The bulls varied in fertility, and highly significant month-to-month variations in fertility were observed. When the data for the ejaculates of the two breeds were combined, the semen characteristics correlated with fertility were concentration of spermatozoa, percentage of abnormal spermatozoa, and methylene blue reduction time. The correlation coefficients, though mathematically significant, were small. In spite of a highly significant breed difference in the proportion of morphologically abnormal spermatozoa, the variations in semen quality noted between the Holstein and Guernsey bulls are believed to be insufficient to explain all the difference in fertility between these breeds.

27. The Relation of Certain Objective Measurements to Weights of Beef Cattle.

J.J. Wanderstock and G.W. Salisbury

A study was made of the relationship between the heart girth measurement and the body weight of 100 Aberdeen-Angus and 45 Hereford good-to-choice fat yearling steers ranging in weight from 640 to 1,150 pounds. Also, data were obtained on height at withers, patella or round, and body length for 36 of the Aberdeen-Angus steers and for 30 of the Hereford Steers. Heart girth measurements and body weights were also obtained on 27 Aberdeen-Angus heifers and cows of the University herd, ranging in age from two to twelve years. The coefficients of correlation between heart girth and body weight were 0.91 for the Hereford steers, 0.89 for the Aberdeen-Angus steers, 0.88 for all steers, and 0.93 for the heifers and cows, all highly significant statistically. A calculation of the regression of the logarithms of actual live weight on the logarithms of heart girth measurements, indicating a straight line relationship, showed that the Aberdeen-Angus steers were lighter than the Hereford steers in relation to their heart girth measurements. The regression line for the heifers and cows indicated that they were heavier than the steers in relation to their heart girth measurements, especially at the larger tape measurements.

28. The Glycolysis, Livability, and Fertility of Bovine Spermatozoa as Influenced by Their Concentration.

G.W. Salisbury

Bull spermatozoa diluted with yolk-citrate decreased in livability during storage at 5°C with each increase in dilution. Dilution rates varying from 1:2 up to 1 part of semen to 100 parts of yolk-citrate have had no influence on the fertility of bull semen stored for a limited period of about 4 days. Apparently minimum fertility threshold values of activity were not reached in this period of storage. Dilution does not depress glycolysis as determined by lactic acid production in yolk-citrate or in yolk-citrate plus glucose. The apparent glucose utilization is much higher relatively for the diluted semen than for the more concentrated material. The percentage of glucose utilized which is recovered as lactic acid is directly related to the concentration. The oxygen consumed by washed spermatozoa is markedly effected by the concentration. Here again livability is poorest at the low concentrations. This suggests that bull spermatozoa in low concentrations are harmed by oxygen. Sulfanilamide not only controls bacteria in diluted semen, but depresses general oxidative mechanisms. It promotes livability, promotes lactic acid production and appears to limit glucose utilization to glycolytic pathways.

29. Effect of Techniques of Preparing Semen Smears for Staining on the Morphology of Bull Spermatozoa.

E. Mercier and G.W. Salisbury

In three experiments conducted to determine the effects of the methods used in making, fixing, and clearing smears and the effect of shaking semen on the proportion of tailless heads and on the proportion of abnormally-formed spermatozoa in smears made with fresh and stored semen of fertile bulls it was found that: 1) The "pulling" and "drop" methods of making semen smears gave the same results when the smears were of uniform thickness. 2) Fixing of smears by heat or by albumin did not influence the proportion of tailless heads or true abnormals.

3) Clearing of smears with 1.0 percent chlorazene was responsible for producing most of the tailless heads and increased the proportion of true abnormals. Normally-formed tailless heads may be artifacts and, consequently, should be included among the true abnormals only when the staining procedures do not produce them. 4) Spermatozoa in fresh semen were much more fragile and more subject to breakage during the clearing process than stored spermatozoa. 5) With the semen of fertile bulls the staining of thin, uncleared smears eliminated most of the tailless heads. This method of preparation of smears for staining should eliminate the tailless heads which are artifacts, and should enable the investigator to distinguish those tailless heads which are truly abnormal.

30. Morphology of Spermatozoa from Different Levels of the Reproductive Tract of the Bull.

C. Branton and G.W. Salisbury

Studies were made on the morphological characteristics of spermatozoa from the reproductive tracts of 21 bulls of different ages. Fertility of only 3 of these bulls was known. Information on the source of morphologically abnormal spermatozoa and the relative proportions of morphologically abnormal spermatozoa, excluding tailless spermatozoa, and spermatozoa with attached protoplasmic droplets was secured. It was found that the various levels of the reproductive tract of the bull did not differ significantly in the proportions of abnormal spermatozoa and that the predominant types of abnormals were those affecting the heads of the spermatozoa. It was, therefore, concluded that the testis is the original source of morphologically abnormal spermatozoa. All bulls studied had three types of spermatozoa as to the location of the protoplasmic droplet. There was no significant statistical difference between bulls as to the proportion of spermatozoa with attached droplets at any given level of the tract. However, the droplet was usually located on the neck of spermatozoa from the head of the epididymis, and was usually absent from ampullar spermatozoa. It was concluded that these were morphological stages in the development of the spermatozoa and that all normal bulls show the same stages.

31. The Effect of Sulfanilamide in the Diluent Upon Fertility of Bull Semen.

G.W. Salisbury and C.B. Knodt

Three separate experiments involving a total of 8,498 inseminations were conducted to determine the effect on fertility of bull semen of adding sulfanilamide to the yolk-citrate diluent at the rate of 300 mg per 100 ml. In the first experiment no benefit was observed. In the next two investigations, where the citrate buffer containing sulfanilamide was protected from direct light rays, an increase in fertility by use of the sulfanilamide was obtained. This improvement amounted to 6.1 per cent of the cows inseminated in the second experiment and 4.5 per cent in the third. The sulfanilamide appeared to influence all semen samples in the same direction, for, in the third experiment, no significant interactions were observed between the treatments and either bulls or first and second ejaculates. These results are interpreted as indicating that the beneficial effects of sulfanilamide on fertility largely are metabolic ones, rather than due to bacterial control alone.

32. Seasonal Variations in Hours of Daylight Associated with Fertility Level of Cattle Under Natural Breeding Conditions.

E. Mercier and G.W. Salisbury

In a study of three herds of cattle located at different latitudes in Eastern Canada, the lowest per cent of successful services was obtained during winter and spring and the highest during summer and fall. The differences in fertility level between herds were not significant statistically but those between seasons were significant at the 5 per cent level of probability. The average monthly conception rate of the three herds was significantly correlated with the monthly average length of daylight, there being a lag of approximately 1 to 2 months before the maximum effect was reached. Temperature changes had no measurable direct effect on fertility level under the existing conditions. The authors believe that at high latitudes variations in length of daylight measurably influence the fertility level in cattle.

33. **Fertility Level in Artificial Breeding Associated with Season, Hours of Daylight, and the Age of Cattle.** E. Mercier and G.W. Salisbury

In an investigation on the seasonal variations in fertility level of about 125,000 cows and 71 bulls of various ages bred artificially within the last 6 years in New York State, it was found that: 1) With respect to fertility level, cattle of various ages responded differently and consistently to seasons of the year, the younger and older cattle being influenced more readily than mature cattle. 2) Winter was the poorest breeding season of the year during this period. 3) The fertility level of all bulls kept at this Station but artificially bred to cattle in all sections of the State from December, 1945, through November, 1946, was significantly correlated with the length of daylight, there being a lag of 1 or 2 months before the effect of daylight reached its maximum. For two groups of bulls less than 6 years of age, the correlation coefficients were not statistically significant. Thus, young bulls probably are more subject to other extraneous influences on fertility than are those from 6 to 10 years of age. It is concluded that variations in the hours of daylight influence the fertility level of dairy cattle at this latitude, and that the response to light varies with the age of the animals involved.

34. **Total Digestible Nutrients and Protein Levels for Dairy Bulls Used in Artificial Breeding.** C. Branton, R.W. Bratton and G.W. Salisbury

Using 18 bulls, 9 Holstein-Friesians and 9 Guernseys, studies were conducted relating measurable semen characteristics, relative fertility, and body weight changes to T.D.N. intake levels of approximately 100, 120, and 140 per cent of recommended maintenance requirements for dry dairy cows of equivalent weights fed simultaneously with concentrate mixtures containing 12, 16, and 20 per cent total protein. These relatively wide ranges of T.D.N. intake, accompanied by considerable shifts in body weight, can be fed mature bulls for at least 90 days without influencing semen production or fertility. Under the conditions of this experiment, 12 per cent of protein in the concentrate mixture fed with mixed hay, containing approximately 10 per cent of legumes, supplied enough protein for semen production and the maintenance of fertility. Based on the results of this experiment, a tentative feeding standard for mature dairy bulls used routinely in artificial insemination is proposed. In terms a of a practical feeding schedule, such a standard reduces itself to approximately 1 lb of hay and 0.4 to 0.5 lb. concentrate mixture daily per 100 lb. of body weight.

35. **The Freezing Point Depression of Bull Semen and its Relation to the Diluter Problem.** G.W. Salisbury, C.B. Knodt and R.W. Bratton

From cryoscopic data it was found that fresh, normal bull semen has an osmotic pressure approaching that of cattle blood. The osmotic pressure of bull semen increased with length of storage in the excurrent ducts of the male or outside the body. The semen of two low fertility bulls depressed the freezing point more than did that of normal, fertile bulls. Heating sodium citrate solutions increases their osmotic pressure. For heated solutions, 2.9 grams of $Na_3C_6H_5O_7 \cdot 2H_2O$ per 100 ml of water distilled in glass is isotonic with blood.

36. **The Effect of Pyridium, Penicillin, Furacin, and Phenoxethol Upon the Livability of Spermatozoa and Upon the Control of Bacteria in Diluted Bull Semen.** R.H. Foote and G.W. Salisbury

The possible usefulness of pyridium was limited by its low solubility. Maximum attainable concentrations produced no noticeable effects on spermatozoan livability or on bacterial growth. Two commercial penicillins were equally effective in controlling bacterial growth and were slightly superior to sulfanilamide in this respect. Neither penicillin proved to be beneficial to the spermatozoa, and one in particular was toxic even when present in small amounts. Furacin and phenoxethol proved to be highly bactericidal, and at the same time were spermicidal at most of the levels tested. Organisms of the pseudomonas group, especially, were resistant to the lower concentrations studied. Throughout these experiments only sulfanilamide, the positive

control, actually improved livability of the spermatozoa.

37. The Effect of Sulfonamides Upon the Livability of Spermatozoa and Upon the Control of Bacteria in Diluted Bull Semen.

R.H. Foote and G.W. Salisbury

Twelve sulfonamides were added to bull semen diluted with citrate-phosphate and stored at 20°C. At the level of each drug determined to be optimum for spermatozoan survival, nine of the 12 drugs increased the livability of the spermatozoa over that observed when no sulfonamide was added to the diluent. Of these nine, only two sulfonamides, sodium sulfamethazine and carboxysulfathiazole, were significantly superior to sulfanilamide in maintaining motility of the sperm cells, but they were inferior in controlling bacterial growth. Sulfanilamide slightly decreased the rate of motility, and N^1-benzoylsulfanilamide exerted a similar but more pronounced effect. No consistent pattern was established by the other sulfonamides. At 20 and 37.5°C all of the sulfonamides were effective in reducing bacterial growth at levels which were not harmful to the spermatozoa. *Pseudomonas pyocyaneus* was not inhibited at these levels. At 5°C the sulfonamides were only slightly effective in controlling bacterial growth because the pseudomonas group of organisms predominated in the bacterial flora surviving at this temperature. Combinations of sulfonamides and other antibacterial agents may control fastidious organisms.

38. Breeding Behavior, Spermatogenesis, and Semen Production of Mature Dairy Bulls Fed Rations Low in Carotene.

R.W. Bratton, G.W. Salisbury, T. Tanabe, C. Branton, E. Mercier and J.K. Loosli.

Six dairy bulls were fed dry roughages low in carotene and a concentrate deficient in carotene and vitamin A for a period of 16 months without inducing clinical manifestations of vitamin A deficiency or noticeable impairment of semen production. Subsequent changes in the roughage component of the ration of three of the bulls from hay and/or straw to dried beet pulp plus the same concentrate mixture resulted in the development of incoordination, edema of the extremities, papillary hemorrhage, a gradual increase in the per cent of abnormal spermatozoa and a decrease in the per cent of motile spermatozoa, but no consistent change in the number of spermatozoa per milliliter of semen within a period of 40 to 120 days. These three bulls were unable to mount but still retained an unusual amount of libido. This inability to mount was manifested before the changes in semen production. Typical patterns of degeneration of the germinal epithelium of the seminiferous tubules were found in the three bulls on the carotene-deficient rations. There were few spermatogonia, spermatocytes, spermatids or maturing spermatozoa in the lumen of the tubules. While supplementing the carotene-deficient ration of the other three bulls with carotene in oil prevented the degeneration of the germinal epithelium, which characterized those bulls not receiving the supplement of carotene in oil, it produced no consistent changes in semen production which reasonably could be attributed to the carotene. Liver carotene and vitamin A levels of the carotene-deficient bulls were of the order of 30 to 50 γ and 5 to 13 γ per g. of fresh liver, respectively.

39. Fertility Level of Bull Semen Diluted at 1:400 with and Without Sulfanilamide.

G.W. Salisbury and R.W. Bratton

In one investigation it was found that the practical limit of dilution rate was about 1:100 when the yolk-citrate diluter was used. In another experiment involving 7,343 inseminations when sulfanilamide was added to the yolk-citrate diluter at the rate of 300 mg per 100 ml., no difference was found in fertility level between dilution rates of one part of semen to 100, 150, 200, 300 and 400 parts of the yolk-citrate-sulfanilamide diluter. However, a trend downwards amounting to 0.8 per cent in fertility level for each decrease of 1 million spermatozoa inseminated was observed, over the range of 2.36 to 15.30 millions of spermatozoa inseminated. The probable reasons for the different results of the two experiments are discussed. With present handling and insemination techniques, it is suggested that the minimum number of spermatozoa consistent with

optimum fertility rests at 5 to 10 millions from bulls of known fertility.

40. Semen Production and Fertility of Dairy Bulls as Related to Frequency of Ejaculation.

E. Mercier, R.W. Bratton and G.W. Salisbury

Three groups of four bulls each were ejaculated according to the following schedule during a period of 216 consecutive days: one ejaculate every six days; two ejaculates, taken within a few minutes of each other, at 12-day intervals; and three ejaculates, taken within a few minutes of each other, at 18-day intervals. The volume of semen per ejaculate averaged 7.9, 6.6, and 5.3 ml for the 6-, 12- and 18-day intervals respectively. Differences in the group means for sperm concentration, percentage of motile spermatozoa, and their rate of motility and methylene blue reduction time were too small to have important practical consequences separately. However, when considered together and along with volume as a basis for selecting ejaculates for use, approximately 45 percent more shippable semen was produced by those bulls ejaculated at six-day intervals than from those ejaculated less frequently. From these data it appears reasonable to conclude that the six-day interval between ejaculations is not detrimental to spermatogenic activity as judged by these criteria. The fertility of the semen from the bulls ejaculated at 12-day intervals was significantly higher than that from the bulls in either of the other two groups. However, the differences observed were believed to reflect unfortunate groupings of the bulls rather than true treatment effects.

41. Semen Production and Fertility of Dairy Bulls Fed Rations Containing Proteins of Plant and Animal Origin.

C. Branton, R.W. Bratton and G.W. Salisbury

Using 18 bulls, 11 Holstein-Friesians, 6 Guernseys and 1 Ayrshire, studies were conducted relating differences in measurable semen characteristics and relative fertility to concentrate mixtures containing corn gluten feed, skim milk powder or soybean oil meal as the protein supplement when fed with timothy hay as the only roughage. The results, as judged by the averages for volume of semen per ejaculate, per cent of motile spermatozoa and their rates of motility, the number of spermatozoa per mm^3 of semen, the methylene blue reduction time and the per cent of usable samples during a 120-day period, showed that corn gluten feed, skim milk powder and soybean oil meal were approximately equal in value as protein supplements in the concentrate mixture. Based on 60 to 90 days non-returns to first service cows, the average fertility levels of the semen produced when the bulls were fed corn gluten feed, skim milk powder and soybean oil meal were 63.5, 61.6 and 65.7 per cent, respectively. While the average per cent non-returns was significantly higher during the periods when soybean oil meal was fed as compared to the periods when skim milk powder was fed, the difference was relatively small and it is doubtful wither any real advantage lies with the soybean oil meal. In these studies, animal protein was not superior to the plant proteins. The monetary economy of the particular plant protein sources used in these trials was much greater than for the animal protein source. A T.D.N. intake of 110 percent of Morrison's recommended requirements for the maintenance of dry dairy cows of equivalent weight resulted in consistent body weight increases for all bulls during the first 240 days but consistent decreases during the last 120 days of the experiment. While the decreases occurred during the summer and early fall months, it was not definitely established whether the observed decreases represented a true cause and effect relationship.

42. Damage to Bull Spermatozoa and its Prevention by Catalase.

N.L. VanDemark, G.W. Salisbury and R.W. Bratton

Bovine spermatozoan activity was studied in the egg yolk-citrate diluent during incubation at 46.5°C and storage at 5°C under air, oxygen and nitrogen, under air and oxygen with and without added catalase, and during storage when subjected to minimum and routine mixing procedures with and without added catalase. Oxygen produced deleterious effects which were manifested by decreased motility and livability of the spermatozoa and a reduction in their ability to convert sugar to lactic acid. Oxygen damage to spermatozoa was largely eliminated when catalase was added to the diluted

semen. Gentle mixing shortened the life of stored bull spermatozoa in the yolk-citrate diluent, but the presence of added catalase in mixed samples obliterated the harmful effects of mixing. The effectiveness of catalase in preventing the harmful action of oxygen and shaking on bull spermatozoa in the egg yolk-citrate diluent led to the conclusion that increased aeration and higher oxygen tensions speed the production of hydrogen peroxide by the spermatozoa and this in turn produces a toxic environment, shortening the life of the spermatozoa. The results suggest that a minimum of air space be left above the diluted semen that is stored and shipped in routine artificial breeding and than semen samples should be subjected to a minimum of mixing.

43. Livability and Fertility of Bovine Spermatozoa in Different Diluents.

R.W. Bratton, R.H. Foote, S.D. Musgrave and N.L. VanDemark

By means of a 6 × 6 latin square design, six bovine semen diluents were compared. Sixty semen samples from ten Holstein bulls were subdivided into six portions and each portion diluted at a rate of 1:200 with one of the six diluents. Based on the per cent 60- to 90-day non-returns to approximately 1850 first service cows per diluent, the mean fertility level for each diluent was as follows: phosphate-yolk, 50.5, 3.6 citrate-yolk, 50.5; 3.6 citrate-sulfanilamide-yolk, 55.3; 2.9 citrate-sulfanilamide-yolk, 56.5; Ortho tablet-yolk, 56.4; and Ortho liquid, 55.0 The average non-returns for the sulfonamide-containing diluents (3.6 citrate-sulfanilamide-yolk, 2.9 citrate-sulfanilamide-yolk, Ortho tablet-yolk and Ortho liquid) was 5 percentage units higher than for those diluents not containing sulfonamides (3.6 citrate-yolk and phosphate-yolk). This difference was significant at the 1 per cent level of probability. Livability of spermatozoa during storage at 5 °C was satisfactory for all six diluents.

44. The Relation of Pedigree Promise to Performance of Proved Holstein-Friesian Bulls.

F.E. Eldridge and G.W. Salisbury

The average butterfat production of the daughters of 207 proved Holstein-Friesian bulls was studied in relation to the average butterfat production of the mates of these bulls and that of their direct and collateral female relatives. Two series of multiple regression equations were calculated depending on the records of production available: A for 158 bulls, B for 141 bulls, of which 92 bulls were common to each group. A multiple correlation coefficient of approximately + 0.37 was found in both cases between the direct pedigree estimate and average production of the daughters of the bulls, accounting for approximately 14 per cent of the variance among the daughter groups in their butterfat production. When average butterfat yield of the bulls' mates (the dams of the daughters) was added to the information from the pedigrees the correlation coefficients were approximately +0.7 and accounted for from 47.5 to 49 per cent of the variance between the butterfat averages of the daughters of the bulls. Non-genetic correlations, presumably due to environmental similarities between herds, contributed to the relationships found. The study indicates that for selection of young dairy bulls, records of performance of females in their pedigrees are of importance in the following order: (a) the average production of the paternal half-sisters of the bull, (b) the average production of the dams of the paternal half-sisters of the bull, and © the average production of the paternal half-sisters of the bull's dam. The average production of the bull's own dam or of his maternal half-sisters showed no relationship to the average production of his daughters. However, since the dams of the bulls were a highly selected group, the only general conclusion that could be made was that among bulls whose dams average above 450 lb. of butterfat, 2X, M.E., D.H.I.A. conditions, the differences between the records of such dams are of little significance.

45. The Fertility of Bovine Semen Cooled With and Without the Addition of Citrate-Sulfanilamide-Yolk Extender.

R.H. Foote and R.W. Bratton

Sixty-four ejaculates of bovine semen were divided and cooled from 30 °C to 5 °C in 75 minutes with and without the addition of citrate-sulfanilamide-yolk extender prior to cooling. Based on 60- to 90-day non-returns to 8,518 first and second service cows, the fertility level of the pre-extended semen (semen cooled in extender) was 59.3 percent and that of the post-extended semen

(cooled without extender), 52.8 per cent. The difference between treatments of 6.5 percentage units was highly significant statistically. Motility estimates made after 3, 24, 48, 72 and 96 hr of storage indicated that the samples cooled without extender had a definitely lower percentage of motile spermatozoa. However, by using covariance analysis the higher per cent non-returns for the pre-extended semen could not be accounted for on the basis of more motile spermatozoa per insemination.

46. The Curvilinearity of Heritability of Butterfat Production.

J.P. Beardsley, R.W. Bratton and G.W. Salisbury

A statistical study of butterfat records of the progeny and mates of 176 proved sires of the Guernsey, Holstein-Friesian, and Jersey breeds has been made. Each bull was represented by at least five daughter-dam comparisons in each of two or more herds. Curvilinear regression of daughter on dam within breeds, within sires and within herds accounted for a larger portion of the daughter variance than did linear regression. The difference, however, was not quite large enough to be statistically significant. The heritability of butterfat yield calculated by doubling the linear regression of daughter on dam within breeds, within sires and within herds was 27.4 per cent. Estimates of heritability on the basis of curvilinear regression gave values decreasing with increased butterfat yield. The pattern described by these estimates appears to hold a valid relation to the problems faced by breeders of dairy cattle and to the results of some experimental studies on similar problems.

47. Motility of Bovine Spermatozoa in Buffered Whole Egg.

H.O. Dunn and R.W. Bratton

Spermatozoan motility at 5 °C was as satisfactory in extenders composed of three parts of a 2.9 per cent sodium citrate dihydrate buffer containing 0.4 per cent of succinylsulfathiazole or 0.4 per cent succinylsulfanilamide and one part of liquid whole egg as in the standard 2.9 per cent citrate-sulfanilamide-yolk extender. The costs of the whole egg extenders were approximately 25 per cent of that of the yolk extender. The former could be prepared more quickly and glassware coming in contact with them was much easier to clean. The spermatozoa were more visible and more active in the whole egg extenders, thereby facilitating microscopic examinations.

48. Fertility and Motility of Bovine Spermatozoa in Buffered Whole Egg Extenders.

H.O. Dunn, R.W. Bratton and W.J. Collins

By means of the split sample technique, comparisons were made between the standard 2.9 and 3.6 per cent citrate-sulfanilamide-yolk extenders and whole egg extenders composed of three parts of either a 1.9 or a 2.9 per cent citrate buffer, containing 400 mg. per cent of succinylsulfathiazole and one part of blended liquid whole egg (yolk + albumen). On the basis of approximately 11,000 first- and second-service cows inseminated artificially with each of these two types of extenders, the 60- to 90-day non-returns averaged 62.3 per cent for the citrate-sulfanilamide-yolk formulae and 61.6 per cent for the citrate-succinylsulfathiazole-whole egg formulae. Spermatozoan motility was satisfactory in both types of extenders.

49. Motility of Spermatozoa and Control of Bacteria in Bovine Semen Extenders Containing Sulfanilamide, Polymyxin and Aureomycin.

R.H. Foote and R.W. Bratton

The effect of polymyxins D, B and E and aureomycin on the motility of bovine spermatozoa and upon the control of bacterial growth in bovine semen extended with citrate buffered yolk and stored at 5 °C was investigated. It was found that 2000 μg of polymyxins D, B or E, or 100 μg of aureomycin could be added per ml of extender without spermicidal effects. These levels of antibiotics were highly bacteriostatic and/or bactericidal. The polymyxins appear to be especially promising for use with other antibacterial agents in semen.

50. **The Fertility of Bovine Semen in Extenders Containing Sulfanilamide, Penicillin, Streptomycin and Polymyxin.** R.H. Foote and R.W. Bratton

By use of the split sample technique the fertility of bovine semen extended in 3.6 per cent citrate-yolk containing no antibacterial agent, sulfanilamide, penicillin, streptomycin, polymyxin or a combination of these four antibacterial agents was studied. Semen was used from bulls with histories of low fertility, as well as from bulls with histories of high fertility. The per cent 60- to 90-day non-returns to first and second service cows for the treatments, no antibacterial agent, sulfanilamide, penicillin, streptomycin, polymyxin, and sulfanilamide plus penicillin plus streptomycin plus polymyxin were, respectively, for the high fertility bulls, 63, 64, 70, 69, 68, and 67; for the low fertility bulls, 58, 60, 67, 66, 59, and 69 and for both the high fertility and the low fertility bulls combined, 61, 62, 68, 68, 64, and 68. On the bases of these results and those reported by other workers, it is concluded that the addition of penicillin, streptomycin or a combination of these plus polymyxin and sulfanilamide to present day extenders may be expected to increase the over-all fertility level of bovine semen used for artificial breeding.

51. **The Fertility of Bovine Semen in Citrate-Yolk Extenders Containing Added Catalase.** N.L. VanDemark, R.W. Bratton and R.H. Foote

Using the split sample technique, the spermatozoan livability and the fertility of 47 semen samples from 24 Holstein bulls were studied when extended to contain approximately 11.9×10^6 motile spermatozoa per milliliter in citrate-yolk and citrate-sulfanilamide-yolk extenders with and without added catalase. On the basis of the average per cent 60- to 90-day non-returns to service to approximately 1,700 first and second service cows per treatment, the extenders compared as follows: 3.6 citrate-yolk without added catalase, 61.4; 3.6 citrate-yolk with added catalase, 60.7; 3.6 citrate-sulfanilamide-yolk without added catalase, 62.2; and 3.6 citrate-sulfanilamide-yolk with added catalase, 61.7. Differences in spermatozoan livability and fertility in the various extenders were not significant.

52. **Motility of Bovine Spermatozoa and Control of Bacteria at 5 and 25°C in Extenders Containing Sulfanilamide, Penicillin, Streptomycin and Polymyxin.** R.H. Foote and R.W. Bratton

The feasibility of storing bovine semen at 25°C for use in artificial insemination to eliminate the expense of refrigerating the semen at 5°C was investigated. Sulfanilamide, penicillin, streptomycin, polymyxin and a combination of these were added to 3.6 percent citrate-yolk extender. The citrate-yolk extender containing no sulfanilamide or antibiotics served as the control. Eighteen semen samples were stored in each of the six extenders at 5°C and at 25°C. The per cent of motile spermatozoa after 24 hr of storage was lower when the semen was stored at 25°C than when it was stored at 5°C except in the extender containing the combination of antibacterial agents. In nearly all samples, this combination of sulfanilamide and antibiotics completely inhibited bacterial growth at both temperatures. This combination of antibacterial agents gives promise of making possible the development of an extender for bovine semen which will not require refrigeration.

53. **The Relationship of Semen Production to Sexual Excitement of Dairy Bulls.** W.J. Collins, R.W. Bratton and C.R. Henderson

In two experiments with 11 bulls each, restraining the bulls to induce sexual excitement was accompanied by highly significant ($P < 0.01$) increases in semen volume per ejaculate and in numbers of spermatozoa per milliliter of semen. The number of motile spermatozoa per ejaculate was 41 per cent greater ($P < 0.01$). These results emphasize the practical importance of procedures designed to induce and control sexual excitement in dairy bulls at the time of semen collection.

54. **The Bull as One Cause of Delayed Returns to Service in Artificial Breeding.** G.W. Salisbury, R.W. Bratton and R.H. Foote

The results of statistical studies indicate that the semen of bulls is one of the major sources of origin of the significant differences observed from bull to bull in the incidence of embryonic mortality, as measured by the difference between 1- and 5-month non-returns to service in artificial breeding. The level of embryonic mortality was negatively and highly significantly correlated with the original fertility level of bulls as measured by 1-month non-returns, the correlation coefficients being in the neighborhood of -0.6 to -0.7. A significant effect of the season of the year on apparent embryonic mortality was indicated, the maximum effect being produced from inseminations in January, February and March, the minimum being in May and June.

55. **The Effect of Time and Other Factors on the Non-Return to Service Estimate of Fertility Level in Artificial Insemination of Cattle.** G.W. Salisbury, R.W. Bratton and R.H. Foote

Evidence is presented indicating that in artificial insemination of cattle a significant proportion, varying up to 25 per cent, of the cows thought to be pregnant after one insemination, because they failed to show estrus during a time interval after insemination equivalent to two normal estrous cycles, actually returned for service at a later date. The difference between the percentage of cows that had not returned for reinsemination during 1 full month after the month of insemination (1-month non-returns) and those that did not return to service during the 4 subsequent months (5-month non-returns) was of the order of 12 to 15 percentage units. The magnitude of the difference varied with the age of the extended semen used for insemination and with experience in the artificial breeding program.

56. **The Influence of Antibiotics on Delayed Returns in Artificial Breeding.** R.H. Foote and R.W. Bratton

A study of 112,312 first-service cows bred artificially during an 8-month period immediately prior to the routine addition of 500 units each of penicillin and streptomycin per milliliter of citrate-sulfanilamide-yolk extender revealed that the 28- to 35-day, 60- to 90-day and 150- to 180-day per cent non-returns were 79.1, 64.1 and 60.3, respectively. The corresponding values for 233,354 first-service cows bred during a 17-month period immediately following the change were 82.5, 73.0 and 69.7. The smaller percentage of delayed returns when antibiotics were used is interpreted as indirect evidence for a marked decrease in embryonic mortalities associated with control of infectious agents in semen. Bulls' fertility (28- to 35-day per cent non-returns) before as well as after the use of antibiotics was negatively correlated with the percentage of delayed returns, indicating that the semen per se also is a source of origin of some of these delayed returns. The among-bull variance for non-returns to first-service cows was reduced to less than 10 per cent of its original value by the addition of the antibiotics, penicillin and streptomycin, to the citrate-sulfanilamide-yolk extender. When antibiotics were used, the 150- to 180-day non-return percentages were predicted nearly as accurately from the 28- to 35-day non-returns as from the 60- to 90-day non-returns.

57. **Fertility of Bovine Spermatozoa in Buffered Whole Egg Extenders Containing Penicillin, Streptomycin, Sulfonamides and Added Glucose.** H.O. Dunn, R.W. Bratton and C.R. Henderson

Three experiments were conducted, using the split sample technique, for comparing the fertility of bovine semen extended in 2.9 per cent citrate-sulfanilamide-yolk, 2.9 per cent citrate-succinyl-sulfathiazole-whole egg, and 2.3 per cent citrate-succinylsulfanilamide-whole egg-glucose. Each

extender contained 500 units each of penicillin and streptomycin. The average 60- to 90-day per cent non-returns to a total of approximately 5,000 first services per treatment was 71.5 for the yolk extenders containing antibiotics and 69.4 for the whole egg extenders containing antibiotics. From these results and those previously reported, it appears that the standard 2.9 per cent citrate-sulfanilamide-yolk extender is about 2.0 percentage units superior to the whole egg extenders when measured by the 60- to 90-day per cent non-returns to first services. Furthermore, it is concluded that together, penicillin and streptomycin have similar effects on fertility when they are added to either the yolk or the whole egg formula.

58. Effect of Three Levels of Nutrition on Sexual Development of Young Dairy Animals. R.W. Bratton

From the limited data obtained at Cornell and Penn State, feed intakes providing from 60 to 75 per cent of the total digestible nutrient allowance recommended for the normal growth and development of dairy heifers definitely delays the onset of semen production in young bulls. Feed intakes achieving 120 to 140 per cent of the normal total digestible nutrient allowance will bring young bulls into semen production at an earlier age than may be expected when they are fed normal allowances of total digestible nutrients. The fertility of sires raised on different levels of nutrient consumption is being studied, and initial results indicate that fertility will be unaffected.

59. The Relationship Between Fertility and the Number of Spermatozoa Inseminated. R.W. Bratton, R.H. Foote and C.R. Henderson

Three groups of approximately 4,100 first service dairy cows each were inseminated artificially with three different concentrations of motile spermatozoa, estimated to be 14.3×10^6, 9.5×10^6, and 4.7×10^6; the 60- to 90-day per cent nonreturns were 70.5, 70.9, and 66.7, respectively, $P < 0.05$. A graph is presented which depicts the relationship between sperm numbers and fertility.

60. Semen Production and Fertility of Dairy Bulls Ejaculated Either Once or Twice at Intervals of Either Four or Eight Days. R.W. Bratton and R.H. Foote

A group of 14 dairy bulls ejaculated once at 4-day intervals for 272 days averaged 5.7 billion motile spermatozoa per ejaculate. Another group of 16 bulls ejaculated once at 8-day intervals for 272 days averaged 7.0 billion motile spermatozoa per ejaculate. The average 60- to 90-day per cent nonreturns to more than 25,000 first services per group was 61.8 for the 4-day bulls and 64.8 for the 8-day bulls, a difference that was not statistically significant, $P > 0.05$. During an 88-day period immediately following the 272-day period the 8-day interval bulls were ejaculated twice every eighth day and averaged 5.7 billion motile spermatozoa per ejaculate and 12.8 billion per 8-day period. The 4-day interval bulls, continued on the original interval between ejaculates, averaged 12.6 billion motile spermatozoa on an 8-day basis during this same period. The 60- to 90-day per cent nonreturns to first services was 65.2 for the 4-day bulls and 67.9 for the 8-day bulls. Again, the difference was not statistically significant, $P > 0.05$. It is concluded that one ejaculation every fourth day or two ejaculations every eighth day for periods as long as 1 year are not detrimental to semen production or fertility and that ejaculations at a rate of two per 8-day interval will yield about 60% more motile sperm than will ejaculations at the rate of one per 8-day interval.

61. Semen Production and Fertility of Mature Dairy Bulls Ejaculated Either Once or Twice at 8-Day Intervals. R.W. Bratton, R.H. Foote and C.R. Henderson

A group of five Holstein bulls ejaculated once at 8-day intervals for 360 days averaged 10.6 billion motile spermatozoa per ejaculate. In another comparable group ejaculated twice at 8-day intervals during the same period of time the first ejaculates averaged 10.3 billion and the second ejaculates 6.9 billion motile spermatozoa per ejaculate. The average 60- to 90-day per cent nonreturns to first-service

cows was 73.3 for the one-ejaculate bulls and 73.6 for the first ejaculates and 73.4 for the second ejaculates from the two-ejaculate bulls. It was estimated that on the basis of the spermatozoa output of the two-ejaculate bulls the ten bulls in the experiment had a breeding potential under present day operational procedures of nearly three-quarters of a million services and nearly a quarter of a million female progeny. Such potentials emphasize the need for applying the best methods now available for testing and selecting the sires to be used so heavily.

62. Preliminary Fertility Results With Frozen Bovine Spermatozoa.

R.W. Bratton, R.H. Foote and J.C. Cruthers

The fertility of bovine spermatozoa frozen in a concentrated form (200×10^6 motile spermatozoa per milliliter of extender), thawed at 5°C at the bull stud, further extended to approximately 10×10^6 motile spermatozoa per milliliter and used for breeding 24 to 60 hours later, was compared with that of semen frozen in single-breeding vials. The latter were thawed at the farm just prior to insemination. The split sample technique was used with two ejaculates from three different bulls. Prefreezing processing and freezing procedures were slightly modified from those reported by Polge et al. and Dunn et al. Unfrozen semen processed in the conventional manner was used for the control breedings. All semen was extended to provide 8 to 10 million motile spermatozoa per insemination, though estimates made later indicated that actual numbers varied between 4 and 14 million. The 60- to 90-day nonreturns to first services were as follows: unfrozen control, 459 services, 74.5%; frozen "concentrated," 449 services, 51.7%; frozen as single breeding samples and stored one day at -79°C, 55 services, 72.7%; frozen as single breeding samples and stored 103 days at -79°C, 92 services, 77.0%.

63. Fertilization and Embryonic Mortality Rates of Bulls with Histories of Either Low or High Fertility in Artificial Breeding.

H.J. Bearden, W. Hansel and R.W. Bratton

On the basis of 3-day post-estrus slaughter data the fertilization rate was 76.9% for 26 heifers bred to bulls with histories of "low" fertility in artificial breeding and 96.6% for 29 heifers bred to bulls with histories of "high" fertility in artificial breeding. The percentage of normal embryos from the same number of heifers bred to the low fertility bulls and slaughtered 33 days post-estrus was 57.7 and the percentage of normal embryos from the same number of heifers bred to the high fertility bulls and slaughtered 33 days post-estrus was 86.1. The percentage of heifers not pregnant, presumably because of embryonic mortality, was 19.6 for the low fertility bulls and 10.5 for the high fertility bulls. The difference of 9.1 percentage units between the two groups of bulls is not statistically significant. It would appear from the data that with high fertility bulls, instances of repeat breedings are nearly always embryonic mortalities, whereas with bulls of low fertility both nonfertilization and early embryonic mortality occur.

64. The Effect of Plane of Nutrition on the Mineral Composition of Blood Serum, Liver, and on the Growth of Bone.

E.J. Thacker, M.L. Alderman and R.W. Bratton

In a slaughter experiment with Holstein bull calves, the effect of age and plane of nutrition was studied on the growth and development of bone and the micronutrient element content of blood serum and liver tissue. The calves were provided with 60 to 75, 100 and 140 to 160% of Morrison's higher total digestible nutrient allowances for growing dairy heifers; and were slaughtered at 16, 32, 48, 60 and 80 weeks of age. A parabolic relation of the physical and chemical characteristics of bone to body weight was observed. Bone from calves of the same body weight were in the same stage of development regardless of age or plane of nutrition fed. Normal bone growth continued in the underfed animals to the final slaughter age. The blood serum level of copper (60.0 ± 5.8 mcg. per 100 ml), molybdenum (5.0 ± 0.88 mcg. per 100 ml.) and manganese (0.59 ± 0.17 mcg. per 100 ml.) did not change with age. Serum copper levels increased with a higher rate of copper consumption. The concentration of iron (184 ± 20.8 mcg. per gm.), molybdenum (4.7 ± 0.35 mcg. per gm.) and manganese (8.9 ± 0.55 mcg. per gm.) in the liver dry matter was not affected by age of the calf or plane of nutrition. The copper concentration

in the liver dry matter decreased progressively with age up to 48 weeks of age, and increased at the higher level of copper consumption. The water content of the liver decreased progressively as the animal aged.

65. Testing Bulls in Artificial Insemination Centres for Lethals, Type and Production.

R.H. Foote, C.R. Henderson and R.W. Bratton

Physiological research on the extension and preservation of semen has been applied more rapidly than genetic research. To insure that only genetically superior bulls are used extensively the best sire selection techniques geneticists can devise should be adopted. Selection for several traits reduces the rate of progress that can be made in any one trait. Therefore, only heritable traits of major economic importance such as production and type should be selected for. It appears that selection for production alone will not appreciably affect type, and vice versa. Emphasis should be on production, but bulls in the sampling programme found to be siring seriously defective progeny, lethals, etc. should be culled. The genetic improvement due to the testing programme depends upon the percentage of tested bulls saved for replacements, the number of tested daughters obtained per bull, and the fraction of the total breedings to bulls being tested versus those already tested. A programme combining an optimum balance between these three factors, and conducted so as to sample the bulls and appraise their progeny as quickly as possible offers the greatest opportunity for increasing the rate of genetic improvement.

66. The Relative Usefulness of Combinations of Laboratory Tests for Predicting the Fertility of Bovine Semen.

R.W. Bratton, R.H. Foote, C.R. Henderson, S.D. Musgrave, R.S. Dunbar, Jr., H.O. Dunn and J.P. Beardsley

Statistically significant "gross" linear correlations were obtained between the fertility of bovine ejaculates extended 1:100 and 1:300 and the concentration of spermatozoa in the ejaculate, the per cent of motile spermatozoa, the methylene blue reduction time, the pH, the percent of unstained spermatozoa, the per cent of morphologically abnormal spermatozoa, the livability of spermatozoa at 5°C., and the oxygen uptake of spermatozoa at 37.5°C. The average 60- to 90-day per cent nonreturns to 7,168 first services with semen extended 1:100 and 5,785 first services with semen extended 1:300 in 3.6% citrate-sulfanilamide-yolk were 56.6 and 52.8, respectively. From among the semen tests that may be performed quickly after the ejaculate is collected, spermatozoan concentration and the per cent of abnormal spermatozoa make substantial contributions to the prediction of per cent non-returns at extension rates of either 1:100 or 1:300. However, in the routine examination of semen for large scale artificial breeding operations, the culling of ejaculates on the basis of their predicted nonreturns from the number of motile spermatozoa per milliliter of extended semen appears to be more feasible. Rejecting 50% of the ejaculates by this procedure and extending the remaining ones 1:300 resulted in a predicted average nonreturn percentage equal to that predicted when no ejaculates were rejected and all were extended 1:100. The practical consequence of the use of such a culling and extending procedure would be to make available for breeding purposes 50% more extended semen without sacrificing fertility. Results of further studies of this problem under present day operating conditions and fertility levels will be reported later.

67. Fertility of Bovine Spermatozoa Stored at Minus 79°C for One Week and For Seventeen Weeks.

R.W. Bratton, J.C. Flood, R.H. Foote, S. Wearden and H.O. Dunn.

In a split-ejaculate experiment involving 75 ejaculates from eight Holstein sires, the 60- to 90-day percentage nonreturns was 71.0 for 1,278 first services to unfrozen semen, 73.2 for 1,151 first services to frozen semen stored 1 week at -79°C and 69.8 for 1,094 first services to frozen semen stored 17 weeks at -79°C. These differences, which were not significant, confirm the previous results with frozen semen reported from this laboratory. The average spermatozoan survival rate for all ejaculates was 77% after 1 week storage and 62% after 17 weeks storage at -79°C. The estimated number of motile spermatozoa per milliliter of extended semen

at the time of breeding was 8.6×10^6, 11.6×10^6, and 9.3×10^6 for the unfrozen, 1-week frozen, and 17-week frozen semen, respectively. Within treatments, there were no significant and useful correlations between the number of spermatozoa inseminated and fertility. A frozen semen kit capable of maintaining a temperature of approximately -79°C for 6 days with a loss of 2 lb of Dry Ice per day is described.

68. **Breeding Difficulties in Dairy Cattle.** S.A. Asdell, K.O. Pfau, V.J. Yates, W.N. Plastridge, A.H. Frank, J.P. Mixner and R.W. Bratton

Although the studies on TDN and feed intake will require several more years to complete, the results to date provide no basis for the earlier and long-held belief that the fertility of the germ cells may be seriously impaired by uncommonly high or low feed and nutrient intakes during early growth and development of the animal. Likewise, the results with low trace-mineral-content rations for dairy cows and rations with different qualities of protein for young breeding bulls support this general conclusion. In contrast to these findings it is equally clear that the onset of heat and ovulation in young heifers, and of semen production in young bulls, is definitely and positively correlated with the level of nutrient intake and rate of body growth. If feed or nutrient intake is too low for normal rates of growth and development the onset of the reproductive function is delayed.

69. **Fertility as it Relates to Genetic Improvement.** R.H. Foote and R.W. Bratton

Artificial insemination in dairy cattle provides an opportunity of using males so extensively that their fertility can be estimated with considerable accuracy. This widespread use, when combined with careful selection of the genetically superior sires, offers an opportunity of making considerable genetic improvement. The over-all genetic effect of a sire on the population is a product of the number of progeny produced times the amount genetically that those progeny are above the average of the population. In turn, the number of progeny obtained by artificial insemination depends on the number of cows which can be inseminated with the quality of semen produced, the proportion of this semen which is actually used, and the fertility of this semen. Since environment rather than heredity accounts for most of the differences in fertility, fertility is primarily a management problem rather than one of genetic selection. Selection should be practiced for more highly heritable traits of economic importance, such as milk production. This requires an objective and adequate testing program as a basis for selecting sires to be used extensively. Under good management conditions, semen from the best bulls can be extended at a higher rate than is currently being practiced without seriously lowering fertility. This would require fewer sires to breed the cattle population, and if only those few sires of outstanding genetic merit were used more genetic improvement would be possible.

70. **The Evaluation of Semen Quality in the Dog and the Effects of Frequency of Ejaculation Upon Semen Quality, Libido, and Depletion of Sperm Reserves.** J.H. Boucher, R.H. Foote and R.W. Kirk

Methods of collecting and evaluating semen were studied using 125 ejaculates of semen obtained from 25 dogs. The methods of collection compared were (1) use of an artificial vagina without a teaser bitch, (2) hand manipulation without a teaser, and (3) hand manipulation with a teaser. For the three methods, respectively, the ejaculate averages were as follows: volume, 4.1 ml, 7.0 ml, and 5.0 ml; percentage of motile sperm, 10, 81, 75; pH, 6.59, 6.52, and 6.72; concentration per $mm^3 \times 10^3$, 71, 62, and 148; total sperm per ejaculate $\times 10^6$, 289, 314, and 528; total motile sperm per ejaculate $\times 10^6$, 65, 260, and 396; and percentage of sperm morphologically normal, 90, 91, and 86. The slow ejaculation of dog semen over the warm latex surface of the artificial vagina definitely impaired the motility of the spermatozoa. Use of a teaser, along with a glass funnel and glass tube, subsequently was adopted as standard. The optical density, as measured photoelectrically with a Klett-Summerson colorimeter, was highly correlated ($r = +0.95$) with sperm concentration determined with a hemocytometer. A photelometer was calibrated for the rapid estimation of sperm counts in dog semen. Obtaining estimates of ejaculate volume, sperm motility, and sperm

concentration permits the calculation of the very important criterion, total motile sperm per ejaculate. The effects of frequency of collection upon semen quality, libido, and depletion of sperm reserves were studied using 248 ejaculates collected from eight beagles. The four frequencies of collection were once every 3.5 days (twice a week), once every 2 days, once every day, and twice a day. The average values obtained were, respectively: volume, 4.0 ml, 5.5 ml, and 3.7 ml (data for the twice-a-day frequency are not given here or under sperm concentration because the third fraction was eliminated); percentage of motile sperm, 78, 77, 77, and 76; sperm concentration per $mm^3 \times 10^3$, 200, 133, and 98; total sperm per ejaculate $\times 10^6$, 580, 548, 268, and 147; motile sperm per ejaculate, $\times 10^6$, 444, 413, 208, and 112; total sperm obtained per day $\times 10^6$, 166, 274, 268, and 293; and motile sperm obtained per day $\times 10^6$, 127, 207, 208, and 225. Thus, it is apparent that collecting from dogs more often than once every 2 days results in a decreased sperm output per ejaculate. As many sperm were obtained per unit of time with this collection frequency as were obtained on the more frequent regimes, indicating that one ejaculate every 2 days is sufficiently frequent to prevent wastage of sperm. From these results it would appear that high-quality semen is obtained and maximum utilization of sperm is achieved by using a dog at stud every 2nd day.

71. Sperm Output and Fertility of Rabbits Ejaculated Either Once a Week or Once a Day for Forty-Three Weeks. A.T. Gregoire, R.W. Bratton and R.H. Foote

Semen was collected with an artificial vagina from each of two groups of four Dutch-type rabbits for a period of 13 weeks. Following this 13-week preliminary period one group was ejaculated once each day and the other group continued to be ejaculated once each week for 43 additional weeks. The motile sperm output per week adjusted for group differences during the preliminary period was 93×10^6 for the bucks ejaculated once a week and 351×10^6 for the bucks ejaculated once a day. There were no significant differences between the average conception rate or average littler size for the eight does inseminated with approximately 20 million motile sperm from the bucks ejaculated once a week and the 12 does inseminated with approximately the same number of sperm from the bucks ejaculated once a day. The body weights of the bucks in both treatment groups remained essentially constant throughout the 56 weeks of the study. Post mortem weights and histological section of the testes and adrenals revealed no signs of tissue damage in the two groups. This study shows that rabbits may be ejaculated as frequently as once a day for as long as 10 months without impairing their libido, sperm production or fertility. The response of dairy bulls under similar conditions is being investigated.

72. Estimation of Some Variance Components of Bovine Semen Criteria and Their Use in the Design of Experiments. H.D. Hafs, R.W. Bratton, C.R. Henderson and R.H. Foote

Semen production records of 68 bulls from five dairy breeds in four studs were used to estimate the bull, ejaculate, breed, season, and interaction variance components of five criteria used to measure semen production. The means and standard deviations of individual observations for the five criteria were: ml of semen per ejaculate, 8.0 ± 2.4; per cent motile sperm, 66 ± 9.0; billions of sperm per ml of semen, 1.71 ± 0.62; billions of motile sperm per ejaculate, 8.9 ± 3.9, and billions of total sperm per ejaculate, 13.6 ± 6.0. Component of variance analyses showed that the bull and ejaculate components accounted for more than 90% of the total variance in all criteria except per cent motile sperm. Consequently, these two variances were taken into account in estimating the power of the test by Tang's method. This estimate showed that there was little increase in sensitivity to be gained by taking more than ten ejaculates per bull. It was calculated that to have a 75% chance of detecting a treatment difference of 50% of the mean of motile sperm per ejaculate at the 5% level of significance would require about ten bulls per treatment and five ejaculates per bull.

73. **Technics For Semen Collection; Semen Production in Young Boars.**
A.R. Turkheimer, D.C. Young and R.H. Foote

Procedures for collecting semen from 22 boars, starting at 4 months of age, were studied. A short artificial vagina of the general bovine type, which permitted grasping the glans penis through the collection cone, was found to be satisfactory. A gilt, barrow, or another small boar restrained in a breeding crate was found to be more of a stimulus in inducing mounting than a dummy. Four of the boars produced first ejaculates between 129 and 133 days of age. Most boars demonstrated interest in the teaser by 6 and 7 months of age, and all but five were ejaculated by 10 months of age. Semen production increased rapidly as the boars approached 1 year of age. Six of the boars were housed individually during the period from 47 to 52 weeks of age. Separate pens are recommended for boars after reaching puberty in order to prevent pederasty. The average values for the different semen criteria for ejaculates collected from these boars were as follows: total volume, 245 ml; strained volume, 177 ml; gel volume, 68 ml; sperm concentration $\times 10^6$ per ml, 275; percentage of motile sperm, 58; total sperm per ejaculate $\times 10^9$, 47; motile per sperm ejaculate $\times 10^9$, 27; and pH, 7.50. Semen from this group of boars used for artificial insemination was found to be fertile.

74. **Estimation of Bull Sperm Concentration by Packed Cell Volume.** R.H. Foote

The hematocrit method for measuring red blood cell concentration was adapted for estimating sperm cell concentration. Inasmuch as many clinical laboratories have equipment for hematocrit work no additional equipment would be required. The simultaneous duplicate measurement of multiple samples also is rapid. Repeatability of measurements was high (> 0.99) and the correlation with sperm concentration measured by optical density was 0.97. However, each percentage unit of packed cell volume represented 206 million sperm, so the sensitivity of the method limits its application to clinical situations where semiquantitative approximations are adequate.

75. **Causes and Prevention of Reproductive Failures in Dairy Cattle. I. Influence of Underfeeding and Overfeeding on Growth and Development of Holstein Heifers.**
A.M. Sorensen, W. Hansel, W.H. Hough, D.T. Armstrong, K. McEntee and R.W. Bratton

This bulletin provides detailed data of studies on 64 female Holstein calves raised on three diets on which control animals consumed 93 of Morrison's TDN standards and two other levels consumed 61 and 129% of the control intake. They were randomized into groups of 4 animals each and slaughtered at 0-1, (4 animals), 16, 32, 48, 64 and 80 weeks of age. Body weight and height at withers, along with sexual behavior were monitored. At slaughter vital organs and reproductive organs were weighed and sections removed for histological study. Development proceeded inversely proportional to TDN intake with the average age at first estrus in the low, medium and high plane of nutrition groups being 72.0, 49.1 and 37.4 weeks, respectively. First estrus could be predicted from skeletal height. Details are given in 18 figures, 16 tables and 5 appendix tables.

76. **Libido, Sperm Characteristics, Sperm Output, and Fertility of Mature Dairy Bulls Ejaculated Daily or Weekly for Thirty-Two Weeks.**
H.D. Hafs, R.S. Hoyt and R.W. Bratton

Ten aged dairy bulls were used to measure the effects of prolonged daily ejaculation on libido, sperm output, sperm characteristics, and fertility. The ten bulls were ejaculated once a week for a 4-wk preliminary period after which five were chosen at random to be ejaculated once a day and five once a week for a 32-wk experimental period. The bulls ejaculated once a week required an average of 9.2 min of active sexual stimulation and 2.9 false mounts before each ejaculation, as compared to an average of 16.0 min and 3.7 false mounts for the bulls ejaculated once a day. The weekly bulls yielded an average of 9.5 ml of semen per ejaculate, 63% motile sperm, 1.89 billion sperm per milliliter of semen, 17.8 billion total sperm per ejaculate (per week), and 11.1 billion motile sperm per ejaculate

(per week). The bulls ejaculated once daily yielded an average of 6.2 ml of semen per ejaculate, 69% motile sperm, 0.81 billion sperm per milliliter of semen, 4.8 billion total sperm per ejaculate, 3.4 billion motile sperm per ejaculate, 33.8 billion total sperm per week, and 23.6 billion motile sperm per week. All of the differences between the two frequencies of ejaculation, with respect to the sperm output criteria, were statistically significant ($P < .05$) except that for volume of semen per ejaculate. Determinations of the per cent vitally stained sperm and the per cent of sperm with morphological abnormalities and estimation of the per cent of motile sperm in extended semen after three intervals of storage at -79, 5, and 15-25°C revealed no significant differences between the two frequencies of ejaculation. Measured by the per cent of cows not returning to service within 60 to 90 days after first insemination, the average fertility of 70% for 42,136 inseminations with sperm from the bulls ejaculated once a week was not significantly different from the average of 73% for 7,108 inseminations with sperm from the bulls ejaculated once a day. The results of this experiment indicate that daily ejaculation of aged bulls for as long as 8 mo is not harmful, either to the bulls themselves or to the quality of the sperm they produce. Ejaculating bulls two or three times on each of two or three days a week and storing the sperm in an improved extender, such as CUE, makes it possible for an artificial breeding organization to provide unfrozen semen with satisfactory fertility at all times from most stud bulls.

77. Development of A.I. and Progeny Testing in Denmark.

K. Rottensten and R. Foote

Denmark has adopted artificial insemination essentially 100%. Special progeny test stations test groups of 20 daughters per bull calving in a restricted period of time and they are compared with 20 daughters of other bulls. Dairymen are able to visit the test stations and see the daughters. A major disadvantage of the stations is that the environment surrounding one group of daughters is different from another group, and proofs obtained do not rank bulls reliably on their genetic merit.

78. Causes and Prevention of Reproductive Failures in Dairy Cattle. II. Influence of Underfeeding and Overfeeding from Birth to 80 Weeks of Age on Growth, Sexual Development, and Semen Production of Holstein Bulls.

R.W. Bratton, S.D. Musgrave, H.O. Dunn and R.H. Foote

Three experimental feeding levels -- high, medium and low -- were planned to provide 140 to 160, 100 and 60 to 75 percent of the upper range of Morrison's recommended TDN allowances for growing dairy heifers. Four bulls were slaughtered within each feeding level at 0, 16, 32, 48, 64 and 80 weeks of age. The bulls reared on the high level of feeding consumed approximately 130 percent of Morrison's recommended TDN allowances and came into semen production at an average age of 37 weeks and an average weight of 644 pounds. The control bulls, reared on a medium level of feeding came into semen production at an average age of 43 weeks and an average weight of 578 pounds. The bulls on the low level of feeding came into semen production at an average age of 51 weeks and an average weight of 519 pounds. Body length at onset of semen production was less variable among the 3 feeding levels than were body weight, circumference of the heart girth, or body height. The average calculated TDN intakes from birth to onset of semen production were 1729, 1650, and 1516 pounds for the high, medium, and low feeding levels, respectively. The relative weights of the testes and seminal vesicles taken at 16-week intervals followed the same general pattern as body weights. Microscopic examinations of the seminiferous tubules revealed a state of active spermatogenesis at 32 weeks of age in 2 bulls on the high feeding level. All bulls on each feeding level slaughtered at 48, 64, and 80 weeks of age showed very active spermatogenetic tissue. By 48 weeks of age there were many sperm in the epididymides of the bulls on the high and medium levels of feeding and in 3 of the 4 bulls on the low feeding level slaughtered at this age. However, on the low level of feeding, 2 of the bulls in the 48-week slaughter group, 2 in the 64-week group, and 1 in the 80-week group had not produced an ejaculate of semen up to

48 weeks of age, and 1 bull in the 80-week age group had not produced an ejaculate of semen when slaughtered at 80 weeks of age. Variations in the concentrations of hemoglobin, plasma protein, calcium, inorganic phosphorus, carotene, and vitamin A from birth to 80 weeks were found to be unrelated to the development of the reproductive organs and the onset of semen production. On the basis of current feed prices the relative costs of the feed required to raise Holstein bulls to semen producing age were, in round numbers, $164, $84, and $60, for the high, medium, and low levels, respectively. It is concluded from these studies that, 1) body development and the onset of semen production in young bulls may be accelerated by a high level of feeding and retarded by a low level of feeding during early calfhood and, 2) a planned TDN intake as low as 60 percent (actual intake, 57%) of Morrison's upper range of recommended allowances does not result in damage to the sperm-producing tissue or permanently delay the onset of semen production.

79. **Collection, Preservation and Artificial Insemination of Boar Semen.** R.H. Foote, D.C. Young, A.R. Turkheimer and R.W. Bratton

Yorkshire boars were trained to serve the artificial vagina at 4 months of age and the number of sperm per ejaculate at 18-33, 34-41 and 42-52 weeks of age was 23, 25 and 47 billion. Ejaculating 10-month old boars daily resulted in 68 billion sperm in 3 days compared with 45 billion sperm per ejaculate collected at 3-day intervals. More than 200 different types of semen diluters and storage temperatures were studied.

80. **A Photoelectric Method for Estimating the Concentration of Sperm in Boar Semen.** D.C. Young, R.H. Foote, A.R. Turkheimer and H.D. Hafs

The turbidometric method for estimating sperm concentration in ram and bull semen was adapted to boar semen using a Cenco-Sheard-Sanford Photelometer. Correlations of 0.95 or higher were obtained between hemocytometer counts of sperm concentration and Photelometer readings expressed as 2 - log of the Photelometer reading. Semen dilution rates of 1:9 and 1:19 gave readings, most of which were within the more sensitive range on the Photelometer scale. No significant differences were found between the average sperm counts made by the hemocytometer, or between transmittancy values of samples taken from the top and the bottom of the ejaculate on day 0 and day 1. The regression equations for estimating sperm concentration ($\hat{Y}$) in millions per ml in boar semen were, for the 1:9 dilution rate, $\hat{Y}=862.1X - 72.4$, and for the 1:19 dilution rate, $\hat{Y}=1328.9X - 21.0$, where X equals 2 - log of the Photelometer reading. As with ram and bull semen this photelometric procedure saves considerable time in evaluating boar semen.

81. **Motility and Fertility of Progesterone- and Estrogen-Treated Bovine Semen.** R.E. Staples, W. Hansel, R.H. Foote and H.O. Dunn

The fertility of bull semen was not affected by the addition of progesterone and estrogen in amounts as high as 5,000 and 200 μg per milliliter of extended semen, respectively. No effect could be demonstrated on the motility of semen treated with these hormones up to two days of storage at 5°C. Increased levels of progesterone (10,000 μg per milliliter of extend semen) reduced the motility of sperm after two days of storage. There was some indication that adding 5,000 μg of progesterone in combination with 200 μg of estrogen per milliliter of extended semen may have altered the sex ratio of the resulting calves, increasing the proportion of males to 55% (277 males: 223 females, $P < 0.05$). However, this finding should be reinvestigated before drawing conclusions.

82. **Survival Rates of Rapidly Frozen Bovine Spermatozoa.** J.J. Kennelly, R.S. Hoyt, R.H. Foote and R.W. Bratton

Subsamples of semen from each of two ejaculates from each of 12 bulls were extended with a yolk-citrate-glycerol extender, equilibrated 5 to 6 hr at 5°C and placed directly in a freezing bath at temperatures of +5, 0, -5, -10, -15, -20, -25, -30, -35, and -40°C. The freezing bath was cooled at the rate of 0.8°C per minute from +5 to -15°C, at a rate of 5°C per minute from -15 to -40°C, and at the rate of

10°C per minute from -40 to -75°C. The percentage of motile spermatozoa in ampules subsequently stored in a mechanical freezer at -85°C for 60 days were, in order of decreasing temperature of the freezing bath at the time of transfer, 29, 21, 24, 27, 33, 36, 35, 35, and 35. From these data it is concluded that time can be saved and a higher percentage of motile spermatozoa recovered by transferring the semen abruptly from +5°C to a freezing bath ranging from -20 to -40°C.

83. Survival of Bovine Spermatozoa Stored at 5 and 25°C in Extenders Containing Varying Levels of Egg Yolk, Glucose, Glycine, Glycerol, Citrate, and Other Salts.

R.H. Foote and R.W. Bratton

Combinations of 20% egg yolk with 1.5 to 3.0% solutions of glycine resulted in poor survival of spermatozoa. Inclusion of 10% glycerol resulted in sperm survival comparable to that obtained in 50% egg yolk-glycine extenders with or without glycerol. A 20% egg yolk extender containing 1.16% sodium citrate dihydrate, 0.75% glycine, and 1.0% glucose was evolved. Twenty ejaculates of semen stored in this extender averaged 41% motile spermatozoa after 12 days of storage at 5°C. Results at 5°C were superior to those at 25°C ($P < 0.01$), even when 0.3% sulfanilamide was added. A yolk-citrate-glucose-glycine-sulfanilamide-antibiotic extender, designated as CU-16, was compared with one designated as CUE, which contained in addition sodium bicarbonate, potassium chloride, and citric acid. In two experiments the percentage of motile spermatozoa over a 12-day storage period at 5°C in CU-16 averaged 49 and 52, and in CUE averaged 50 to 52 ($P > 0.05$). The high sperm survival in both of these extenders at 5°C indicated they might be useful in preserving the fertility of sperm stored for several days at this temperature.

84. Fertility of Bull Semen Stored Up to Four Days at 5°C in 20% Egg Yolk Extenders.

R.H. Foote, L.C. Gray, D.C. Young and H.O. Dunn

Three fertility trials comparing experimental extenders CUE and CU-16 with a standard 50% egg yolk-citrate-sulfanilamide control were conducted in 1957 and 1958. Semen was extended to ten million motile spermatozoa per milliliter of extender in all trials. The first trial included four bulls, and the semen was used for insemination primarily one and two days after collection. A second field trial was designed to use semen extended with CUE and CU-16 more extensively three to four days after collection, and a third field trial, conducted on a larger scale, was designed to obtain results with all three extenders over a four-day storage period. The combined number of first services and weighted mean percentages of 60- to 90-day nonreturns for all three trials was 6,069 and 73.4 for the control 2.9 CSAY, 6,280 and 76.6 for the CUE, and 6,145 and 76.0 for the CU-16. The adoption of CUE for routine use by the local artificial breeding cooperative resulted in an immediate and consistent increase of about two percentage units, bringing the 60- to 90-day nonreturn rate to an average of 74.9% for nearly 300,000 first inseminations.

85. Effect of Promazine Hydrochloride and Chlorpromazine Hydrochloride on the Motility and Fertility of Bovine Semen.

R.H. Foote and L.C. Gray

Promazine hydrochloride (Sparine) and chlorpromazine hydrochloride (Thorazine) were incorporated in 20% egg yolk extenders of the CU-16 type at levels as high as 2,000 μg per milliliter of extender. None of the levels of promazine tested improved sperm survival, and the highest levels of both drugs were spermicidal ($P < 0.01$). Survival of spermatozoa was improved by the replacement of sulfanilamide (SA) with 200 μg chlorpromazine per milliliter in CU-16 extender, but chlorpromazine was only partially effective in replacing SA in CUE extender. The same level of chlorpromazine was slightly spermicidal when added to a 50% yolk-citrate-sulfanilamide extender, and markedly spermicidal when added to heated skimmilk. The 60- to 90-day nonreturns for semen extended in the CU-16 extender, modified by replacing SA with 200 μg of chlorpromazine per milliliter, averaged 73.9% compared to 70.3% for the control 50% yolk-citrate-sulfanilamide extender. Subsequently, this modified CU-16 extender was compared with the sulfanilamide-containing standard CU-16 and CUE extenders. The number of first services and 60- to

90-day per cent nonreturns for the three extenders, respectively, were 4,796 and 73.7, 4,871 and 74.9, and 4,650 and 74.9 ($P > 0.05$). While all three extenders gave highly acceptable fertility, there appears to be no advantage in replacing sulfanilamide with chlorpromazine in the extenders used.

86. Causes and Prevention of Reproductive Failures in Dairy Cattle. III. Influence of Underfeeding and Overfeeding from Birth Through 80 Weeks of Age on Growth, Sexual Development, Semen Production and Fertility of Holstein Bulls.

R.W. Bratton, S.D. Musgrave, H.O. Dunn and R.H. Foote

Three groups of 10 young bulls each were raised on either a high, medium, or low feeding level, from 1 through 80 weeks of age. These 3 feeding levels were planned to provide 160, 100, and 60 percent, respectively, of the upper range of Morrison's recommended TDN allowances for growing dairy heifers. The effects of these feeding levels on body growth, sexual development, semen production, and fertility of the young bulls were compared. The bulls raised on the high level of feeding came into semen production at an average age of 39 ± 4.6 weeks and an average weight of 704 ± 67 pounds. The bulls on the medium level (control group) came into semen production at an average age of 46 ± 11.6 weeks and an average weight of 619 ± 135 pounds. The bulls on the low level of feeding came into semen production at an average age of 58 ± 5.7 weeks and an average weight of 517 ± 40 pounds. The differences among group averages for age and weight were highly significant, statistically. The differences among group averages for height at withers, heart girth, and body length followed patterns similar to those for body weight. The calculated average pounds of TDN consumed from approximately one week of age to onset of semen production of the bulls on the high, medium, and low levels of feeding were 1826 ± 315, 1778 ± 777, and 1552 ± 200, respectively. Since these averages were not significantly different statistically, they suggest that the total amount of TDN required to develop young Holstein bulls to semen-producing age approaches a constant, and that the magnitude of this constant is of the order of 1700 pounds. The principal effect on the reproductive function of bulls receiving less than normal amounts of feed appears to be restriction of sperm output as a consequence of slower development of the testes, until feed intake is increased to normal, after which sperm output also increases gradually to normal levels. The average 60 to 90-day percent nonreturns for a total of approximately 82,000 first services for the bulls raised on the high, medium, and low levels of feeding were, 74, 75, and 74, respectively, emphatic evidence that these feeding levels have no effect on fertility. Furthermore, the blood values for the concentration of hemoglobin, plasma protein, carotene, vitamin A, calcium, and inorganic phosphorus showed no relationship to semen production or fertility. On the basis of local prices during this study, the average feed costs to onset of semen production for the bulls on the high, medium, and low levels of feeding were, $131, $86, and $62, respectively, and to the end of the sampling period for each group were, $357, $254, and $238, respectively. These results show that Holstein bulls may be raised to semen-producing age cheaply on limited amounts of low quality feeds or expensively on *ad libitum* feeding (high level) of high quality feeds, without impairing fertility. *Ad libitum* feeding, however, makes it possible to prove Holstein bulls about 4 months earlier than with normal (medium level) feeding, but it costs approximately $100 more for feed.

87. Survival of Bull Sperm in Milk and Yolk Extenders With Added Catalase.

R.H. Foote

After 12 days of storage at 5 C the percentage of motile sperm in CUE containing 0, 1, 10, 100, and 200 µg of catalase per milliliter of extender was 28, 37, 44, 44, and 45, in CU-16 was 35, 43, 47, 52, and 50, in CU-16 plus chlorpromazine hydrochloride was 29, 35, 35, 41; and 43, and in 20% yolk-citrate was 24, 34, 38, 37, and 34, respectively. All levels of catalase improved sperm survival significantly ($P < .01$), but little additional response was observed with catalase levels higher than 10 µg/ml of extender. The concentration of catalase required to produce a maximum response was much greater than that theoretically required to eliminate any hydrogen peroxide produced. No beneficial effect of adding 20 µg of catalase per ml of skimmilk or skimmilk

containing glycerol was found. The percentage of motile sperm after 12 days of storage at 5 C averaged 47 for CU-16 extender with catalase, 11 for skimmilk, and 12 for skimmilk with catalase. The reason for the differential response to catalase in egg yolk versus milk has not been elucidated.

88. The Use of Radioactive Isotopes to Study Spermatogenesis and Transport of Spermatozoa in Mammals. A Review. R.H. Foote

This paper is concerned with the use of radioactive isotopes in conjunction with histological techniques to study quantitative aspects of the number of spermatozoa formed, the time required for the various steps to take place, and to relate this to sperm output data obtained by collecting semen samples regularly. The frequency with which the samples are obtained, and the sexual stimulation prior to ejaculation influence the number of spermatozoa obtained per ejaculate and per unit of time. Even with frequent semen collection this technique does not necessarily indicate how many spermatozoa are actually produced by the testis, for it does not take into account the possible losses of spermatozoa through dissolution and resorption, passage out the urethra with urine, adherence to the measuring equipment, etc. Presumably sperm losses are minimized by frequent semen collection, and are considerably larger during periods of infrequent semen collection. Sperm production by the testes can be estimated directly from histological studies of the spermatogenic elements combined with estimates of the time involved in each stage of development. Certain radioactive isotopes which are incorporated into deoxyribonucleic acid (DNA) can be used to time the whole cycle. There appears to be no metabolic turnover of a structural component of the DNA. Therefore, the cells remain labeled throughout spermatogenesis and transport of the spermatozoa through the excurrent ducts of the male. It is desirable that the radioactive isotope chosen for this purpose give high resolution autoradiographs, and under certain conditions it is desirable to be able to isolate different components and measure specific activity. Originally P^{32} was used but it gives poor resolution with autoradiography. Adenine-8-C^{14} has been used, and a compound specific for DNA such as thymidine-H^3 is available. Studies with mice indicate that the first radioactive sperm appear in the ejaculate 30 days, after injection of the radioactive material, in the rabbit about 40 days, in the ram about 44 days, and in the bull about 50 days after injection. Through the use of specimens obtained by biopsy, castration and sacrifice the overall time intervals can be subdivided into the time required for various stages of spermatogenesis, spermiogenesis and transport to and through the epididymis. Labeled spermatozoa subsequently can be followed during transport in the female reproductive tract to fertilization of the ovum. By knowing the time required for each stage of the cycle (for example, the number of days the cells exist as primary spermatocytes), the total number of spermatocytes per testes, the percentage of spermatocytes which complete spermatogenesis, and the number of spermatozoa derived from one spermatocyte, it is possible to estimate the potential daily sperm production per testis. The difference between average estimated production and average actual number of spermatozoa obtained by frequent semen collectors is presumed to represent losses though dissolution and absorption, losses in the urine, adherence to collecting equipment, etc.

89. Production Testing of Dairy Bulls Used in Artificial Breeding, Giving Consideration Also to Type and Lethal Factors.

R.H. Foote and K. Rottensten

The development of artificial insemination with the result that progeny in thousands of herds can be produced by a single sire each year has greatly increased the responsibility of those selecting and testing bulls to insure that bulls of high genetic merit are available for extensive use. The records of production accompanying the growth of artificial insemination have provided the necessary data to develop effective methods for the testing and selection of bulls. Selection should be for traits of economic importance that are inherited. Milk production would rank first. Research on solids-not-fat indicates that it may soon be practical to test for under field conditions. Type should be emphasized only to the extent necessary to maintain a sturdy conformation, in order to achieve as much genetic improvement as possible in production. Bulls carrying recessive genes causing lethal or semi-lethal effects can be detected by an active program to

survey the progeny resulting from the production-testing of young bulls, when the gene frequency exceeds about one percent in the population. Any genes occurring less frequently than this would be of little economic importance. Non-specific temporary infertility has a heritability of essentially zero, and consequently, selection for its is a wasted effort. In progeny testing bulls and selecting the parents of each bull generation special testing stations do not appear to offer any advantage over field records of production when the latter are properly corrected for known environmental effects. Essentials in a program of progeny testing bulls for production include the following: a) Select young bulls for sampling which as a group have a high genetic potential; b) Select many more bulls for sampling than are needed for replacements; c) Obtain enough tested daughters (20-50) to minimize errors in evaluating individual bulls; d) Determine the optimum relationship between b and c within the limits of the tested cattle available, so that about 1/3 of the breedings are to young sires and about 2/3 to sires already selected on the basis of their progeny test; e) Select the bulls as young as possible, and reduce to a minimum the time required to progeny test. This will reduce the cost of testing and far more important it will increase the genetic gain per year and per generation. The genetic gain is permanent. It is in addition to the temporary and often spectacular gains resulting from improved management that can accompany an educational artificial breeding program.

90. Motility and Fertility of Bull Semen Extended at High Rates in Yolk Extender Containing Catalase.

R.H. Foote and H.O. Dunn

Sperm survival in 20% yolk extender (CUE and CU-16) during a 12-day storage period was improved by catalase. The improvement was greater for semen extended to five million motile sperm per milliliter than for semen extended to ten million ($P < .01$), thus reducing the dilution effect on motility. However, fertility tests of semen used for insemination within a few days of collection showed no beneficial effect of catalase on fertility. In two trials the control CUE extender averaged 71.0 and 76.9% 60- to 90-day nonreturns compared with 71.5 and 74.6% nonreturns for CUE containing 20 μg of catalase per milliliter. When bull semen was extended to five million motile sperm vs. ten million motile sperm per milliliter of CUE extender, no difference in fertility was observed ($P > .05$). In two trials totaling 64,930 inseminations, the 60- to 90-day percentage of nonreturns for 5×10^6 motile sperm were 74.6 and 75.2, and for the control 10×10^6 motile sperm were 76.1 and 76.1. These results clearly indicate that the usefulness of genetically superior sires can be increased by reducing the number of sperm per insemination.

91. Catalase Content of Rabbit, Ram, Bull and Boar Semen. R.H. Foote

Catalase content of 100 ejaculates of Dutch-Belted rabbit semen averaged 275 mcg/ml. Catalase was not correlated with sperm concentration, and it was high in the supernatant of centrifuged semen and in the seminal plasma produced by vasectomized bucks. Catalase added to rabbit semen did not prolong sperm survival. A high degree of similarity between littermates suggested that catalase concentration in semen may be partly affected by heredity. Catalase content of semen collected from Dorset, Corriedale and Hampshire rams was much lower than rabbit semen, averaging 12 mcg/ml in 62 samples. The correlation between catalase and sperm concentration of ram semen was 0.71, and 66% of the catalase was in the sperm fraction of centrifuged semen. Catalase levels in bull semen averaged only 3 mcg/ml for 46 samples collected from Holstein, Guernsey, Jersey, Ayrshire, Brown Swiss, and Angus bulls. Added beef liver catalase was not appreciably taken up by bull sperm, but did prolong survival of bull sperm stored at 5°C. Catalase in boar semen was too low to estimate reliably, but eight samples averaged 0.2 mcg/ml.

92. Survival of Bovine Spermatozoa at Room Temperature in Citrate and Cornell University and Tris Extenders Containing Whole and Fractionated Coconut Milk.

R.H. Foote, R.W. Bratton, C.R. Henderson, E.M. Shantz and J.K. Pollard

The average percentage of motile sperm over an eight-day storage period in a citrate buffer alone or with the addition of 15% by volume of whole coconut milk (CM), neutral fraction (NF), and active

fraction (AF), for carrot explants, or NF + AF was 19, 34, 42, 22, and 36, respectively. Corresponding values in Cornell University Extender (CUE) were, respectively, 44, 42, 44, 42, and 42% and for Tris (hydroxymethyl) aminomethane (Tris), 31, 32, 24, 32, and 30%. Whole coconut milk and the neutral fraction equally improved sperm survival in citrate buffer, but did not improve sperm survival in the other buffers. Removal of anions and cations from the NF reduced this response, but carbohydrates or other material, or both, remaining in the NF produced some effect. The fraction active in producing cell division in carrot explants was ineffective for sperm. In general, the CUE buffer was equal or superior to the other treatments tested, indicating that coconut milk had no special properties due to unknown substances. Sperm survival in all buffers was improved by the addition of egg yolk.

93. Livability of Bovine Spermatozoa at 5 C in Tris-Buffered and Citrate-Buffered Yolk-Glycerol Extenders.

I.S. Davis, R.W. Bratton and R.H. Foote

When bovine spermatozoa were stored in 20% egg yolk-glycerol extenders buffered with .15, .2, and .25 M Tris (hydroxymethyl) aminomethane and adjusted with citric acid to pH's of 6.50, 6.75, and 7.00 optimum sperm survival was found in the 0.2 M extender with a pH of 6.75. After eight days of storage at 5 C the motile sperm in this extender averaged 42%, compared with 35% in CUE and 18% in a citrate-yolk-glycerol extender used routinely for freezing bovine spermatozoa.

94. Effects of Dyes on Survival of Bull Spermatozoa Stored in Yolk and Milk Extenders at 5 and -79 C.

L.L. Clamohoy and R.H. Foote

Strawberry Shade Red, Emerald Shade Green, Purple Shade Grape, Brown, Lemon Yellow, and Blue certified food colors added to yolk-citrate-glycerol and skimmilk-glycerol extenders at the rate of 0.6 mg/100 ml of extender had no effect on sperm survival during a 30-day storage period at -79 C. The same concentration of all food colors excepting brown had no harmful effect on bull sperm stored for 12 days at 5 C in CUE, 50% yolk-citrate and skimmilk extenders. Brown food color was spermicidal ($P < .01$). When 0.2 ml/100 ml of brown food color was added, the percentage of motile sperm after 1, 4, 8, and 12 days of storage in CUE was 62, 31, 7, and 3; in 50% yolk-citrate it was 65, 37, 12, and 5; and in skimmilk it was 64, 49, 15, and 4, respectively. These results were significantly lower than the for controls in CUE and 50% yolk-citrate ($P < .01$), but were an improvement over the higher concentration of brown food color. All food colors, at the levels tested, can be used as an aid in identification of frozen semen, but brown food color may be injurious to bull sperm at 5 C, unless the concentration of this color is reduced to less than 0.2% by volume.

95. Ovulation Rates and Litter Sizes in Sexually Receptive and Nonreceptive Artificially Inseminated Rabbits Given Varying Dosages of Luteinizing Hormone.

R.H. Foote, H.D. Hafs, R.E. Staples, A.T. Gregoire and R.W. Bratton

At any one time a large proportion of individually caged, sexually mature female rabbits failed to copulate. Intravenous administration of purified pituitary luteinizing hormone (PLH) (Armour) produced ovulations in sexually receptive and nonreceptive does alike. Does with histories of infertility ovulated after the injection of PLH. The routine administration of 2.5 mg of PLH to fifty-seven virgin and multiparous Dutch-Belted does averaging 2 to 3 kg, followed by artificial insemination, resulted in 91% of the does kindling and 307 young being born. Administration of either 0.5 mg or 1 mg of PLH per kg to random members of fourteen pairs of does, accompanied by artificial insemination, resulted in no difference between the number of ovulations or the number of young per treatment ($P > 0.05$). Fertility and litter size were normal when sixteen does were reinjected with PLH and inseminated after weaning their previous litters.

96. Effect of Sperm Losses in Semen Collection Equipment on Estimated Sperm Output by Bulls.

R.H. Foote and A. Heath

Several experiments were conducted to measure the number of sperm that could be recovered from the rubber liners of the artificial vagina (A.V.) after removing the semen collection tube, as in 1948 it was noted (unpublished data) that about 1 ml of semen was retained. In the current study 12% of the sperm in 60 ejaculates was found adhered to the rubber liner. It was found that treating the liners and moistening them with harmless buffer just before semen collection reduced the loss by more than one-half. The quality of sperm washed from the A.V. liners was lower than in the bulk of the semen. However, prior treatment of the A.V. should result in reduced loss of sperm without loss in sperm quality.

97. Livability of Bovine Spermatozoa at 5, -25, and -85°C in Tris-Buffered and Citrate-Buffered Yolk-Glycerol Extenders.

I.S. Davis, R.W. Bratton and R.H. Foote

Survival of bovine spermatozoa in 0.2 and 0.25 M Tris-buffered yolk-glycerol (TYG) extenders was generally superior to that in a standard citrate-yolk-glycerol (CYG) extender at 5, -25, and -85°C. The 0.1 and 0.15 M Tris-buffered extenders were inferior, $P < .01$, and the bending of the sperm's tails in these two extenders suggested that these levels of Tris were hypotonic. Twenty ejaculates of semen, averaging 62% motile sperm initially, averaged 50 and 48% after eight days' storage at 5°C in 0.2 M and 0.25 M TYG, respectively. These same extenders averaged 31% or less motile sperm after one day of storage at -25°C, indicating that this temperature was unsatisfactory for sperm storage with the extenders used. At -85°C, nearly 90% of the sperm motile before freezing were still motile immediately after freezing in 0.2 M and 0.25 M TYG, but this percentage had dropped to 66% sixty days after freezing. This latter survival rate was higher, $P < .05$, than that observed in the standard CYG. The accidental exposure of stored samples from eight ejaculates for a brief period to a temperature warmer than -85°C suggested that temperature changes were less critical in their effects on sperm survival in the Tris-buffered than in citrate-buffered extenders.

98. Cytology and Kinetics of Spermatogenesis in the Rabbit.

E.E. Swierstra and R.H. Foote

The cycle of the seminiferous epithelium of the rabbit was divided into eight stages, using as criteria the shape of the spermatid nucleus, the location of the spermatids and spermatozoa in regard to the basement membrane, the presence of meiotic figures and the release of spermatozoa from the lumen. The relative duration (frequency) of Stages 1 to 8 were 27.7, 13.4, 7.3, 11.0, 4.1, 15.7, 12.2 and 8.6%, respectively. Each stem cell (Type A spermatogonium) divided to produce two Type A spermatogonia. One of these was the starting cell for the next generation, while the other gave rise to two intermediate-type spermatogonia. Three more spermatogonial divisions followed, producing sixteen primary spermatocytes from one Type A spermatogonium, as is characteristic for the bull and the ram, but unlike the rat, mouse and hamster. It was estimated that only 3.1 spermatids were generated from one primary spermatocyte, suggesting that in the rabbit there is considerable degeneration of spermatogenic cells during the two maturation divisions.

99. Effect of Tranquilizers on Libido, Sperm Production and In Vitro Sperm Survival in Dogs.

R.H. Foote and L.C. Gray

Additions of promazine or chlorpromazine in concentrations below 200 μg/ml of media suitable for prolonged storage of dog sperm were without effect on sperm motility. Higher levels of these drugs were spermicidal. Oral administration of chlorpromazine to 8 dogs (4.4 mg/kg of body weight) every other day or daily for 9 days did not reduce the libido of dogs trained to routine semen collection procedures. Motility of the sperm collected was unaffected, and sperm output was inconsistently higher in the treated group. It is concluded that the in vivo levels of chlorpromazine studied did not alter the normal hypothalamic-pituitary-gonadal relationship.

100. **The Influence of pH, Osmotic Pressure, Glycine, and Glycerol on the Survival of Dog Sperm in Buffered-Yolk Extenders.**
R.H. Foote and E.P Leonard

Systematic studies with dog semen extended from 1:10 up to 1:20 and stored at 5 C have shown that 20 percent egg yolk combined with a buffer is superior to 50 percent egg yolk. Sodium citrate buffer is superior to a phosphate buffer. Replacement of part of the sodium citrate with glycine prolonged sperm survival. Milk extenders were less satisfactory. Tonicity of the extenders was optimal when the FPD's were between -.52 C and -.66 C. When the pH readings of the yolk-citrate-glycine media were adjusted to 5.9, 6.6, and 7.3, optimal survival occurred at a pH of 6.6. For prolonged storage at 5 C the addition of glucose was desirable. The best extender tested for dog semen contained, in the final extender, 20 percent egg yolk, 1.16 percent sodium citrate dihydrate, 0.75 percent glycine, and 1 percent glucose, to which was added 1,000 units of penicillin and 1,000 μg of dihydrostreptomycin per mil of extender. The pH of this extender was 6.6, and the freezing point depression was -.65 C. When nine freshly collected semen samples were extended 1:20 with this extender, the average percentages of motile sperm after 1, 2, 4, 8, and 13 days of storage at 5 C were 78, 76, 69, 56, and 40 respectively. The results are encouraging, especially in view of the fact that a similar extender preserves the fertility of bull sperm. Glycerol can be added to this extender before semen is added as satisfactorily as it can be added in increments after the sperm is extended. When 8 percent glycerol by weight was included, sperm motility was reduced by 13 percent over a 12-day storage period.

101. **The Influence of Frequency of Semen Collection, Fractionation of the Ejaculate, and Dilution Rate on the Survival of Stored Dog Sperm.**
R.H. Foote

Semen from nine beagle dogs trained for routine semen collection was used to compare survival of extended sperm during storage from 8 to 16 days at 5 C. The following were compared: (1) the first two fractions of semen vs. three fractions, (2) semen obtained after dogs were sexually rested for 1 vs. 5 days, (3) first versus third ejaculates collected within 24 hours, and (4) an extension of one part of semen to three parts of extender vs. 1:30. The third fraction did not affect the survival of dog sperm in most extenders but was of possible benefit when the pH of the extender was below 6.0. Sperm in semen samples obtained after 5 days of sexual rest appeared to maintain a higher percentage of motility during storage than did those obtained after a 1-day rest period. However, the sperm concentration was higher in samples collected infrequently, and when this was taken into consideration in extending the semen to standard numbers of sperm for storage little difference was observed. The first ejaculate in a series of three collected in a 24-hour period averaged 66, 61, 57, and 29 percent motile sperm when examined after 1, 4, 8, and 12 days of storage at 5 C. Corresponding values for the third ejaculate were 74, 67, 60, and 23 percent. Sperm motility was higher during storage when semen was extended 1:30 to give 20 million sperm per ml than when extended 1:3 to give 200 million sperm per ml ($P < .01$). The average percentage of motile sperm when nine ejaculates of semen were extended 1:30 with a yolk-citrate-glycine-glucose extender was 71, 70, 69, 62, and 43 after 1, 2, 4, 8 and 16 days of storage, respectively.

102. **The Effects of Electrolytes, Sugars, Glycerol, and Catalase on Survival of Dog Sperm Stored in Buffered-Yolk Mediums.** R.H. Foote

Dog semen was extended 20 times with a 20% yolk-citrate-glycine-glucose medium at 5 C. Low levels of K ion could be substituted in the medium for Na ion. The replacement of glycine with a bicarbonate-phosphate buffer, or the citrate ion by chloride ion, was detrimental. Arabinose, which is not metabolized, was not a satisfactory substitute for glucose. When 8% glycerol by weight was included in the extender, the percentage of motile sperm after 1, 4, 8, and 12 days of storage at 5 C was 64, 52, 43, and 19, respectively, compared to 60, 44, 34 and 16 for the conventional method of adding glycerol slowly to the diluted semen. Catalase content of 10 samples of dog semen averaged 5 μg/ml. The addition of beef liver catalase to stored semen did not

improve sperm survival. Dog sperm survived poorly at 25 C in extenders containing 0, 1, and 20% yolk. Best survival in the 1% egg yolk at 25 C was markedly inferior to the best survival at 5 C obtained with 20% egg yolk.

103. Extenders for Freezing Dog Semen.

R.H. Foote

Dog semen was extended with 20% buffered-yolk mediums containing varying levels of glycerol, frozen at rates of 0.8 to 3 C per minute, and stored at -79 or -195 C. Survival in mediums with glycine was poor. Best survival was obtained in a 0.20 M tris-buffered-yolk-glucose extender containing 11% glycerol. In this extender, the motility of 9 ejaculums averaged 41% after freezing. A 2.17% sodium citrate-buffered yolk-glucose extender containing 8% glycerol averaged 27% following freezing. No decline in motility was observed during a 30-day storage period at -195 C. As the concentration of the buffer was increased the concentration of glycerol required to give maximum protection increased.

104. Action of Amphotericin B (Fungizone) on Spermatogenesis in the Rabbit.

E.E. Swierstra, J.W. Whitefield and R.H. Foote

Quantitative and qualitative analysis of the seminiferous epithelium of rabbits treated with Amphotericin B (Fungizone) revealed that ten intravenous injections of Fungizone, at a dose level of 2.0 mg Fungizone/kg body weight/day, had no effect on the duration of the stages of the cycle of the seminiferous epithelium, but affected the spermatozoa prior to their release from the seminiferous tubules. Fungizone decreased the rate at which the spermatozoa migrated from the Sertoli cells towards the lumen. Thus, spermatozoa which normally are released prior to Stage 1 were retained in some Stage 1 tubules of injected animals. A single local injection of Fungizone, at a dose level of 0.2 mg/kg, into the cava vaginalis did not result in any observable change of the seminiferous epithelium. Unilateral castration had no effect on spermatogenesis in the remaining testis.

105. Sampling Boar Testes To Study Spermatogenesis Quantitatively and to Predict Sperm Production.

J.J. Kennelly and R.H. Foote

The volumetric proportions of eight testicular structures in boar testes were estimated histologically. Four predetermined areas were sampled to determine if each area were representative of the entire organ. Six 2-year-old boars (group I) and nine 1-year-old boars (group II) were sampled. Boars in group I differed significantly with respect to spermatogonia, unclassified nuclei, total tubular cytoplasm, total tubule, intertubular tissue and artifacts. Similarly, the four locations sampled differed significantly with respect to lumen and total tubule. Boars in group II differed significantly with respect to primary spermatocytes, total tubule, non-tubular tissue and artifacts. Left and right testes did not differ in either group of boars, indicating that boar evaluation can be done by properly sampling one testis. A formula is proposed for converting the volumetric proportion of primary spermatocytes to predicted maximum daily sperm production (PMDSP). For group-I boars PMDSP was 31.3×10^9 and the corresponding value for group-II boars was 13.4×10^9. The daily sperm output for group-I boars including estimated sperm losses in the collection procedure, was 19.7×10^9 sperm per day. The total sperm collected per day equalled 63.0% of the predicted values based on the histological data. The difference between these two values is considered to be primarily a function of in vivo sperm resorption, and losses in the urine.

106. Water: The Chief Ingredient in Semen Extenders. R.H. Foote

Water is the chief component of any extender used to preserve sperm. Tap water was distilled and redistilled by multiple procedures and used to prepare semen extenders containing 20% by volume egg yolk. There was no difference in sperm survival during storage at 5 °C, indicating that with egg yolk present to protect against any residual ions after distillation, any distillation procedure carefully done should safeguard against the presence of potentially damaging salts.

107. **A Comparison of Several Photoelectric Procedures for Estimating Sperm Concentration in Dog Semen.** R.H. Foote and J.H. Boucher

Photoelectric methods for estimating sperm concentration in dog semen were tested with 3 colorimeters and a spectrophotometer. A 2.9% sodium citrate diluent was found to be more satisfactory for making optical density measurements than higher concentrations of this salt. The optimum dilution rate varied with the initial concentration of sperm in the semen samples and the equipment used. Dilution rates of 1:4, 1:9, and 1:16 gave optical density values which were highly correlated ($r \geq 0.92$) with sperm counts measured with the hemacytometer. The optical density values for all instruments were highly correlated ($r=0.99$). After the regression equations to predict sperm concentrations from absorbance or transmittance measurements were calculated, routine estimation of the sperm concentration in dog semen by this method proved to be rapid, repeatable, and accurate.

108. **An Improved Artificial Vagina for Collecting Rabbit Semen.** P.J. Bredderman, R.H. Foote and A.M. Yassen

An inexpensive artificial vagina has been developed which can be cleaned and sterilized without disassembly, thereby simplifying semen collections in rabbits. Quantitative semen studies are facilitated because sperm losses in collection can be virtually eliminated. The design of the artificial vagina is illustrated.

109. **Influence of pH on Survival and Fertility of Bull Sperm.** R.H. Foote

The mean percentages of motile spermatozoa over a 12-day storage period for 5 C for Illinois Variable Temperature (IVT) and Cornell University Extenders (CUE) with added CO_2, and IVT and CUE with citric acid added to reduce pH by an equivalent amount, were 51, 53, 54, and 58, respectively. Corresponding values at 25 C were 51, 48, 54, and 55. The small difference in favor of citric acid was significant ($P < .05$). Sperm metabolism was inhibited to a greater extent by the addition of CO_2. The rise in pH which resulted from CO_2 loss and caused death of the sperm cell under certain storage conditions could be detected in unopened containers by the inclusion of innocuous levels of bromthymol blue. The fertility of bull sperm in CUE with citric acid added to adjust pH to approximately 6.80, 6.65, and 6.50 was tested in two field trials totaling about 85,000 first inseminations. The corresponding 60- to 90-day nonreturn rates were 72.6, 74.6, and 73.9 ($P = .10$) in the first trial, and 74.5, 74.7, and 74.7 ($P > .10$) in the second trial. These results demonstrate that the pH of extender reduced to 6.5 had no harmful effect.

110. **Effect of Catalase and Anaerobic Conditions Upon the Post-Thawing Survival of Bovine Spermatozoa Frozen in Citrate- and Tris-Buffered Yolk Extenders.** J. Steinbach and R.H. Foote

Semen samples from six different bulls were extended in citrate-fructose-yolk-glycerol (CFYG) and Tris-fructose-yolk-glycerol (TFYG) extenders with and without catalase, sealed under a high partial pressure of purified nitrogen or argon gas, and equilibrated with glycerol for 6 or 18 hr, and then frozen at rates of 0.8, 3.0, and about 8.0 C per min in the critical region from 5 C to -15 C. The mean percentages of motile cells in TFYG and CFYG were, respectively, 36 and 31 immediately after thawing and 31 and 22 after a post-thawing storage period of 24 hr at 5 C. Thus, the percentage of motile cells in TFYG 24 hr after thawing was equal to that found in freshly thawed CFYG. Neither catalase nor the inert gases exerted a significant influence upon post-thaw recovery of motile spermatozoa, although in the absence of catalase argon gassing showed a positive effect during the period of liquid storage. The slight differences between the periods of glycerol equilibration and among freezing rates were not significant.

111. **Evaluation of a Testicular Biopsy Technique in the Rabbit.** A.F. McFee and J.J. Kennelly

A simple and convenient open-surgery procedure for testicular biopsy was tested on rabbits at 1,

3, 5 and 11 months of age. Six rabbits in each group were anesthetized and injected with procaine at the base of the scrotum. An avascular area on the tunica albuginea was selected for biopsy after making a small incision in the scrotum and tunica vaginalis. The technique yielded a representative sample of testicular material while damaging less than 1% of the remaining testicular tissue. At least two biopsies can be taken from the sufficiently separated areas of a testis at either 2- or 14-day intervals without the second sample being affected by the first.

112. **Post-Thaw Survival of Bovine Spermatozoa Frozen by Different Methods in Buffered-Yolk and Skimmilk Extenders.**
J. Steinbach and R.H. Foote

Semen samples were extended in citrate-yolk-glycerol (CYG), Tris-yolk-glycerol (TYG), and skimmilk-glycerol (SMG), frozen in a dry ice-alcohol bath and transferred to liquid nitrogen for storage at -196 C. Glycerol levels (v/v) of 4.0, 5.6, and 7.2% in CYG, 6.4, 8.8, and 11.2% in TYG and 9.0, 13.0, and 17.0% in SMG, the addition of 1.0% fructose, equilibration times of 1, 6, and 18 hr, freezing rates requiring 4, 8, 16, and 34 min, and storage for 1, 7, or 40 days were studied. The TYG extender consistently resulted in a higher percentage of sperm surviving freezing than did the CYG or SMG extenders ($P < .005$). The optimum level of glycerol in TYG was about 8.8%, in CYG and about 7.2%, and in SMG it was less than 9%. Fructose added to CYG and SMG was beneficial. Sperm motility did not decrease during storage at -196 C. Bulls differed significantly ($P < .005$) and a large bull × extender interaction was found. Many other statistically significant interactions ($P < .05$) suggest that optimum conditions at one step in freezing are altered by changes in other steps.

113. **Sperm Losses From Semen Collection to Insemination**. R.H. Foote

Up to 20% of the sperm collected from bulls, boars, dogs and rabbits with the artificial vagina may be left adhering to the collection equipment. This loss may be reduced by altering the equipment and collection procedure. In the artificial breeding of dairy cattle up to 10% of the sperm may be left in the ampule, and 10 to 39% left in the catheter, depending upon the extender and rate of expulsion of semen into the uterus. Reduction of this loss would be of economic importance and could result in more efficient use of superior sires.

114. **Are You Throwing Away Fertile Sperm From Good Bulls?** R.H. Foote

Losses of valuable sperm are considerable in the artificial vagina, and proper sized A.V.'s should be used to minimize this loss. Large numbers of viable sperm may be thrown away with the discarded ampule or inseminating catheter. Thawing and opening the ampule in an upright position and removing the extended semen carefully minimized the loss from the ampule. Slow steady expulsion of the semen over a period of several seconds will deliver much more of the sperm to the cow than a fast jerky expulsion. Without these precautions as much as 40% of the sperm a bull ejaculates are accidentally discarded. At the present time it is not possible to state accurately how many motile sperm must be inseminated to give a maximum conception rate. Individual packages of sperm that can be transferred into the cow are needed to eliminate field losses.

115. **Duration of Spermatogenesis and Spermatozoan Transport in the Rabbit Based on Cytological Changes, DNA Synthesis and Labeling with Tritiated Thymidine.**
E.E. Swierstra and R.H. Foote

The duration of spermatogenesis in the rabbit was determined by injecting in a single dose 1 millicurie of thymidine-methyl -H^3 per kg into the marginal ear vein of each of 14 Dutch-belted bucks. At different intervals, biopsies and unilateral castrations were performed on nine of the 14 bucks to time the stages of spermatozoan formation in the testis and transport though the epididymis. The remaining five animals were ejaculated for 63 days to determine the interval between the thymidine-H^3 injection and the appearance of the isotope in the ejaculated semen. Histological sections, 8 μ thick, were stained with the Feulgen technique and coated with NTB 3

nuclear emulsion. The autoradiographs showed that the leptotene primary spermatocytes were the most advanced spermatogenic cells that incorporated the label. This agreed with the microspectrophotometric determinations of DNA which showed this to be the last stage at which DNA was synthesized in spermatogenesis. Therefore there appears to be no DNA exchanged during spermiogenesis. Uptake of tritiated thymidine by spermatogenia also was in accordance with measurements of DNA synthesis and content of these cells. Thirty-one days post-injection the label had progressed from leptotene primary spermatocytes to spermatozoa leaving the testis. At this time a few labeled spermatozoa also were present in semen smears taken from the caput epididymis. Epididymal transport time was eight days for one rabbit and ten days for four rabbits. The duration of one cycle of the seminiferous epithelium based on 55 estimates was 10.9 ± 0.1 days. The lifespan of type A spermatogonia, type 1 intermediate spermatogonia, type 2 intermediate spermatogonia, type B spermatongonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa was estimated to be 3.4, 0.6, 2.8, 1.1, 16.5, 0.8, 10.0 and 15.6 days respectively. The estimated total duration of spermatogenesis in the rabbit depends on the point chosen as the onset of spermatogenesis. If spermatogenesis is considered to begin with the first of the series of spermatogonial divisions leading to the production of primary spermatocytes then about four cycles of the seminiferous epithelium or 4 × 10.9 = 43.6 days are required. However, if one assumes that spermatogenesis starts with the formation of spermatogonial stem cells and that the lifespan of these stem cells is one cycle of the seminiferous epithelium then spermatogenesis extends over about 4.75 cycles, or 51.8 days.

116. Superovulatory Response of Pre- and Post-Pubertal Rabbits to Commercially Available Gonadotrophins.

J.J. Kennelly and R.H. Foote

The superovulatory response of Dutch-belted does injected with several standard gonadotrophin preparations twice daily for 3 days at 12, 16 and 20 weeks of age and in adult animals was examined to determine the relationship between age, dose and response. The effect of age and the age × treatment interaction were highly significant ($P < 0.01$). At 12 weeks of age 0.31 mg and 0.50 mg FSH/injection resulted in overstimulation without ovulation. Pre-priming with progesterone apparently counteracted high FSH levels at this age because 0.125 mg FSH with and without progesterone resulted in 16.8 and 1.8 ovulation points respectively. Similar results were obtained at 16 weeks of age, but at 20 weeks of age the number of ovulation points was lower in the progesterone-treated groups. Adult rabbits responded with a mean of 53.7 ovulations following administration of 0.50 mg of FSH. Overstimulation reduced the rate of egg recovery. This was attributed partially to the effect of endogenous oestrogen production on the rate of egg transport and partially to eggs being trapped in follicles.

117. Estrous Cycle Synchronization and Fertility of Gilts Fed Progestational and Estrogenic Compounds.

W.G. Pond, W. Hansel, J.A. Dunn, R.W. Bratton and R.H. Foote

Eighty-five Yorkshire and Berkshire × Yorkshire gilts were used in three experiments to determine the effect of the addition of a progesterone or estrogen derivative to the feed on the estrous cycle and on subsequent fertility. In experiment 1 the addition to the ration of 0.66 or 1.1 mg of AMP per kg of body weight for a 15-day period was effective in inhibiting estrus in all gilts during the feeding period. Twenty-one of 22 treated gilts showed signs of estrus within 1 week after hormone withdrawal. Some of the gilts in each group slaughtered 2 or 4 days following artificial insemination had a high incidence of cystic ovarian follicles, especially gilts on the 0.66 mg level. The estrous cycle was synchronized to some degree at the second cycle following hormone withdrawal (21- to 33-day cycles). Conception at this estrus using natural service was 83.3%. Average litter size for the six gilts that farrowed in each of the control, 0.66-mg and 1.1-mg groups was 8.7, 10.2 and 7.8 live pigs, respectively. In experiments 2 and 3 the feeding of 16 mg, of MEE per gilt daily for 9 days, followed by feeding 1.32 mg of MAP per kg of body weight daily for 9 days, inhibited estrus in 29 of 31 gilts. Treated gilts showed typical signs of estrus, including swollen vulvas and behavioral changes, 3 to 8 days after hormone withdrawal in experiment 2, and 4 to 10 days in

experiment 3. However, 2 of 11 treated gilts in experiment 2 and 5 of 20 in experiment 3 refused to accept the boar. First service conception of mated gilts was 81.8% for controls and 77.8% for treated gilts in experiment 2 and 100% for controls and 93.3% for treated gilts in experiment 3. Corresponding average litter sizes (live + stillborn pigs) were 8.1 and 8.9 in experiment 2 and 10.4 and 11.7 in experiment 3.

118. Oocytogenesis in Rabbits. The Role of Neogenesis in the Formation of the Definitive Ova and the Stability of Oocyte DNA Measured with Tritiated Thymidine.

J.J. Kennelly and R.H. Foote

A series of six thymidine-methyl-H^3 (thy-H^3) injections, one every 12 hours, was administered to 35 Dutch-belted female rabbits (does) commencing on the day of their birth. Seventeen of the does were unilaterally ovariectomized as follows: five does at 4 and 12 weeks of age, five does at 4 and 20 weeks of age, five does at 12 and 20 weeks of age and two does at 4 and 40 weeks of age. Autoradiographs of ovarian tissue were prepared, up to 600 oocytes per ovary were randomly chosen and the silver grains associated with the oocyte nuclei recorded. The mean grain counts decreased significantly ($P < 0.05$) from 4 to 12 weeks, 13.5 vs 10.1, and from 4 to 20 weeks, 14.7 vs 11.4. However, the mean grain counts did not differ significantly ($P > 0.1$) between 12 and 20 weeks of age, 10.5 vs 9.6. Although the mean grain count decreased between 4 to 40 weeks of age 21.5 vs 14.6, the number of replications was too few to detect significant differences ($P > 0.1$). At 40 weeks of age 91% of the oocytes were still labeled. The reduction in grain counts from 4 to 12 weeks of age was attributed to the non-random degeneration of the older, more highly labeled oocytes located deep in the cortical zone. The lack of significant differences between grain counts at 12 and 20 weeks of age suggests that (1) during this interval the desoxyribonucleic acid (DNA) is metabolically stable and (2) significant de novo oocyte formation did not occur. Nine rabbits were superovulated at 20 weeks of age and of the 217 ova recovered 82.5% were radioactive. This was similar to the 89.8% radioactive oocytes observed in the ovaries of the same animals. Artificial insemination of the remaining nine does at 35 and 60 weeks of age resulted in normal litters and superovulation and artificial insemination of this group at 52 weeks of age produced 73.8% radioactive ova. Five Dutch-belted does injected at four weeks of age with thy-H^3 at the same rate and interval as the day-old rabbits showed a slight incorporation of the isotope into both oocyte nuclei and cytoplasm suggesting that most of the incorporation was not related to any synthesis of new chromosomal DNA. A single thy-H^3 injection into three 20-week-old does did not result in appreciable uptake of the isotope by oocyte nuclei, although many nuclei of parenchymal cells in the ovary were highly labeled. The results conclusively support the view that most, if not all definitive ova are formed at birth and de novo oocytogenesis does not occur in the post-pubertal rabbit.

119. The Effect of Plastic Devices in the Uterine Lumen on Pregnancy and Parturition in the Rabbit.

E. Brown and R.H. Foote

The rabbit was selected to test the hypothesis that they could be used as a model to test the contraceptive effectiveness of intrauterine devices (IUD's). Various lengths of plastic material were fabricated and implanted in the uteri (one side only or both sides). Fourteen does had IUD's inserted and at laparotomy on Days 11-13 there were many implants, but fewer than on the control side. Based upon kindling data, there was little suppression of reproduction by the IUD's in the rabbit.

120. Bovine Male Anatomy and Spermatogenesis. R.H. Foote

The reproductive system of the bull is highly developed and coordinated to provide a place for the birth and nurture of spermatozoa. The testes are factories capable of mass production (up to 10,000,000,000 units per day) of a highly complicated and beautifully assembled product--the spermatozoan! By means of ducts, particularly in the epididymis, spermatozoa are conveyed, matured and stored. During this process some spermatozoa may pass their peak in vitality, die, be reabsorbed or otherwise lost. Much is yet to be learned about this. A great challenge to the animal breeding industry is to learn how to harvest all the spermatozoa in the

best condition possible through sexual preparation and frequent semen collection, and to preserve and use the spermatozoa in this condition in the field.

121. Osmotic Pressure and pH Effects on Survival of Frozen Bovine Spermatozoa.

J. Steinbach and R.H. Foote

Semen from ten bulls was extended in egg yolk extenders buffered with five concentrations of Tris adjusted to four pH levels, and containing two levels of glycerol, to study their interactions during semen freezing. Spermatozoa in these 40 media (5 × 4 × 2) were equilibrated for 1.5 and 6.0 hr, frozen at 0.8 and 3.0 C/minute in the critical range, and stored seven days at -196 C. Duplicate estimates of the percentage of motile spermatozoa made immediately after thawing (6,400 observations) indicated that the freezing rate and equilibration time had little effect on survival. The percentages of motile spermatozoa for the 6.4 and 8.8% glycerol levels were 30 and 28, for the 0.15, 0.20, 0.25, 0.30, and 0.35 M Tris-buffered yolk extenders 37, 40, 37, 28, and 5; and for the pH's of 6.0, 6.5, 7.0, and 7.5, 25, 29, 31, and 31, respectively. These effects were statistically significant ($P < .005$). Several main factors interacted in opposing ways, thus reducing their overall differences. The highest percentage of motile spermatozoa was observed in the extender adjusted to pH 6.50 containing 0.20 M Tris, 6.4% glycerol and 1% fructose; motility averaged over the two equilibration periods was 43% upon thawing and 33% after an additional storage period of 24 hr at 5 C. This extender without glycerol was slightly hyperosmotic to sperm, as evidenced by a freezing point depression of 0.62 C.

122. Ovarian Response and Cleavage Rate of Ova in Control and FSH-Primed Rabbits Receiving Varying Levels of Luteinizing Hormone.

N.B. Varian, R.R. Maurer and R.H. Foote

The superovulatory response of Dutch-belted does receiving 0.10, 0.25, 0.50, 1.00, 2.00 and 4.00 mg of LH/kg of body weight following priming with twice daily injections of 0.50 mg of FSH each for 3 days was examined. The does were inseminated immediately following LH injection. Rabbits given the lowest level of LH did not ovulate, and an average of 1.0 ovulation points was obtained following the 0.25 mg level of LH. All does receiving ≥ 0.50 mg of LH ovulated, with an average of 33.7, 40.0, 45.1 and 35.6 ovulation points for the 0.50, 1.00, 2.00 and 4.00 mg levels, respectively ($P > 0.10$). Some large unruptured follicles remained in all groups after they received LH. The proportion of cleaved ova collected from FSH-primed does 26, 28 and 30 hr after LH administration was 68, 86 and 91% respectively, whereas in non-primed controls all fertilized ova appeared to have cleaved by 26 hr. This difference is attributed to the longer time required for ovulation to be completed in FSH-primed animals rather than a delay in cleavage rate following fertilization.

123. Efficiency of Liquid Scintillation Counting and Autoradiography for Detecting Tritium in Spermatozoa.

W.L. Hunt and R.H. Foote

Assays by autoradiography (grain counts) and liquid scintillation (disintegrations per minute, dpm) were made on semen samples collected from 5 rabbits for 63 days following injection with H^3-thymidine. The mean grain counts and mean dpm determined on semen samples containing radioactive spermatozoa were highly correlated ($r = 0.93$, $P < 0.001$). With this high relationship it was possible to calculate a meaningful regression of mean grain counts on dpm. It was found that 5.8 disintegrations were required per grain observed in NTB-3 emulsion for an autoradiographic efficiency of 17.3%. Utilizing direct sample preparation, liquid scintillation counting was demonstrated as a rapid method to detect initial appearance of H^3-labeled spermatozoa. The correlation coefficient calculated for counts obtained on duplicate subsamples was 0.99. The efficiency of the procedure was estimated by means of external standards to range from 9.8 to 12.5%. Inclusion of thixotropic gel reduced errors in counting, but actual solidification of the mixture was not necessary, as nongelled and gelled samples averaged 1293 and 1202 dpm, respectively.

124. **Freezability of Bovine Spermatozoa in Tris-Buffered Yolk Extenders Containing Different Levels of Tris, Sodium, Potassium and Calcium Ions.**
A.M. Yassen and R.H. Foote

This study was designed to investigate the effect of different species of ions and osmotic pressure on survival of bull spermatozoa during freezing to -196 C. Replacing 20 to 80% of the Tris-buffered 20% yolk extender with isosmotic NaCl, KCl, or $CaCl_2$ solutions was found to be harmful. Potassium was more harmful than Na or Ca at the highest level of replacement. When either 10 or 20% by volume of a 0.25 M Tris-buffered 1% yolk extender was replaced with isosmotic Na and K chloride and citrate salt solutions, survival of spermatozoa during freezing was not affected. Replacement of 50% or more of the Na citrate with K citrate in 1 and 20% yolk-citrate extenders reduced sperm cell survival before and after freezing. These results indicate that the optimum levels of Na and K in extenders for freezing spermatozoa are less than the normal intracellular concentrations of these ions. Osmotic pressure of a 0.25 M Tris-buffered 20% egg yolk extender (freezing point depression, 0.55 C) was increased by adding NaCl, KCl, and $CaCl_2$ singly and in all combinations. As the osmotic pressure increased, motility of the spermatozoa after freezing declined in a linear manner, regardless of the tonicity contributed by each cation.

125. **Relationship of Ion Concentration to the Critical Temperatures During Freezing of Bull Spermatozoa.**
A.M. Yassen and R.H. Foote

The over-all osmotic pressure of the extender is an important factor affecting the critical temperatures during which most of the damage to motility of bull spermatozoa occurs during freezing. For a 0.10 M Tris-yolk extender containing various levels of NaCl, KCl, or $CaCl_2$ the critical temperature begins at -30 to -35 C, and for a 0.30 M Tris solution containing corresponding salts this temperature begins at -10 to -15 C. Post-thaw survival of spermatozoa was higher in 0.3 M than in 0.1 M extender. Addition of $CaCl_2$ was beneficial and addition of KCl was harmful.

126. **Factors Prolonging Survival of Unfrozen Bovine Spermatozoa.**
R.H. Foote

Bovine spermatozoa were extended with Cornell University Extender (CUE) and other buffered yolk extenders, placed in glass ampules and sealed, or gassed with various combinations of air, O_2, CO_2, and N_2 before sealing. Inclusion of catalase was highly beneficial to spermatozoa stored in air or a CO_2-O_2 atmosphere. Sperm cell survival in CUE containing catalase with air in the gas phase was equal to survival observed in the presence of any gases used. Up to 42% of the spermatozoa were motile after 30 days of storage at 5 C.

127. **Sodium and Potassium Content of Bull Spermatozoa During Processing Prior to Freezing.**
A.M. Yassen and R.H. Foote

The average values in mcg/ml for the whole bull semen, spermatozoa and seminal plasma for Na were 1,678, 1,027 and 1,768; corresponding values for K were 1,187, 1,887 and 1,095. The Na and K concentration in semen varied greatly between bulls. Extending the semen with Tris-buffered egg yolk at room temperature caused the cells to outflux 85% of their Na and 75% of their K within 15 min after extension. This resulted in an equilibrium between intra- and extracellular Na, but intracellular K remained three times as high as K in the extracellular fluid. When spermatozoa were extended and stored at room temperature or cooled to 5 °C, only moderate changes took place following the decrease of intracellular Na and K at the time of extension. Na and K levels tended to rise ($P < .05$) during cooling and storage except when glycerol was added. Intracellular K concentration was maintained at a higher level than intracellular Na during a 4-hr period, but neither ion concentration was as high as intracellular levels in fresh bull semen.

128. **Influence of Light and Agitation on Bovine Spermatozoa Stored with Protective Agents.** R.H. Foote

Bovine spermatozoa were stored at 5 C in the Cornell University Extender (CUE) consisting of

20% egg yolk medium and exposed to 2,150 lux of illumination. Viability of spermatozoa following agitation and exposure to light was determined in a series of factorially arranged experiments combining the effects of catalase, N_2 gassing, spectral filtering of the light, and the radiation-protective agent, β-mercaptoethylamine (MEA). Both light and agitation were spermicidal ($P < .01$). The two factors interacted dramatically, causing rapid immobility of spermatozoa stored in the air, whereas progressive motility in other treatments was retained for more than 12 days. Replacement of air with N_2 in sealed glass ampules was highly effective and catalase was partially effective in negating this harmful effect on motility of spermatozoa ($P < .01$). Attempts to remove all traces of O_2 with MEA were thwarted by toxicity of MEA to spermatozoa. These results indicate that only part of the damaging effect of O_2 is catalase specific (H_2O_2 formation), and that other harmful mechanisms may be inhibited by replacement of air with N_2. Spectral filtering of white light revealed that the blue region was particularly damaging to spermatozoa and to catalase. The spectral characteristics of the cytochromes suggest that these heme proteins, like catalase, may be particularly sensitive to the blue light and serve as photon acceptors, thus initiating a series of photochemical reactions damaging to the cell.

129. **Influence of Light on Nuclear Size and Deoxyribonucleic Acid Content of Stored Bovine Spermatozoa.** S.K. Paufler and R.H. Foote

Feulgen-positive material, assumed to be deoxyribonucleic acid (DNA), was determined by microspectrophotometry for 790 spermatozoa from 17 bulls stored in Cornell University Extender (CUE) at 5 C in sealed ampules. Factorially arranged treatments included light and dark, air and N_2 gassing, and addition of β-mercaptoethylamine (MEA). Light was associated with reduced motility and increased nuclear area, the correlation between the two variables being -0.51 ($P < .05$). Light also reduced the DNA content from 4.52 to 3.70 relative units ($P < .01$). The correlation between motility and total DNA content corrected for nuclear area was 0.70 ($P < .01$). N_2 was more effective in preserving sperm motility than in preventing a decrease in DNA in sperm exposed to light. Bulls differed in size of the sperm nuclei (range 24.9 to 31.5 μ^2), and in DNA content, but there was no significant relationship between either variable and fertility. Addition of 0.5 M MEA was toxic to spermatozoa and caused complete disappearance of the nuclei exposed to light in an air atmosphere. In N_2 many nuclei were shrunken. Other nuclei in the presence of MEA were swollen or ruptured, or both, as were the nuclei of spermatozoa stored in the dark with air or N_2.

130. **Freezability of Bull Semen in Centrifuged or Dialyzed and Reconstituted Buffered Egg Yolk Extenders.** A.M. Yassen and R.H. Foote

Bull spermatozoa were frozen with equal success in a 0.25 M Tris-buffered extender containing either the supernatant or precipitated portion of centrifuged egg yolk. Washing the precipitate with distilled water to remove additional ions and other water-soluble materials had little effect on the osmotic pressure of the washed egg yolk precipitate reconstituted with Tris, but viability of spermatozoa both before and after freezing was reduced. Egg yolk was dialyzed against distilled water until the Na and K levels were reduced to 12 and 10 μg per ml, respectively. Dialyzed and non-dialyzed egg yolk were combined with Tris buffer, and sperm cell survival following freezing was higher than in the Tris-yolk control ($P < .01$). Post-thaw motility of spermatozoa ranged from 80 to 90% of the prefreeze motility. The results indicate that high survival of bull spermatozoa can be achieved with an extender low in all ions except those supplied by Tris.

131. **Research on Converting Bulls to Blue Chip Stock.** R.H. Foote

This paper describes the procedures to follow to obtain maximal use of genetically superior bulls. These include selection of bulls with large testes, harvesting sperm often and preceding semen collection with intensive sexual preparation. Objective semen evaluation followed by the best processing procedures, using sufficient sperm without wastage should be other goals, along with training of personnel handling semen in the field.

132. **Artificial Insemination.**
R.H. Foote and G.W. Trimberger

This chapter plus later editions by the senior author describe artificial insemination from management of the male, semen collection, evaluation and processing and finally insemination. Species discussed include cattle, swine, horses, sheep, goats and poultry.

133. **Artificial Insemination of Dogs.**
R.H. Foote

This chapter includes a diagram of the semen collection equipment for dogs. The simple funnel and collection tube was superior to more complex artificial vaginas with various rubber components. Semen was collected directly into the collection tube without loss or drying as occurred on the latex liner of the complex A.V.'s. Semen could easily be collected in three fractions (pre-sperm fraction, the sperm fraction and prostatic fluid in the third fraction). Sperm motility was higher using this equipment than when using the latex liner and latex cone attached to a collection tube.

134. **Repeated Superovulation Following Administration of Exogenous Gonadotrophins in Dutch-Belted Rabbits.**
R.R. Maurer, W.L. Hunt and R.H. Foote

Repeated superovulation and in-vivo collection of ova were carried out with thirty does divided into three groups of ten does each. One group received FSH-LH, a second group received PMSG-HCG and the controls received only LH. Injections of FSH-LH at three 16-week intervals followed by one 8-week interval resulted in 46.5, 35.4, 25.2 and 18.0 ovulation points/doe. This decrease with time was significant ($P < 0.05$). Corresponding values for the PMSG-HCG group which, however, received FSH-LH for the final superovulation were 13.6, 5.7, 6.2 and 20.3. The LH controls averaged 7.8, 7.0, 5.1 and 5.6 ovulation points. Overall treatment differences were highly significant ($P < 0.005$). From the 1920 ovulation points 1593 ova (83%) were recovered, of which 83.1% were cleaved. Young born from unrecovered ova accounted for 3.1%. Control kindlings by the same does at regular periods resulted in normal litter size but in fewer does kindling as the experiment progressed. Results of two- bioassays for antihormones suggested that this decrease was due to hormonal refractoriness which was most pronounced in the PMSG-HCG group.

135. **Developmental Potential of Superovulated Rabbit Ova.**
R.R. Maurer, W.L. Hunt, L.D. VanVleck and R.H. Foote

Female rabbits were superovulated with either FSH-LH or PMSG-hCG and embryos recovered and transferred. Also animals bearing young without embryo transfer were included for comparison. The overall percentage of pregnant recipients following embryo transfer was 24/26 (92%) for the FSH-LH group, 12/17 (71%) for the PMSG-hCG group and 20/23 (87%) for the LH alone group. Young from all groups grew similarly and could be cross-fostered successfully when litters were large.

136. **Survival of Bull and Ram Spermatozoa in Preparations from Skimmilk.** R.C. Jones

Media containing the nondialyzable components of skimmilk were prepared in dialysis sacks by placing the sacks filled with skimmilk in buffered saline and storing at 5°C for 48 hours. Similarly, media containing the dialyzable components of milk were prepared by placing dialysis sacks of buffered saline in skimmilk. When these preparations (alone and in combination) were mixed 1:1 with a solution of 185 mM lactose and electrolytes, bull and ram spermatozoa survived at 37°C better in the nondialyzable than in the dialyzable preparations, and either of these preparations was better than 1:1 mixture of both. Heating the dialyzable preparation or mixtures of both preparations before use increased survival rates, but heating rendered the lactose solution harmful to bull and ram spermatozoa. When the concentration of sodium chloride was varied in buffered saline diluents containing heated dialyzable and unheated and heated nondialyzable milk preparations, it was found that media containing the nondialyzable preparations had a higher tonicity (measured by the survival of bull spermatozoa) than

the dialyzable preparations. Heating the non-dialyzable preparations improved the survival of spermatozoa. Both preparations, either heated or unheated, were lyophilized and used in a factorial experiment at concentrations of 0.5 and 1.0% w/v in media of relative tonicities 0.8 and 1.0. Scores of survival of spermatozoa made after incubation at 30°C for eight hours were lowered by increasing the concentration of the lyophilized preparations and increasing the tonicity of the diluents. However, mean scores of per cent motile spermatozoa in samples from media containing unheated dialyzable and nondialyzable preparations were 25.4 and 24.6, respectively; for media containing preparations heated before lyophilization, the means were 18.8 and 33.8.

137. **Semen Extenders and Sperm Requirements.** R.H. Foote

This paper discusses storage of sperm at ambient temperatures, 5°C and frozen sperm. Requirements for sperm and how to meet these requirements are suggested. It is clear that knowledge of the crucial mechanisms involved is lacking and most of the progress in improving sperm survival has been made by a trial and error method.

138. **Evaluate the Whole Semen Processing Procedure Periodically.** R.H. Foote

Personnel and other factors which affect the nonreturn rate in any A.I. organization include: Bulls and bull handlers, semen collectors, laboratory personnel, field distributors, inseminators, and cows and cow managers. Immediate benefits can be expected by developing computer-assisted quality control systems which optimize the multiple use of existing tests of semen quality so that semen samples are selectively extended and inferior samples culled. Periodic spot checks should be made of all important steps in the semen handling system. With subjective tests such as motility the identity of the material must be unknown to the evaluator. Long range evaluation should include such concepts as the possibility of storing "sperm in waiting" instead of "bulls in waiting." Proper environmental housing of bulls and sperm during this period may essentially eliminate present undesirable detectable effects of season and senescence of old Ferdinand himself.

139. **Standards for Sperm Concentration: Polystyrene Latex Particles as An Aid in Quality Control.** R.H. Foote

A series of suspensions of polystyrene latex particles are simple to prepare and are technically suitable as standards for making turbidity (optical density) measurements photometrically. These suspensions should be of considerable value in calibrating and checking routinely photometers used for estimating sperm concentration. Preliminary work on collecting semen directly into optically clear diluents indicates that these optical methods of estimating sperm concentration are still valid. Once the data are transferred to cards or tape, computer programs can be prepared easily to (a) transform the data as necessary, (b) compute the appropriate correlations and regressions and (c) print out complete tables listing the proper extension rate to use for any combination of information on initial sperm concentration, % motile sperm and desired final sperm concentration per ml of extender or per insemination dose.

140. **In-vitro Culture of Rabbit Ova From Early Cleavage Stages to the Blastocyst Stage.** H. Onuma, R.R. Maurer and R.H. Foote

Rabbit embryos (1285 total) from the 2- and 4-cell stages were cultured to the hatched blastocyst stage in both rabbit and bovine serum. The bovine serum was superior. The addition of glucose did not increase the proportion of blastocysts formed, but the addition of 1% pronase for 5 minutes 52 to 60 hours in culture weakened the zona pellucida and promoted hatching (83%, 29/35, either hatching or hatched).

141. **Maturation Changes in Bull Epididymal Spermatozoa.** G. Igboeli and R.H. Foote

Spermatozoa recovered from the caput epididymides of four bulls were immotile, whereas sperm from the cauda epididymides averaged 41% progressive motility. The percentage of unstained or morphologically normal spermatozoa did not differ between the caput and cauda epididymides. When

caudal sperm were used to inseminate 100 cows, 69% did not return for reinsemination in 60 to 90 days.

142. Morphology, Motility and Fertility of Spermatozoa Recovered from Different Areas of Ligated Rabbit Epididymides.

S.K. Paufler and R.H. Foote

Forty-three males were divided into three experimental groups and ligatures placed unilaterally as follows: (1) on the ductus deferens, (2) on the ductus deferens and corpus epididymidis, and (3) on the ductus deferens, corpus epididymidis and ductuli efferentes. Semen was collected six times a week from all males before ligation and for as long as 12 weeks thereafter. Spermatozoa transported normally from the caput to the cauda epdidymidis in non-ligated controls were characterized by rapid migration of the protoplasmic droplets, a decrease in swollen acrosomes and other abnormalities, an increase in the percentage of motile cells and a striking increase in fertility. Ligation of the ductus deferens only had little effect upon any of these changes. Also, considerable motility and fertility was maintained for 12 weeks following single ligation in contrast to a reduction after 4 weeks in the group with the isolated cauda epdidymidis. This suggests that considerable mixing of spermatozoa normally can occur in the cauda. The proportion of abnormal forms, particularly decapitated spermatozoa, increased considerably in the isolated cauda by 8 weeks. In the isolated caput abnormal spermatozoa increased rapidly and motility decreased. The protoplasmic droplet movement was delayed, as 54% had droplets on the midpiece after 4 weeks in contrast with 16% on spermatozoa which migrated normally to the cauda. Severe degeneration and disappearance of spermatozoa followed after 4 weeks, indicating that the caput may have dissolution properties. Litter size, based on all does inseminated, averaged only 0.5 for caput spermatozoa compared with 5.0 for both caudal and ejaculated spermatozoa. The morphological and fertility data indicate that extrinsic factors as well as intrinsic ones are required for complete development of the fertilizing capacity of rabbit spermatozoa.

143. Sizing of Aging Bull Spermatozoa with an Electronic Counter.

R.H. Foote and P.J. Bredderman

Cell size was evaluated as a criterion of the functional state of spermatozoa. Packed cell volume of 13 samples of bull semen stored at room temperature for 1, 3, 5, 7, 9 and 11.5 hours averaged 8.7, 10.6, 11.2, 12.0, 12.1, and 12.2%. Corresponding mean window values obtained with a Coulter Counter were 12.3, 14.3, 15.6, 16.0, 16.1, and 15.9. Correlations between these two types of measurements ranged from 0.87 to 0.99 ($P < 0.01$) for 11 of the 13 bulls. Spermatozoa killed by freezing and thawing did not swell. Mixtures of live and dead spermatozoa stored at 7 C show a bimodal distribution formed by the two types of cells. Following death of all cells, a single distribution remained. In a study of size changes during storage of 87 ejaculates from six bulls, bulls were found to differ ($P < 0.05$). Samples high in sperm concentration swelled less and for a shorter period; motility declined more rapidly during storage. Size changes measured in living cells with this technique offer an approach to studying maintenance of integrity of the cell in a variety of environments.

144. Inheritance of Fertility in Cattle Inseminated Artificially. J. Hahn

Higher conception rates were obtained in both virgin heifers and cows when the following estrus characteristics were present at insemination: good standing heat, presence of heat mucus, intense red color of the internal portion of the vagina, easy cervical passage with a pipette, good uterine contractions, normal ovaries and follicles, and soft follicles. Precise determination of these estrus characteristics was difficult, and heritability estimates ranged from 0 to 0.24. Consequently, an improvement in fertility by selection of these characteristics would be uncertain. Heritability and repeatability estimates of nonreturn rates after first insemination in virgin heifers in herds with low fertility were 0.17 and 0.23, respectively, and nearly zero in herds with good fertility. Thus, the use of nonreturns to first insemination for virgin heifers in herds with low fertility appears to be more valuable for fertility improvement through selection, because 1) these

data can be obtained very easily and 2) this measurement determines the fertility complex better than other traits evaluated.

145. **Ultrasonic Treatment of Rabbit Testes.** J. Hahn and R.H. Foote

Ultrasonic waves were applied to one testis of each of 25 sexually mature Dutch-belted rabbits with the contralateral testis serving as a control. For all treatments transducers with the same frequency of 2.25 MHZ and the same acoustic power of 1 mW/cm^2 were used. Exposure time varied from 2 min to 10 min per location for each testis. The rabbits were sacrificed 1, 2 and 4 wk after treatment. Testis size was unaffected by treatment, and there was no difference between treated and untreated sides. Diameter of the seminiferous tubules and spermatogenic activity, as indicated by the proportion of Stage 8 tubules, was the same in the control and treated testes. The percentages of motile, unstained and normal sperm ejaculated or recovered from different parts of the epididymides also were unaffected by ultrasonic radiation. Therefore, ultrasonic evaluation of the testis appears feasible without risk of injury to the tissue.

146. **Volume of Stressed Bull Spermatozoa and Protoplasmic Droplets, and the Relationship of Cell Size to Motility and Fertility.** P.J. Bredderman and R.H. Foote

An electronic sizer (Coulter Counter) was utilized to determine the effects of four solutions of different osmolarities on cell volume of spermatozoa from two ejaculates from each of 17 bulls. Spermatozoa showed a decrease in volume in the hypertonic medium. A portion of the population of cells showed a marked increase in cell volume in the hypotonic medium. This increase was associated with live cells. Sperm cells killed by freezing and thawing did not show osmotic behavior. The absolute mean volume for spermatozoa at the time of measurement in iso-osmotic saline or Tris, hypo-osmotic saline, hypo-osmotic saline with killed spermatozoa, hyperosmotic Tris and hyperosmotic Tris with killed spermatozoa were estimated to be 25.2, 23.1, 32.4, 20.8, 20.0 and 20.4 μ^3, respectively. Since dead cells change little, swelling of live cells can be much greater than mean values indicate. These results clearly show the need to control osmolarity in studies of sperm cell size. Protoplasmic droplets were found to be osmotically reactive. Their mean volume in 0.87% saline was estimated to be 8.3 μ^3. The droplets appeared to rupture in hypo-osmotic media or upon freezing, leaving only debris visible microscopically. Thus, they may contribute materials to seminal plasma, which could alter its composition, unless the droplets are removed promptly after ejaculation. Bulls contributed the major source of variation excepting in treatments in which the spermatozoa were frozen before sizing. The significant bull effect in the four sizing media was significantly correlated with the motility at the time of sizing, but not with the bulls' fertility.

147. **Repeated Superovulation in Calves.** H. Onuma, J. Hahn, R.R. Maurer and R.H. Foote

Holstein calves were raised from birth under standard conditions of feeding and management. Gonadotropins were administered at 9 to 11 weeks of age followed by a second and third course of injections 8 and 16 weeks later. Injection of 50 mg of FSH, divided into five equal daily doses of 10 mg each, was much more successful in promoting follicular development than administration of 50 mg given as two equal doses (47.3 vs 1.0 follicles $\geq$ 1.0 cm in diameter). Injections of LH, following priming with FSH, resulted in only 3.8 ovulations, compared with an average of 15.4 ovulations when LH followed a single injection of 2,000 I.U. of PMS. Follicular and ovulatory response appeared to decline with repeated administration of gonadotropin. This decline is believed to be due both to the development of a refractoriness to the gonadotropins and to the development of adhesions around the ovary following repeated surgery. Injection of 17β-estradiol benzoate did not alter follicular response. None of the 38 ova obtained were cleaved. This may have been due to the difficulty of depositing semen beyond the entrance to the cervix in these calves. Thus, while a satisfactory ovulation rate was achieved with the best hormone treatment, and the calves readily survived the repeated laparotomies, procedures for insuring fertilization and efficient recovery of ova require further development.

148. **Estimation of Testicular Size in the Live Rabbit**. S.K. Paufler, L.D. Van Vleck and R.H. Foote

The total length (L) and maximum width (W) in centimeters of the testes, plus the thin scrotum, in 26 mature live Dutch-belted rabbits were measured with fine calipers. The 52 testes from these animals were removed, weighed, and the volume measured. From the measurements in vivo, testis volume and weight were calculated. The regression equation derived to predict testicular volume in milliliters was $\hat{Y}_v = -0.2699 + 0.3654\ LW^2$, and for testicular weight in grams it was $\hat{Y}_w = -0.2315 + 0.3731\ LW^2$. The correlation coefficients between predicted volume and actual volume and predicted and actual weight were both 0.90. Thus, the technique described for measuring testicular size in live animals permits accurate monitoring of changes associated with growth, environmental influences, or with experimental treatment without damage to the animal. Furthermore, the design of experiments can be made more efficient and costs of experimentation can be reduced.

149. **Physiological Aspects of Artificial Insemination**. R.H. Foote

This chapter provides an overview, with numerous references, detailing development of sexual function, and onset of puberty. This is followed by sections on libido of bulls, sexual preparation and semen collection. The evaluation and processing of liquid and frozen semen as well as reports on freeze-drying of sperm are included. The physiology of the cow relative to an artificial insemination program, estrous cycle regulation and factors affecting fertility are discussed.

150. **Motion Pictures of Ejaculation in the Bull**. G.E. Seidel Jr. and R.H. Foote

Four striking features were uniquely revealed by high speed photography. (1) A large volume of semen was sprayed over the inner surface of the A.V., indicating the need for proper length of A.V. to obtain a maximum proportion of the sperm in the collection tube. (2) A counterclockwise coiling of the glans penis (as much as 360°C) was found to be a normal occurrence in breeding bulls. (3) Sexual behavioral patterns, such as wiggling of the dew claws during mounting, were revealed. (4) Patterns of incomplete ejaculation associated with improper tactile stimuli were photographed.

151. **Maturation and Aging Changes in Rabbit Spermatozoa Isolated by Ligatures at Different Levels of the Epididymis**. G. Igboeli and R.H. Foote

Maturation and aging changes of epididymal spermatozoa were studied in 28 sexually mature Dutch-belted buck rabbits by retention of their sperm with ligatures for varying periods of time. Spermatozoa held in the caput epididymis by a ligature on the corpus epididymis for 4 days showed an increase in motility followed by a significant decrease in motility and percentage of live (unstained) cells after longer intervals. The percentage of abnormal sperm significantly increased ($p < 0.05$). Sperm from the caput epididymis showed little or no fertility, even after retention for periods of 4, 7, and 28 days. The shorter intervals give sufficient time for sperm to become fertile during normal migration. These results suggest that the caput epididymis was not a suitable site for sperm maturation, and that some extrinsic factors associated with more distal parts of the epididymis are required for sperm to achieve full fertility in the rabbit. There was a gradual loss of quality when spermatozoa were retained in the cauda epididymis, and by 21 days of storage the percentage of normal, live (unstained), motile, and fertile sperm had declined significantly ($p < 0.05$). However, some sperm were still fertile when examined 35 days after isolation. No difference was observed in the fertility of cauda epididymal spermatozoa obtained by mincing the cauda epididymis in saline and sperm obtained by ejaculation. Insemination of aged sperm 4 hr after HCG injections to reduce the period required for survival in the female prior to ovulation favored ova cleavage, but not the total number of young born. Ligatures on the vasa efferentia or corpus epididymis for 4 or more days restrict testicular output of fluids. This resulted in edema, increased size of the testis initially, and eventual atrophy of the testis followed by partial regeneration. The seminiferous tubules showed sloughing of spermatogenic elements, shrinkage, and absence of tubules in Stage 8. Ligating the corpus epididymis

caused an increase in the size of the caput epididymis, apparently as a result of accumulation of fluid from the testis.

152. Superovulation in Prepuberal Calves on Two Levels of Nutrient Intake.

H. Onuma and R.H. Foote

Fourteen Holstein calves were raised from birth through 8 weeks of age on a standard regimen. During the next 8 weeks, six heifers were randomly assigned to receive a ration consisting chiefly of hay, and the other eight heifers were placed on a high concentrate ration. During the 17th week, all heifers received 2,000 I.U. of PMS intramuscularly, followed by 25 mg of LH intravenously, 5 days later. TDN and protein intake, and average daily gain during this second 8-week period was approximately 50% higher in the high plane group than in the controls. No differences in ovarian response or size of the reproductive organs were found between groups ($P > .05$). The mean number of developed follicles and number of ovulations was 64 and 37 for the controls, and 69 and 34 for the high plane group, respectively. The pituitary weights for the controls averaged 0.73 gm and for the high plane group 1.06 gm ($P < .01$). These results indicate that high average rates of ovulation can be obtained in young dairy calves with the PMS-LH combination tested. However, considerable individual variation occurred. Since the two groups differed widely in energy and protein intake without differing in average response, it appears that under the usual feeding conditions factors other than nutritional status primarily are responsible for the large individual variation observed.

153. The Survival of Frozen Bovine Spermatozoa Following Minimum Exposure to Glycerol.

W.E. Berndtson and R.H. Foote

Bull spermatozoa in several buffered egg yolk media and in skim milk were exposed at 5°C to varying levels of glycerol for 6 hrs, 30 min, and approximately 10 sec prior to freezing. Freezing was done by placing droplets of the extended semen in small holes on blocks of Dry Ice. Highly significant improvement in sperm motility following freezing and thawing was obtained with the 10-sec exposure in comparison with the 30-min and 6-hr exposures, which did not differ from each other. Part of this difference can be attributed to the slightly harmful effect of glycerol on spermatozoa during the longer exposure periods at 5°C before freezing. However, the survival rate during freezing was improved by the 10-sec treatment, suggesting that the conditions provided by the brief exposure to glycerol more fully protected the spermatozoa than did prolonged exposure. Motility following freezing and thawing also was affected by the thawing medium, which indicates that this may be an important consideration in thawing material which is to be diluted at the time of thawing.

154. Spermatogenesis in the Rabbit Following Ligation of the Epididymidis at Different Levels.

S.K. Paufler and R.H. Foote

Ligatures were placed unilaterally on the ductus deferens (experiment 1), on the ductus deferens and the middle of the corpus epididymidis (experiment 2) and on the ductus deferens, corpus epididymidis and ductuli efferentes (experiment 3) in 39 rabbits. The untreated contralateral side served as a control. Vasectomy alone appeared to have no effect upon the testes, or upon spermatozoa accumulated proximal to the ligature during a period of 12 weeks. In experiment 2 severe disruption of spermatogenesis occurred. Diameter of the seminiferous tubules decreased, testes atrophied, and the stages of the seminiferous epithelium were abnormal and often not classifiable. Some regeneration was observed after 12 weeks. In experiment 3 there was only a moderate and more transient disturbance of spermatogenesis. This occurred despite an accumulation of fluid in the testis due to the ligature on the ductuli efferentes, which prevented testicular effluent from reaching the caput epididymidis. These results suggest that when testicular effluent is allowed to reach the caput, but nonresorbed residues are prevented from further transport (experiment 2), a feedback to the testis occurs which is more harmful than that produced by preventing tubular contents from leaving the testes.

155. **Influence of Semen Collection Interval and Tactile Stimuli on Semen Quality and Sperm Output in Bulls**. G.E. Seidel, Jr. and R.H. Foote

Most semen characteristics of eight Holstein bulls 28 to 36 months old were significantly affected ($P<.01$) by intervals between semen collections. First and second ejaculates, and ejaculates after five and two days of sexual rest were, respectively: ejaculate volume, (ml), 5.55, 5.24, 5.69, and 5.09; sperm concentration (10^9/ml), 1.72, 1.08, 1.53, and 1.27; initial sperm motility (%), 52.5, 52.3, 52.2, and 52.6; post-thaw motility (%), 36.5, 37.5, 36.7, and 37.3; and total sperm per ejaculate (10^9), 9.59, 5.55, 8.76, and 6.38. Bull differences were highly significant ($P < .01$) for all criteria studied. The length and temperature of the artificial vagina had little effect on any characteristics studied. Sperm output per week averaged 30.3 billion, and when adjusted for losses averaged 35.4 billion. Sperm output was positively correlated with maximum testes-scrotal circumference, $r = .76$ ($P < .05$). A concurrent experiment showed that ejaculation of sperm directly into a centrifuged tris-egg yolk extender caused a slight but significant ($P < .001$) increase from 36.2 to 37.5% in motility after freezing and thawing.

156. **Influence of Semen Collection Techniques on Composition of Bull Seminal Plasma**.
G.E. Seidel, Jr. and R.H. Foote

Seminal plasma from semen collected with an artificial vagina at 55°C contained slightly higher fructose concentrations ($P < .01$) and slightly lower chloride concentrations ($P < .05$) than plasma from semen collected at 38°C. However, neither artificial vagina temperature nor length had any significant effect on glycerylphosphorylcholine concentration of seminal plasma, freezing point depression, or the total amounts of chloride, fructose, or glycerylphosphorylcholine per ejaculate. First ejaculates and semen collections after five days of sexual rest were higher in glycerylphosphorylcholine and lower in chloride concentration than corresponding second ejaculates or collections after two days of sexual rest. There was a high positive correlation between sperm and glycerylphosphorylcholine concentrations ($r = .84$ on a within bull-experimental day basis) and negative correlations existed among glycerylphosphorylcholine, fructose, and chloride concentrations of seminal plasma. These relationships were expected on the basis of sources of these components in semen. No significant relationships between levels of any of the constituents and sperm motility before or after freezing were found.

157. **In Vitro Development of Ova from Prepuberal Cattle**.
H. Onuma and R.H. Foote

Ova obtained from superovulated and inseminated calves nine or 17 weeks old were cultured in bovine or rabbit serum, follicular fluid, and synthetic media, Krebs-Ringer-bicarbonate solution, and Ham's F10 medium. Of 184 ova cultured, 45% cleaved in vitro. No significant differences were found among media in their ability to support limited early embryo development. One-celled fertilized ova cleaved more times in culture than ova which had cleaved before collection ($P < 0.01$). None of the cultured ova cleaved more than 2.5 times and none cleaved beyond the 16-cell stage. However, this development is equivalent to the best results obtained with cow's ova in vitro and indicates that the potential of ova obtained from young calves is equivalent to those from sexually mature cattle.

158. **Testicular Growth and Related Sperm Output in Dairy Bulls**. J. Hahn, R.H. Foote and G.E. Seidel, Jr.

Scrotal circumference in living Holstein bulls was found to be a better measure of testis size than various linear measurements. This variable was easy to measure, was highly repeatable ($r = 0.98$) when measured by different investigators and was highly correlated with testis weight ($r = 0.92$). The regression equation for estimating testis weight ($\hat{Y}$) from scrotal circumference (X) in mature bulls was $\hat{Y} = -1298.5 + 50.2X$. Scrotal circumference ($\hat{Y}$) and age of bulls in mo. (X) showed a curvilinear relationship described by the multiple regression equation $\hat{Y} = -7.65 + 50.10 \log X - 12.44 (\log X)^2$. Semen was collected twice each Tuesday and Friday for 4 weeks from groups of Holstein bulls (10 bulls per group) which were 17 to 22, 34 to 42, 42 to 53 and 59 to 69 months of age. In addition 15 proven bulls

over 72 months of age, varying greatly in physical condition, were placed on the same schedule after an initial period of depletion. Average sperm output per week in order of increasing age group was 28.4, 41.5, 38.0, 42.7 and 28.2 × 10^9. These values are not corrected for loss in the collection equipment. Corresponding values for sperm output per week per g of testis were 61.2, 58.5, 53.6, 52.8 and 34.4 × 10^6. These data suggest that there is a reduction in spermatogenesis per unit of testis in old bulls, and indicate the desirability of capitalizing upon the large spermatogenic potential of sires 2 to 6 yr old. Correlations between scrotal circumference and sperm output per week were 0.81, 0.72, 0.64, 0.40 and -.22, respectively. The youngest group of bulls was ejaculated a year later and the correlation between scrotal circumference and sperm output per week was 0.69. The correlation between scrotal circumference measurements in consecutive years was 0.91. These results indicate that scrotal circumference is a useful indicator of potential current sperm output and subsequent testicular development in young bulls. However, it appears to be of little value in bulls more than 5 to 6 years of age, where other changes in the testes apparently mask the relationship of testis size to total spermatogenic potential.

159. Effect of Triethylenemelamine (TEM) and Cadmium Chloride on Spermatogenesis in Rabbits.

S.K. Paufler and R.H. Foote

Five groups of sexually mature, Dutch-belted male rabbits were studied. Group I consisted of ten controls. Group II (five males) received one injection of 0.2 mg of triethylenemelamine (TEM)/kg body weight. Group III (two males) received twice the amount of TEM given to Group II, and Group IV (five males) received five weekly injections of 0.4 mg TEM/kg body weight. Group V (five males) received one subcutaneous injection of 0.05 m-mole $CdCl_2$/kg body weight. Spermatogonial divisions were inhibited in TEM-treated animals. In Groups II and III, sperm output reached minimal levels 8 to 9 weeks after treatment. Recovery took place within 6 weeks. In Group IV, sperm output was also reduced to minimal levels by the 8th week. Slow recovery was initiated about 15 weeks after the initial TEM injections. Cadmium chloride caused extensive damage and marked atrophy of the testes, so that extra-tubular tissue accounted for about 75% of the testis. Aspermia was approached within 4 weeks. Little recovery followed during 17 weeks following treatment. The percentage of motile spermatozoa and morphologically normal spermatozoa was markedly reduced in Groups IV and V. All treated groups showed marked changes in the frequency of the eight stages of the cycle of the seminiferous epithelium and depression of fertility. Rapid recovery followed in the TEM-treated groups.

160. Components of Bull Semen Responsible for Tonicity-Induced Turbidity Changes.

W.E. Berndtson and R.H. Foote

Turbidity of semen diluted with saline was increased markedly by dialyzing it against distilled water and thereby decreasing the concentration of diffusible substances in the semen. The turbidity returned to normal when the reverse dialysis against 0.9% saline was employed. Separation of whole semen into sperm cells, seminal plasma, filtered plasma, and protoplasmic droplets revealed that seminal plasma was primarily responsible for the change in turbidity. Droplets were disrupted as the osmolarity of the suspending fluid decreased, but this in itself caused little change in turbidity. Turbidity of seminal plasma diluted with water was highly correlated with plasma protein concentration ($r = 0.80$), indicating that protein is a major cause of turbidity under these conditions. Thus, when estimating sperm cell concentration in semen by turbidimetric procedures, physiological diluting solutions, such as 0.9% NaCl, should be used to avoid possible interference by seminal plasma.

161. Semen Quality and Testicular Function in Rabbits Following Repeated Testicular Biopsy and Unilateral Castration.

S.K. Paufler and R.H. Foote

The effect of biopsying the same testis three times at 4-week intervals and of castration was investigated with Dutch-belted rabbits. Biopsy specimens averaged 22.6 mg and permitted thorough histologic evaluation. The frequency of the eight

stages of spermatogenesis and the diameter of the seminiferous tubules was unaffected by the operation. The proportion of round spermatids showed a significant decrease ($p < 0.05$) after the second and third biopsies, but testis size, sperm output, and sperm motility and morphology did not differ from the controls. Unilateral orchiectomy reduced sperm output to approximately half the previous level. Testis weight appeared to increase about 11% during 4 weeks following removal of the contralateral testis. Since the testes showed no change in weight during the 12 weeks preceding castration, compensatory hypertrophy is suggested.

162. Relationship of in Vivo Gamete Aging and Exogenous Hormones to Early Embryo Development in Rabbits.

R.R. Maurer, R.H. Whitener and R.H. Foote

Oocytes aged before fertilization by delaying insemination for 10 hr resulted in embryonic failure in culture prior to the blastocyst stage, similar to previous studies in vivo. Aging of spermatozoa for 10 hr before inducing ovulation with LH administration reduced fertilization and cleavage rates, and reduced the proportion of embryos that developed into blastocysts. The fertilizable life span of spermatozoa was prolonged by the environment of FSH-primed does. Overall the development in culture of embryos recovered from FSH-primed does was equal to or better than the controls. Some one-celled zygotes along with 2- and 4-celled embryos recovered from both groups developed into blastocysts in culture. Thus, the combined techniques of superovulation and embryo culture appear to be well suited to studies of this kind.

163. Changes in Epididymal Spermatozoa and in the Testes of Rabbits after Experimental Cryptorchidism.

G. Igboeli and R.H. Foote

Surgical cryptorchidism was induced in sexually mature rabbits by a scrotal incision approach or an abdominal approach, and the effects were observed for the next 14 days. Various combinations of ligatures on the corpus epididymidis and ductus deferens were employed to compare effects with and without restricted sperm movement. Neither cryptorchidism nor epididymal ligatures reduced libido. However, ejaculate volume significantly decreased after 14 days of cryptorchidism ($P < 0.05$), and this may have resulted from a reduction in contributions by the epididymis as indicated by oligospermia. Damage due to cryptorchidism was significantly more severe on sperm in the caput than in the cauda epididymis. Four days of cryptorchidism caused an increase in percent of abnormal spermatozoa at all levels of the epididymidis. It did not alter the percent of unstained sperm or fertility of sperm in the cauda epididymidis. Thus, uncapacitated sperm in the male at body temperature retain their fertilizing potential for considerably longer periods than capacitated sperm do in the female. Cryptorchidism for seven or more days resulted essentially in a complete loss of fertility of cauda sperm. Caput sperm usually were infertile initially, and after retention by ligation or after the elevation of temperature by cryptorchidism. Testicular alterations were detected after one or more days of cryptorchidism, characterized by cellular sloughing, giant cells, absence of tubules in stage 8 and testicular and tubular shrinkage. Ligatures on the corpus also caused fluid and sperm accumulation and interfered with spermatogenesis. This fluid accumulation was associated with a significant increase in caput epididymal weights ($P< 0.05$).

164. Tonometer for Measuring Testicular Consistency of Bulls to Predict Semen Quality.

J. Hahn, R.H. Foote and E.T. Cranch

An instrument, called a tonometer, was designed to measure testis consistency in bulls. Two force-deflection readings of the testis were obtained as a measure of consistency. The objective was to relate this to the quality of sperm produced. When the instrument was used by different experienced personnel and applied to the same series of bulls repeatability was high ($r \geq .89$). When 150 bulls were remeasured after 1 yr repeatability was 0.49, indicating that consistency of the testes had changed somewhat during 1 year. This was suggested by manual palpation also. Semen was collected four times per wk for 4 weeks from 64 Holstein bulls ranging from 17 to 150 months of age. Among-bull correlations between the ratio of two tonometer

force-deflection measurements and tests of semen quality such as % unstained sperm, % normal sperm, % motile sperm after 1 day of storage at 5°C, % motile sperm after pellet and ampule freezing and nonreturn rate ranged from 0.59 to 0.94. Nearly all correlations were highly significant statistically ($P < .01$). The results indicate that the tonometer provides a simple quantitative means of predicting semen quality and potential fertility in dairy bulls. Further investigation of the usefulness of the tonometer in evaluating breeding potential in young bulls under range conditions, where it is difficult to obtain representative semen collections, is needed.

165. Sperm Retention and Resorption in Sexually Active Rabbits with Epididymal Ligatures.

S.K. Paufler and R.H. Foote

Possible epididymal sperm resorption in sexually active rabbits (six ejaculates of semen collected per week) was examined under conditions allowing for sperm accumulation in distended epididymides versus retention of sperm in various segments isolated by appropriately placed ligatures. As much as 85% of the sperm in the isolated caput underwent dissolution and resorption within 4 weeks ($P < .01$). In contrast there was no significant decline in the number of sperm retained in cauda epididymides by ligation over a 12-week period. Seventy-six to 89% of the sperm ejaculated from the control nonligated side could be accounted for by sperm accumulated in contralateral epididymides with vasoligations. These results suggest that epididymal resorption may be minimal in the sexually active rabbit.

166. Quality and Freezability of Semen from Growing and Aged Dairy Bulls.

J. Hahn, R.H. Foote and G.E. Seidel, Jr.

Five groups of nine to ten Holstein bulls, over the age range from one and a half to six years, and a sixth group of 15 aged Holstein bulls were ejaculated four times per week for four weeks following an initial adjustment period. Sperm output in the aged bulls (72 to 150 months old) was considerably lower than in the groups ranging in age from 34 to 69 months. The percentage of morphologically normal sperm in semen at the time of collection and of motile sperm after freezing in pellets and ampules was lowest in the aged bulls. Nonreturn data available for this group indicated that the per cent unstained before freezing and the per cent normal and per cent motile sperm after freezing were significantly correlated with fertility ($P < .01$). Since semen quality was highest in bulls not yet old enough to complete the progeny test, freezing and storing selected semen samples from these bulls may be desirable. Semen quality of the youngest group was highly correlated with tests conducted a year later. If this relationship persists for several years, some young bulls with inferior semen quality might effectively be culled before being sampled in progeny test programs.

167. Motion Picture Analysis of Ejaculation in the Bull.

G.E. Seidel, Jr. and R.H. Foote

The following criteria, expressed as average figures, were evaluated from motion pictures of ejaculation in the bull: (1) the length of the penis extension from the entrance of the artificial vagina (AV) was 53.4 cm (2) the time between initial contact of the penis with the AV and the initiation of semen emission was 0.97 sec, and (3) the semen emission time was 0.29 sec. Coiling of the glans penis was observed in about half of the ejaculates, ranging from a slight twist to a 360° counter-clockwise coil 6 cm in outside diameter. There was a rhythmic movement of the fore-limb dew claws during ejaculation which appears to be a characteristic of sexual behaviour in the bull. Various abnormalities were observed in thrust and semen emission patterns using a short, low temperature AV. The large amounts of semen lost on the sides of the experimental AV suggest that an AV of proper length should be used for each bull.

168. Effect of Osmolarity on In Vitro Development of Rabbit Embryos in a Chemically Defined Medium.

D.L. Naglee, R.R. Maurer and R.H. Foote

Rabbit embryos were cultured from the 2- to 4-cell stage in a modified Ham's F-10 defined medium prepared so as to give calculated osmolarities of 230, 250, 270, 290, 310 and 330 mOsm. After culturing for 120 h in culture, the number of embryos reaching

the blastocyst stage in the 270 mOsm medium was significantly ($P < 0.05$) higher than that at 310 and 330 mOsm. The number of blastocysts which had enlarged and partially or completely escaped from the zona pellucida at 270 mOsm was significantly higher than at 230, 310, and 330 mOsm ($P < 0.01$) and 250 mOsm ($P < 0.05$).

169. **Research Techniques to Study Reproductive Physiology in the Male.** R.H. Foote

This chapter provides specific suggestions with references to the literature of techniques useful for studying broad areas of male reproductive physiology as follows: 1) Development of testicular function and related phenomena in the prenatal and prepuberal period. 2) Detailed investigations of spermatogenesis in postpuberal males, including endocrine function and the accessory sex glands. The use of ultrasound was advocated. 3) Semen collection techniques and measurement of sexual behavior. 4) Semen analysis by the usual criteria plus DNA and metabolic studies and sexing sperm. 5) Variation in semen characteristics and experimental design. 6) Semen preservation at different temperatures, artificial insemination and measurement of fertility.

170. **Estimation of Total Solids in Bull Seminal Plasma by Refractometry.** R.H. Foote

About 0.05 ml of semen was required to fill a microhematocrit tube and obtain enough seminal plasma to estimate total solids by refractometry. Repeatability of the method, based on duplicate subsamples from 140 ejaculates, was 0.999. The correlation between refractometer readings and dry matter determinations on 21 samples was 0.997. Bull differences were highly significant ($P < .005$), and pairs of first and pairs of second ejaculates from 19 bulls were highly correlated, $r = 0.71$ and 0.73, respectively. Total solids in seminal plasma from 112 Holstein bulls less than 6 years of age was higher than for 34 older bulls ($P < .005$). Overall averages for 206, 4, 27, 11 and 20 semen samples collected with an artificial vagina from Holstein, Guernsey, Jersey, Brown Swiss and Angus bulls, respectively were 9.6, 9.5, 8.8, 10.0 and 9.6 g of solids per 100 g of plasma. Semen obtained by electroejaculation was consistently lower in solids.

171. **Factors Affecting Superovulation, Fertilization and Recovery of Superovulated Ova in Prepuberal Cattle.** H. Onuma, J. Hahn and R.H. Foote

Holstein calves, reared from birth under standard conditions, were given various combinations of PMSG-LH or PMSG-HCG at 8 to 9 weeks, or at 17 weeks of age. Follicular development was variable, but averaged fifty-three follicles $\geq$ 1.0 cm in diameter following administration of either 1500 or 2000 i.u. of PMSG. Ovulation rate was 79% following 50 mg of LH, but it was only 16% following 1500 i.u. of HCG. The presence of young corpora lutea or the administration of progesterone accelerated transport of ova into the uterus and was associated with a reduced percentage of cleaved ova and a lower recovery rate. Liquid semen was superior to frozen semen in initiating cleavage of ova. Under optimum conditions, approximately 75% of the developed follicles ovulated and a similar proportion of ova was cleaved at the time of collection or cleaved in culture. Recovery rate was usually less than 30%.

172. **Culture of Two- and Four-Cell Rabbit Embryos to the Expanding Blastocyst Stage in Synthetic Media.** M.T. Kane and R.H. Foote

Development of two- to four-cell rabbit ova to the expanding blastocyst stage occurred in a simple glucose-salt solution supplemented with amino acids, and with 1.5% BSA as the only macromolecule. However, optimum growth required the presence of vitamins. These experiments would indicate that although a uterine protein component may be necessary for blastocyst growth and expansion in utero, it is not necessary in the in vitro culture system used. Hypoxanthine and/or thymidine may be inhibitory to good blastocyst development, as the highest percentage of expanding blastocysts (63%) was obtained when the nucleic acid precursors were omitted.

173. Compartmental Analysis of Sources of the Bovine Ejaculate.

G.E. Seidel, Jr. and R.H. Foote

A compartmental analysis model applicable to many experimental situations was used to estimate the volume contributed to ejaculated bull semen by the (1) seminal vesicles, (2) epididymides and (3) other sources. The fluid which drips from the urethra during sexual preparation was assumed to be characteristic of the latter compartment. Fructose, sperm, and chloride (Cl), respectively, were used as markers. Estimates of vesicular fluid fructose concentration ranged from 959 to 1658 mg/100 ml; similarly, estimates of epididymal sperm concentration ranged from 3.35 to 7.10 $\times$ 10^9 sperm/ml. Average urethral fluid Cl concentrations ranged from 487 to 517 mg/100 ml. The uniformity in Cl among bulls supports the postulate that urethral fluid essentially is a filtrate from a rather uniform pool, the blood. For first ejaculates it was estimated that each of the three areas contributed approximately 1.5 ml to the average ejaculate of 17- to 22-month-old bulls and similarly each contributed about 2.0 ml for 3-year-old bulls. For both groups of bulls, epididymal volume contribution to second ejaculates was only about half that of first ejaculates, but volume contributions from the other two compartments were similar to first ejaculates.

174. Culture of Two- and Four-Cell Rabbit Embryos to the Blastocyst Stage in Serum and Serum Extracts.

M.T. Kane and R.H. Foote

Two- and four-cell rabbit embryos were cultured for 4 days in a simple synthetic medium containing 1.5% crystallized bovine serum albumin (BSA) with bovine serum and serum fractions added in order to determine the serum components useful for promoting blastocysts formation. Four experiments, involving 34 treatments and 1875 embryos, were carried out. The minimum level of serum for optimum blastocyst formation was found to be about 5%, and all the components necessary for blastocyst formation were present in the dialyzable portion of serum. With 1.5% BSA in a medium containing the dialyzate no other macromolecules were required to obtain expanding blastocysts. The ability of these compounds to promote blastocyst formation was not destroyed by boiling, freeze-drying, treatment with 1 N HCl or 1 N NaOH, irradiation with UV light nor by adding EDTA to the culture medium. The dialyzate was very soluble in water and relatively insoluble in nonpolar solvents. The nondialyzable serum fraction did not promote blastocyst formation.

175. Viability of Cultured and Transferred Rabbit Embryos.

R.R. Maurer, H. Onuma and R.H. Foote

Rabbit embryos in the two- and four-cell stage were placed in rabbit and bovine serum with and without the addition of 100 mg glucose per 100 ml serum and cultured for 97 hr. Maximum development in culture was to the expanding blastocyst stage. Embryos were transferred to recipient does after 0, 24, 36, 48, 62, 72, 88 and 97 hr in culture. Embryos transferred after 62 hr in culture readily developed into neonates, and one embryo cultured for 88 hr to the blastocyst stage developed into a full-term young. The addition of glucose significantly increased ($P < 0.005$) the number of blastocysts produced in culture.

176. Fractionated Serum Dialysate and Synthetic Media for Culturing 2- and 4-Cell Rabbit Embryos.

M.T. Kane and R.H. Foote

Embryos in the 2- and 4-cell stage were cultured for 4 days in a simple glucose-salt solution with 1.5% crystallized bovine serum albumin (BSA) to which were added fractions of freeze-dried bovine blood serum dialysate separated on G-25 and G-10 Sephadex columns. Development of 1389 embryos was compared in 35 treatments. The fractions which promoted blastocyst formation had a molecular weight of less than 700. Most of the amino acid content of the unfractionated dialysate was found in the active fractions, thus implicating amino acids as being important additions to the simple synthetic medium. In the absence of BSA no blastocysts formed in the simple synthetic medium containing serum dialysate, or in a "complete medium" formed by adding a complement of amino acids, vitamins, and trace minerals to the simple medium. The addition of serum dialysate to the complete medium resulted in 4.5% of the embryos reaching the expanding blastocyst stage as contrasted with 69.8%

when 1.5% BSA was added. The combined addition of serum dialysate and BSA to the simple medium resulted in 22.6% expanding blastocysts. These studies suggest the need for a macromolecular component (BSA) and indicate that the serum dialysate did not provide as optimum a balance of amino acids and other nutrients as were provided by the complete synthetic medium.

177. **Inheritance of Fertility -- Facts, Opinions, and Speculations.**
R.H. Foote

Specific genetic defects affecting reproduction in both males and females are discussed. The traits used to estimate fertility such as percent conception, services per conception and calving interval, all are influenced by the environment and probably are of multigenic nature. Heritability has been approximately zero. The fertility problem of greatest economic importance in herd or population is over-all breeding efficiency. As this has been measured by services per conception, per cent nonreturns, first service to conception, calving interval, etc., additive genetic variance has been very small. Thus, selection for these traits would not be effective and would be at the expense of other traits of economic importance which show a greater response to selection. Infertility in cows appears to be primarily a management problem. There are heritable differences in nonreturn rates of sires used in artificial breeding, but the genetic variance appears to be small. In addition to the natural selection pressure against low fertility, bull studs probably make less use of bulls of low fertility, and fewer sons of such bulls may be selected. In the future, bull-rearing schemes might increase the opportunity of early selection for animals with high potential for sperm production, without reducing selection pressure on productive traits.

178. **Testicular Measurements as Predictors of Sperm Output and Semen Quality.**
R.H. Foote, J. Hahn and L.L. Larson

Instruments to measure testes size and consistency or firmness were developed in an attempt to predict in advance semen quality and fertility of bulls. Scrotal circumference was an excellent indicator of testis size, as well as a useful means of estimating potential current sperm output and subsequent testicular development in young bulls. The tonometer provided a measure of testicular consistency which was highly correlated with concurrent semen quality and fertility. To the limited extent studied it appears to have some predictive value of future performance. As a consequence, these measurements should be useful in evaluating potential bulls for purchase, and before they are used in extensive artificial breeding operations or in natural service. Furthermore, young bulls with predicted inferior reproductive potential might be culled effectively before being sampled in a progeny test program.

179. **Artificial Insemination of Beagle Bitches with Freshly Collected, Liquid-Stored, and Frozen-Stored Semen.** H.P. Gill, C.F. Kaufman, R.H. Foote and R.W. Kirk

Insemination of Beagle bitches with freshly collected semen, with semen extended with a tris-buffered egg yolk extender and used immediately, or with extended semen stored for 24 hours at 5 C resulted in whelping rates of 75 to 80%. In each of the 3 groups, 200 million motile sperm were used for each insemination. This whelping rate compared favorably with that for natural service breeding, using the same stud dogs for all groups. The use of 50 million sperm for each insemination resulted in lower whelping rates and variable litter size. Conceptions did not occur after 8 experiments with frozen semen, although 40 to 50% of the cells were vigorously motile upon thawing. An additional 4 bitches were inseminated with frozen-thawed semen from which glycerin was removed, but none conceived. The length of time that proestrus lasted was not related to the length of time of estrus ($r = -0.19$). The appearance of glucose in the vagina or cervix did not correspond with the estrous period. Stained vaginal smears proved helpful in detecting estrus, but the best sign of the onset of estrus was acceptance of the male by the bitch.

180. Fertility of Bull Semen at High Extension Rates in Tris-Buffered Extenders. R.H. Foote

A 0.20-M Tris-yolk extender buffered to pH 6.50 or 6.75 was superior to other combinations tested in preserving motility of sperm ($P < 0.005$). Inclusion of glycerol in the extender added to semen at room temperature was as satisfactory in maintaining sperm motility as glycerolating semen at 5 C. In 3 trials involving 135,579 first inseminations with unfrozen semen, fertility results in a 0.20-M Tris extender buffered at pH 6.5, and containing 1.0% fructose (w/v) and 6.4% glycerol (v/v) in the final extender, were equal to those obtained with Cornell University Extender. Omission of glycerol resulted in a decrease in 60- to 90-day nonreturns from 73.6 to 71.6% ($P < 0.01$). The nonreturn rate with semen extended to 8 million motile sperm per milliliter was 73.7 as compared to 72.7% when 4 million motile sperm were used ($P > 0.10$).

181. Influence of Extender, Extension Rate, and Glycerolating Technique on Fertility of Frozen Bull Semen. R.H. Foote

In four field trials involving 57,791 inseminations with ampuled frozen semen, there was no difference in motility or fertility when glycerol was included initially in Tris-fructose-glycerol-yolk extender or when it was added slowly after cooling to 5 C. Fertility in citrate-glycerol-yolk extender also was the same when glycerol was added slowly in four steps or in one step at 5 C. Thus, slow addition of glycerol is not necessary. Reduction in pH of the citrate extender from 7.0 to 6.5 did not affect fertility. The 60- to 90-day per cent nonreturns for Tris extender at pH 6.5 and 6.75 were 74.1 and 71.9 ($P = 0.10$). Sperm motility following freezing and thawing in Tris extender was slightly superior to that in citrate extender, but there was no difference in fertility. Fertility was 0.5 and 1.5 percentage units lower in two trials when 12×10^6 motile sperm numbers per ampule before freezing were compared with 24×10^6 motile sperm ($P > 0.10$). After freezing and thawing, motile sperm numbers per ampule ranged from 3×10^6 to 23×10^6. Correlations between these sperm numbers and fertility in three experiments were 0.17, 0.26, and 0.42. Thus, sperm numbers in the range studied accounted for only 3 to 18% of the variation in nonreturn rate. Increasing sperm numbers had little effect on improving fertility of low-fertility bulls.

182. Superovulation, Ovum Collection, Culture and Transfer. A Review. R.H. Foote and H. Onuma

This paper reviews the need for developing methods for nonsurgical recovery of oocytes from females of all ages. Superovulation and fertilization in prepuberal and postpuberal animals is discussed. Embryo culture in cattle, sheep, goats and rabbits is discussed from a research standpoint as well as for practical application. When the paper was written in 1969-1970 the following was concluded. At the present time embryo transfer in cattle is a technique sufficiently developed to be utilized experimentally in expanding knowledge of reproduction. Before it can be used on a practical level repeated nonsurgical recovery of ova, storage methods, and pregnancy rate following nonsurgical transfer need to be improved. The paper contains 128 references, including many describing the excellent progress in embryo transfer research in Japan that, heretofore, were little known in the western world.

183. Duration of Premeiotic Deoxyribonucleic Acid Synthesis and the Stages of Prophase I in Rabbit Oocytes. J.J. Kennelly, R.H. Foote and R.C. Jones

To estimate the duration of oocyte DNA synthesis 36, 3-day-old female rabbits received 3, 6, 9, 12, 15, or 18 injections of tritiated thymidine (thy-^{3}H) at hourly intervals. The ovaries, removed at 1, 10, or 20 days after the first injection, were radioautographed. Counts made of the number of silver grains associated with oocyte nuclei in meiotic Prophase I indicate that the duration of DNA synthesis is between 9 and 12 hr. To determine the length of the stages of meiotic Prophase I, a group of 2-3-day-old rabbits was given a single subcutaneous injection of thy-^{3}H, and the ovaries were removed at hourly and/or daily intervals after treatment. The minimum duration of leptotene was 3 hr and the

maximum duration probably was less than 8 hr. The maximum durations of zygotene, pachytene, and diplotene were estimated to be 44, 216, and 96 hr, respectively. The interval from the end of oogonial DNA synthesis to the beginning of premeiotic DNA synthesis (G_2 + Mitosis + G_1) appeared to be less than 6 hr.

184. **Culture and Transfer of Calf Ova.** G.E. Seidel, Jr., L.L. Larson, C.H. Spilman, J. Hahn and R.H. Foote

Modified Ham's F10 and TCM 199, each with 1.5% bovine serum albumin, were used to culture superovulated ova from pre- and postpuberal heifers. The maximum cleavage in vitro was from the 1-cell to the 12-cell stage. Twenty-two of 30 normal appearing 2-cell ova recovered from calves cleaved further when cultured in chemically defined media, but five calf ova with four or more blastomeres at the time of collection and ova collected 5 days after ovulation did not cleave further in vitro. This suggests that the reproductive tract of the immature animal may be harmful to ova. Four of 14 calf ova cleaved up to three times in the ligated oviduct of pseudopregnant rabbits. A total of 102 ova was transferred to 18 postpuberal recipient heifers. One definite pregnancy was achieved following transfer into the oviduct of one laparotomized heifer about three hours after recovery of the ova. After 275 days the heifer delivered a bull calf weighing 45 kg, showing that some ova recovered from prepuberal heifers are fully capable of normal development. This is believed to be the first calf reported in the world resulting from calf insemination and embryo transfer.

185. **Maternal Ageing and Embryonic Mortality in the Rabbit. I. Repeated Superovulation, Embryo Culture and Transfer.** R.R. Maurer and R.H. Foote

Embryos from ageing (20 to 148 weeks of age) and young (20 to 30 weeks of age) donors were transferred to ageing (52 to 221 weeks of age) and young (18 to 30 weeks of age) recipients to partition the effects of ageing oocytes and uterine environment on embryo mortality. More than 3300 two- to eight-cell embryos collected following superovulation at six 6-month intervals were transferred. The average number of ovulation points per ageing donor doe superovulated at the six intervals declined with age and repeated superovulation. The average number of ovulations for the young donors at the six intervals also differed slightly, the low number (forty-one) for the last group possibly being due to the crossbred strain used. Both embryo recovery and cleavage rates usually exceeded 80% and did not differ between young and ageing donors. The percentage of viable young developing from embryos transferred from ageing and young donors showed that the potential for embryo development had not been impaired during 3 years of ageing. The percentage of viable young developing from embryos transferred to ageing and young recipients indicated that conditions for maintaining pregnancy had been impaired in the ageing recipients. The average number of ovulations for a group of old does superovulated for the first time at 229 weeks of age was fourteen compared to sixty-two for young controls, and only 26% of the embryos transferred from the old does developed into neonates, whereas 45% of those from young donors developed normally. As the female ages, the uterine environment may become less conducive to prenatal development and the oocytes then show the effects of the ageing process directly or as a result of exposure of the oocyte or young embryo to the aging oviduct. At laparotomy 12 days after transfer, the ageing recipients had 45.3% pre- and 14.8% postimplantation mortality. Corresponding values for young recipients were 33.5 and 16.0% respectively. Laparotomy increased embryo mortality in ageing females only. The percentage of embryos which developed to blastocysts in vitro paralleled the development in vivo of embryos in ageing and young donors. The overall sex ratio of 81.6 males/100 females resulting from transferred embryos was significantly different ($P<0.05$) from the expected figure.

186. **Factors Affecting Blastocyst Expansion of Rabbit Zygotes and Young Embryos in Defined Media.** M.T. Kane and R.H. Foote

A total of 939 one-, two- and four-cell rabbit embryos were cultured at 37 C in defined media containing glucose, salts, amino acids, vitamins, trace elements, and bovine serum albumin (BSA). Dow Corning 360 Medical Fluid was introduced to

cover embryos cultured in individual cups in place of oil previously used to cover droplets of media in culture dishes. The addition of thymidine inhibited development of two- and four-cell embryos into expanding blastocysts, but hypoxanthine had no apparent effect. A level of 1.5% BSA was markedly superior to a 0.15% level in the production of expanding blastocysts (67% vs. 12%). Phenol red at levels of 0.012 μg and 1.2 μg/ml of medium had no effect on embryo development. Starting with one-cell embryos 29% developed to the expanding blastocyst stage in the defined medium containing 1.5% BSA as compared to 64% of the embryos cultured from the two- and four-cell stages. However, 48% of one-cell embryos developed into expanding blastocysts in the optimal treatment containing 0.11 mg/ml of sodium pyruvate. The addition of pyruvate had no effect on the older embryos cultured. When the concentrations of methionine, cysteine, serine, threonine and tyrosine were increased tenfold, embryo development was inferior to results obtained in the basal medium containing amino acids at the levels found in Ham's F10 medium.

187. Alteration of Cell Volume in Bull Spermatozoa by Factors Known to Affect Active Cation Transport.

P.J. Bredderman and R.H. Foote

The effects of Na^+, K^+, Li^+, fructose, cyanide, ouabain, and oligomycin on the motility and electronically monitored cell volume stability of bull spermatozoa diluted approx. 1:10,000 and incubated at 35°C in media isosmotic with 0.15 M NaCl buffered to pH 7.0 were studied. When only one type of alkali metal ion was present (Na^+, Li^+ or K^+), sperm motility was poor and sperm started to swell immediately, resulting in apparent lysis after several hours. By incorporating 2.25 mM of K^+ in a medium containing Na^+, onset of sperm swelling was delayed, but motility was not improved. Li^+ was unable to satisfactorily replace Na^+ in maintaining sperm cell volume stability and it abolished sperm motility. Fructose significantly delayed onset of swelling only when adequate levels of K^+ replaced part of the Na^+. Cyanide or oligomycin abolished sperm motility and the delaying effect of K^+ on sperm swelling when no energy source was supplied. Fructose with CN^- and K^+, greatly extended sperm motility and cell volume stability. The latter was abolished by ouabain or oligomycin but motility was little affected, indicating that these compounds inhibit Na^+ transport without blocking glycolysis. Thus, spermatozoa resemble other animal cells in their ability to stabilize cell volume through the active maintenance of intra- and extracellular distribution of Na^+ and K^+.

188. Factors Stabilizing Bull Sperm Cell Volume and Prolonging Motility at High Dilution.

P.J. Bredderman and R.H. Foote

Sperm cell volume changes were monitored electronically, and motility was observed microscopically during incubation at 35°C in chemically defined media isosmotic with 0.15 M NaCl and buffered at pH 7.0. Neither 2.0 mM Pi nor 10 mM Mg^{2+} had any marked effects on cell volume stability or motility when used with different levels of fructose and K^+. Both 3.0 mM K^+ and 10 mM fructose significantly ($P<0.05$) delayed the onset of cellular swelling, but this delay and the effects on motility were minimal in overcoming the 'dilution effect.' EDTA (0.05-1.0 mM) had a marked stabilizing effect on cell volume and essentially doubled the duration of motility. When EDTA was combined with fructose the effects were more beneficial than with either one alone. BSA (1 mg/ml) also stabilized cell volume in the presence and absence of fructose, and markedly prolonged vigorous sperm motility. BSA and EDTA tended to be additive in effect. CN^- markedly improved the otherwise limited beneficial effects of fructose on sperm volume stability and motility even in the presence of oligomycin. Without fructose, CN^- caused early swelling and an apparent eventual cell lysis. CN^- could not abolish the beneficial effects of BSA in the presence of fructose or those of BSA or EDTA during preincubation without fructose. Oligomycin blocked the beneficial response to EDTA or BSA in the absence of fructose, but it did not prevent the improvement and extension of motility caused by EDTA, BSA or CN^- when fructose was included. Oligomycin caused immediate swelling followed by cell shrinkage to less than the initial cell volume. The onset of shrinkage could be delayed by fructose and further delayed if CN^-, EDTA or BSA was also included. The delay in onset of shrinkage was accompanied by prolonged

motility. Spermatozoa, immobilized by preincubation in a fructose-free media containing CN^-, could be activated by the addition of fructose up to the time swelling began. Both BSA and EDTA extended this period during which motility could be restored.

189. Effects of Age and Gonadotropin Treatment on Superovulation in the Calf.

G.E. Seidel, Jr., L.L. Larson and R.H. Foote

Superovulation was studied in 113 Holstein calves using PMSG followed 3 or 5 days later with 50 or 75 mg LH i.v. Calves were inseminated twice into the uterus 0.5 to 2 days after LH. Ova were recovered by sacrifice or laparotomy 2.5 to 5 days after LH. Treatment of calves with 1,500 IU PMSG followed by 50 mg of LH 5 days later produced an average of 0, 9.4 and 28.2 ovulations in calves 0, 1 and 2 months old. By 5 months of age, 1,500 to 2,000 IU PMSG and 75 mg LH produced 77.2 ovulations per calf, but there was considerable variability in response. Over all experiments the number of ova recovered was about one-fifth the number of ovulation points, and the correlation between these two variables was r = .79. Control calves that received only 1,500 IU of PMSG at 2 months of age averaged 46.5 follicles $\geq$ 10 mm in diameter 5 days later. Another control group with no treatment averaged 6.7 follicles $\geq$ 5 mm in diameter, with an occasional follicle $\geq$ 10 mm. Calves given only 50 mg LH i.v. did not ovulate. Injection of 10 mg of estradiol-17β to 2-month-old calves 3 days after PMSG treatment reduced ovarian response to PMSG but increased the percentage of cleaved ova. Recovery of ova 5 days instead of 3 days after LH resulted in more ova in the uterus, a lower recovery rate and an increase in the percent of abnormal ova. The ovaries were larger and had a higher proportion of luteinized follicles at 5 vs. 3 days after LH. Different lots of PMSG did not differ in ability to induce superovulation of calves 2 months old. Supplementation of PMSG with small amounts of FSH or LH did not improve superovulatory effectiveness. It was possible to mimic the action of 1,500 IU of PMSG with 50 mg FSH and 17 mg of LH given s.c. in 10 exponentially decreasing aliquots over a 5-day period.

190. Anatomical and Histological Observations on Reproductive Organs in Superovulated Calves.

L.L. Larson, G.E. Seidel, Jr. and R.H. Foote

Histological and anatomical measurements of ovaries, oviducts, uteri, pituitaries and adrenals are presented for Holstein calves superovulated with PMSG and LH at 0, 1 an 2 months of age. Most large differences between groups of calves were related to age rather than to superovulatory treatment, but ovarian weight and uterine gland proliferation were affected by both. There was a marked increase in uterine gland proliferation between 0 and 2 months of age and a further increase after 2-month-old calves were under the influence of progesterone for 5 to 20 days as a result of extensive luteal tissue formation following superovulation. The average CL weight 3, 10, 15 and 20 days after LH administration was 0.9, 2.8, 2.6 and 1.1 g, respectively. Luteal tissue 20 days after LH injection was extremely variable, with both functional and regressing CLs present in the same calf. Most anatomical measurements were positively correlated with calf weight ($P<.01$) and many of these measurements were significantly correlated among themselves. Uterine weight was correlated with uterine gland proliferation, $r = 0.74$. The immature anatomical and secretory status of the uterus is consistent with poor survival of young embryos in the reproductive system of the calf.

191. What Might Sex Ratio Control Mean in the Animal World?

R.H. Foote and P. Miller

This paper provides data on the inventory and value of livestock and poultry on farms in the U.S. and traits that one sex possesses that are advantageous, such as milk production by the female. Various breeding strategies are presented assuming various efficiencies of sex control. The schemes are theoretical as no effective means of sex control are known. From this discourse the following conclusions seem plausible. Sex control would permit the livestock industry to produce the optimum proportion of males to females to take advantage of sex-limited and sex-influenced traits. The benefits can

result from both phenotypic and genotypic selection, particularly from the former, in producing food for the world more efficiently and biologically. The potential benefits are enormous, but the optimum schemes have not been established. Furthermore, to be applied widely, any sex control technology must be effective, must result in high fertility and must be reasonably inexpensive and convenient to apply in animals. In humans, any predictions of the price people will pay, or the consequences of paying the price are highly speculative, but the possibility of sex control with family size limitations demands careful consideration.

192. The Effect of Calcium Ions on Cell Volume and Motility of Bovine Spermatozoa.

P.J. Bredderman and R.H. Foote

Ca^{2+} was added to a buffered medium containing various combinations of K^+, fructose, CN^-, ouabain, and BSA. Constant osmolarity, pH, and temperature of the incubation media were maintained. Without K^+ in the medium, Ca^{2+} limited the duration of rapid swelling of spermatozoa by causing early onset of shrinkage to about 90% of initial cell volume. Shrinkage occurred sooner as the Ca^{2+} level increased, and was associated with a fuzzy microscopic appearance of the sperm cells. Addition of 2.25 to 3.0 mM K^+, and to a lesser extent 1.0 mM of fructose, delayed the onset of swelling and subsequent shrinkage associated with Ca^{2+} alone. This delay also was observed in a variety of media which promoted good sperm motility. BSA was able to counteract the effects of Ca^{2+}, but this was abolished by addition of CN^-. Fructose overcame the CN^- effect, indicating that the process is energy related. It appears likely that Ca^{2+} enters the cell only after energy sources are depleted, and its presence there causes structural and permeability changes in spermatozoa.

193. Bovine Sperm Cell Volume at Various Intervals after Addition of Glycerol at 5°C.

W.E. Berndtson and R.H. Foote

Sperm cell volume measurements were made by electronic particle sizing equipment and by the determination of packed-cell volume at intervals after glycerol addition to test for glycerol penetration. In the absence of penetration by glycerol, dehydration and shrinkage of the cells through efflux of water would be expected, while if penetration occurred the cells should return to their former size at a rate reflecting the rate of glycerol penetration. No significant changes in cell volume were observed after glycerol addition at 5°C, but changes could have occurred during the 3-7 min before the volume measurements could be completed. The average mean volumes determined electronically were 25 μ^2, vs. 26 μ^3 for sperm cells with and without glycerol, respectively. The corresponding modal volumes were 21 μ^3 and 23 μ^3, respectively. The lack of substantial differences in cell volume in media with and without glycerol, and the absence of size changes with time in the presence of glycerol, suggest that glycerol penetration of bull sperm was very rapid at 5°C, occurring within the 3 min required for the first volume determination to be made. Packed cell volume determinations gave similar results when glycerol was added at 5°C and 25°C. Even when the cells were previously swollen in hyposmotic media so that they had known potential for undergoing shrinkage after glycerol addition, detectable changes in volume were not observed over time. Furthermore, the volume of cells suspended in media with and without glycerol was similar. Thus, glycerol probably penetrated these cells within the 5-7 min required to make the first volume measurement.

194. The Freezability of Spermatozoa After Minimal Pre-Freezing Exposure to Glycerol or Lactose.

W.E. Berndtson and R.H. Foote

Bull spermatozoa were frozen in egg yolk-sodium citrate and egg yolk-Tris media after different lengths of exposure to a range of glycerol levels at 5°C by placing 0.1 ml of extended semen in small holes on a dry ice block. Postthaw motility was significantly greater after glycerol exposure of 10 sec before freezing (shortest time studied) as compared with 2 min of exposure or longer ($P < 0.05$). After 6 min of glycerol exposure no further significant decline occurred. Thus, momentary exposure to glycerol provided the best conditions for sperm

survival during freezing. It is proposed that immediately after glycerol addition dehydration of the spermatozoa occurs and by freezing the cells immediately at a rapid rate, intracellular ice formation and the prolonged influence of high solute concentration may be avoided. Similar studies were conducted by freezing sperm in the presence of a presumably nonpenetrating cryopreservative, lactose, without glycerol. Post-thaw motility was not influenced by the length of prefreezing exposure to lactose at 5 °C. The fact that only minimal exposure to both cryopreservatives is required for optimum survival supports the concept that penetration is unnecessary. The studies with glycerol suggest that penetration may be undesirable for bull spermatozoa.

195. Can Fertility of Sires Used in Artificial Insemination Be Improved?

R.H. Foote, L.L. Larson and J. Hahn

Testicular measurements of size and consistency can be obtained on young postpubertal bulls with a positive relationship between adult sperm producing ability and fertility. Thus, selected young bulls could have semen frozen and the bulls discarded. Several practical conclusions are possible. It is possible to select individual bulls with superior semen quality at the time they are ready for sampling. A portion of this superiority is expected to be maintained subsequently. Semen quality and fertility of Holstein bulls during the period they are waiting to be proven (2 to 6 years) is superior to that obtained after the bulls are proven. During this period most dairy bulls have good libido, are in good physical condition and few have succumbed to natural causes of death. The fertility of sperm cells stored continuously in liquid nitrogen at -196°C remains unchanged for several years, and it is unlikely that this condition will change markedly during considerably longer periods of proper storage. While it is technically feasible to tap this great unused sperm potential of young bulls the circumstances under which it may be desirable to do so, if any, are yet to be determined.

196. In Vitro Progesterone Synthesis by Corpora Lutea Induced in Prepuberal Cattle.

C.H. Spilman, G.E. Seidel, Jr., L.L. Larson and R.H. Foote

Superovulation was induced in prepuberal calves using PMS and LH. The induced CL were collected at 5, 10, 15 or 20 days after the LH injection. Initial progesterone concentrations were greatest at 5 days, but remained high even to day 20. Upon incubation CL obtained after 5, 10 and 15 days synthesized more progesterone ($P < .05$) than 20-day CL in the control medium and in media containing LH or NADPH. The overall stimulation of progesterone synthesis by LH was 27% and by NADPH was 60% when compared to control incubations. All incubated CL utilized glucose, but in no case was glycolysis stimulated by LH. NADPH stimulated glycolysis in 5-, 10- and 15-day CL incubations. It did not stimulate glycolysis at 20 days even though these incubations could still be stimulated to synthesize progesterone by NADPH. The accumulation of lactate in vitro was not affected by LH or by NADPH. Asynchronous regression of CL was observed both within and among animals at 20 days, a fact which may in part be due to different CL ages resulting from variable ovulation times in superovulated animals. Of major interest in these studies was the fact that an abundance of functional luteal tissue was formed in prepuberal calves following superovulation. This tissue responded to LH and NADPH in vitro by increasing progesterone synthesis. These in vitro results were consistent with the in vivo findings of high concentrations of circulating progesterone in superovulated calves.

197. Induced Ovulation and Fertility in Prepuberal Cattle, Sheep and Swine.

R.H. Foote

In cattle, sheep and swine oogenesis occurs early in life. Antra, which appear to be a prerequisite for ovulation, are present neonatally in cattle and sheep and found in swine at 2 to 30 months of age. Tertiary follicles can be stimulated by administration of gonadotropic hormones (FSH, PMS, LH, HCG)

and ovulation induced in calves by 1 month of age, lambs by 2 months and swine at about 3 months of age. Fertilization rates are low in the prepuberal animal with frozen semen. The placement of fresh semen into the uterus may improve conception rates markedly. Ovulatory response, fertilization and embryo recovery rates often are highly variable. Prepuberal females stimulated to ovulate appear to secrete estrogen (show estrus) and corpora lutea formed secrete an abundance of progesterone. The pituitary glands contain considerable amounts of FSH and LH. Nevertheless such females usually do not continue to cycle after the induced ovulation. Pregnancy can be initiated in prepubertal animals with hormone treatment and insemination, but complications can arise during pregnancy if the animal is too small. Selection for early sexual maturity may be useful in some species.

198. Nondialyzable Skimmilk in Diluents for Ram and Bull Semen.

R.C. Jones and R.H. Foote

Bull and ram spermatozoa were stored in synthetic media containing various skimmilk preparations. The reduction in motility of ram spermatozoa diluted 80-fold and incubated at 37 C in synthetic media could be prevented by the inclusion of .8% (w/v) of a lyophilized preparation of nondialyzable skimmilk heated to 92 C for 10 min. Bull spermatozoa incubated at 37 C in buffered saline solutions 60, 80, and 100% of the osmolality of 154 mM sodium chloride, and to which 0, .75, 1.5, 2.25, and 3% (w/v) of the milk preparation were added, survived best in the 80% solution. The detrimental effect of osmolality on sperm motility diminished as the concentration of the milk preparation increased. For either ram or bull spermatozoa, fresh nondialyzable skimmilk was best detoxified by heating after dialysis or by adding .5 or 1.0 mg of cysteine-HCl/ml before dialysis. Dialysis removed excess unbound cysteine-HCl. When the milk preparations were lyophilized for storage and reconstituted as diluents, bull sperm motility was higher during incubation at 5 or 37 C in heated milk preparations than in preparations treated with cysteine-HCl before dialysis. The latter treatment was improved by our removing a precipitate formed when cysteine-HCl was added, but heat treatment still was superior. We concluded that heating and cysteine-HCl addition act differently in detoxifying milk. The most suitable methods of preparing fresh milk diluents may not be satisfactory if the preparations are to be lyophilized for storage before use.

199. Spermatogenesis in the Dog.

R.H. Foote, E.E. Swierstra and W.L. Hunt

The cycle of the seminiferous epithelium of the dog was divided into eight stages, using as criteria the shape of the spermatid nucleus, the location of spermatids and spermatozoa in regard to the basement membrane, the presence of meiotic figures and the release of spermatozoa from the lumen of the tubule. Based upon these criteria, a modification of the eight-stage system of classification of the cycle of the seminiferous epithelium was developed. This classification is relatively simple to use and makes it possible to compare spermatogenesis in the dog with other species where this classification has been used. Cell populations making up each stage are described. The relative frequencies of the stages 1 through 8 were 21.9, 12.7, 2.8, 11.5, 8.3, 15.4, 13.3 and 14.0%, respectively. The duration of one cycle of the seminiferous epithelium was 13.6 days (SE ± 0.7), as determined from cells labeled by tritiated thymidine. The absolute durations of stages 1 through 8 were 3.0, 1.7, 0.4, 1.6, 1.1, 2.1, 1.8 and 1.9 days, respectively. The life span of primary spermatocytes was 20.9 days, of secondary spermatocytes 0.5 days, spermatids with round nuclei 10.5 days, spermatids with elongated nuclei up to the time they are released into the lumen, 10.6 days. Counts of the different types of spermatogenic cells in tubular cross sections revealed little or no germ cell degeneration during the two maturation divisions.

200. Uterine Collagenase and Collagen in Young and Ageing Rabbits.

R.R. Maurer and R.H. Foote

Collagenase activity was measured 66 to 75 hr after parturition in does at 34, 167 and 204 weeks of age. Uteri of ageing does contained less collagenase than those of young does. Uterine collagen content tended to increase with age. At Day 12 of pregnancy, uterine acid-soluble collagen was higher in does 174 weeks old than in does at 38 weeks of age. Higher uterine collagen content and lower progesterone

output in old does may contribute to reduced efficiency of the uterine environment and an increase in embryo mortality.

201. **Aging of Spermatozoa During Storage in Liquid Nitrogen**. R.H. Foote

Semen from 15 bulls was frozen in the summer and in the winter season and used in both seasons. It was stored centrally in ampules immersed in liquid nitrogen at -196°C for 1, 6, 12, 18, and 24 months until time for transfer to the field. The nonreturn rates for 3,222 inseminations with semen stored 18 to 25 months for the 30-60, 60-90 and 150-180-day intervals was 78.6, 72.2 and 68.8%. Corresponding values for 17,820 inseminations with semen stored 1-12 months were 78.2, 71.3 and 66.9%. These experiments demonstrate that there is no impairment in fertilizing ability of sperm stored continuously in the dark at -196°C for up to 2 years.

202. **How to Measure Sperm Cell Concentration by Turbidity (Optical Density)**. R.H. Foote

This paper describes the principles and procedure for determining sperm concentration by optical density. Standards with a known concentration of latex spheres can be prepared and used for a considerable length of time to check the instrument as the phototubes age. When replacing an old phototube it is important to check and be sure that it gives nearly the same readings with the standards as the previous phototube. The largest difference between instruments is due to the phototube, and phototubes should be matched to avoid requiring a new calibration of the spectrophotometer.

203. **Ovarian Function During Pregnancy in Young and Aged Rabbits: Temporal Relationship Between Fetal Death and Corpus Luteum Regression**. C.H. Spilman, L.L. Larson, P.W. Concannon and R.H. Foote

Corpus luteum (CL) progesterone concentration and total progestins in the peripheral plasma were measured at various stages of pregnancy in 18 young rabbits 35 weeks old and 26 aged rabbits 250 weeks old. Fertilization rate, average number of CL, and viable fetuses in the young does were 83%, 7.7, and 5.8, respectively. Corresponding values in the aged does were 81%, 6.2, and 1.1. There were no differences between young and aged does in any of the parameters of ovarian function measured until after Day 12 of pregnancy. This was true even though five of nine aged animals did not have viable fetuses at this time. Significant differences were detected in aged animals without viable fetuses only at Day 24. At this time average CL weight (6.3 mg) total luteal tissue (30.9 mg), and total CL progesterone content (0.49 μg) in aged animals without viable fetuses were significantly ($P < 0.05$) less than corresponding values in aged animals with viable fetuses (15.1 mg, 110.6 mg, 2.55 μg) and in young animals all with viable fetuses (16.5 mg, 120.7 mg, 2.83 μg). These latter two groups did not differ. Peripheral plasma progestins on Day 24 of planned pregnancy in aged animals without viable fetuses (3.2 ng/ml) were also significantly ($P < 0.05$) less than in aged animals with viable fetuses (16.8 ng/ml) and in young animals (11.4 ng/ml). Again, these latter two groups did not differ. It is concluded that the decrease in fecundity in aged animals is not caused by a lack of progestin secretion, but rather the absence of viable fetuses may cause luteal regression. Thus, reproductive failure in aged does appears to be directly associated with uterine dysfunction.

204. **Uterine Blood Flow in Young and Aged Rabbits**.
L.L. Larson and R.H. Foote

Uterine blood flow was measured in 35 rabbits using the gas washout technique of an inert gas (^{85}Kr). Both nulliparous and multiparous does were tested. Uterine blood flow rate in 15 aged does average 18.6 ml/100 g tissue/min, and this was significantly lower than the average rate of 37.8 ml/100 g tissue/min in 20 younger does. It is concluded that reduced uterine blood flow rate is one component of the aging uterus which may decrease reproductive performance in aged rabbits.

205. **Ouabain Effect on Bovine Spermatozoan Motility and Testosterone Binding.**
R.C. Wester and R.H. Foote

Spermatozoan motility of whole bull semen incubated aerobically at 37° in 0.9% NaCl saline was inhibited by 10^{-3} to 10^{-7} M ouabain, an effect opposite to that observed for testosterone. Preincubation for 30 to 45 min with ouabain blocked the effect of testosterone, while preincubation with testosterone only partly blocked the effect of ouabain. When equimolar ouabain and testosterone were incubated together motility of spermatozoa initially tended to be intermediate between the individual effects of ouabain and testosterone. However, by the second hour of incubation ouabain inhibition was similar to that observed in ouabain treatment alone. Ouabain showed a synergistic effect when testosterone was added at only one-third the molar concentration of ouabain. The ability of ouabain and testosterone to block the action of each other suggests that their mechanisms are interrelated. However, they do not appear to have common binding sites because pretreatment of spermatozoa with ouabain (which is irreversibly bound) did not diminish the capacity of spermatozoa to bind testosterone.

206. **Maternal Ageing and Embryonic Mortality in the Rabbit. II. Hormonal Changes in Young and Aging Females.**
R.R. Maurer and R.H. Foote

Progesterone and 20α-hydroxypregn-4-en-3-one (20α-P) synthesis in vivo and in vitro was measured 12 days post coitum in does averaging 32 weeks (young) and 214 weeks old (ageing). Young pregnant does had an average of 7.7 corpora lutea and 7.3 implantations for a mortality rate of 4.3%. Similar values for aging does were 7.7, 5.4 and 29%, respectively. The progesterone content of ovarian venous blood was higher in young pregnant does than ageing does but the 20α-P content did not differ. Administration of LH did not significantly alter progesterone blood levels in either age group but increased 20α-P levels in both groups. Synthesis of progesterone and 20α-P by interstitial tissue in vitro did not differ significantly between ages. Addition of LH to the incubation medium stimulated synthesis of progesterone by tissue from the aging group whereas 20α-P synthesis was increased in the young group. There was a positive relationship between doe age and pituitary weight. The ageing does had heavier pituitary glands than the young does but there was no difference in total LH content. A lower concentration of LH was found in the pituitary gland and a lower progesterone content in the ovarian venous blood of old compared to young pregnant rabbits. While these differences may be partly responsible for the lowered reproductive efficiency of the aging female, the evidence is somewhat equivocal since total pituitary LH and synthesis of progesterone in vitro were similar in the two age groups.

207. **Uterine Uptake of Progesterone and Estradiol in Young and Aged Rabbits.**
L.L. Larson, C.H. Spilman and R.H. Foote

Eighteen young female rabbits 6-13 mo old, and 28 aged rabbits 49-72 mo old, were randomized into three treatment groups. Group 1 does were ovariectomized and sacrificed 14 days later. Groups 2 and 3 were sacrificed 12 and 24 days post-coitum (pc), respectively. Net progesterone-7-^{3}H and 17β-estradiol-4-^{14}C uptakes were determined for uterine tissue strips incubated at 37° for 0, 2 and 4 hr by correcting for skeletal muscle incubated similarly. Net uterine uptakes of progesterone-7-^{3}H and 17β-estradiol-4-^{14}C were greatest in the ovariectomized does and decreased particularly in the young does with increased time pc. Of most significance was the two- to four-fold greater uptake of both steroid hormones by uteri from young does than old does after 2 and 4 hr of incubation in the ovariectomized group and after 4 hr of incubation in the 12 day pc group. By 24 days pc steroid hormone uptake was low in both groups and not significantly different. The reduced capacity of uterine tissue in aged rabbits to take up steroid hormones is interpreted to be one of the uterine factors limiting reproductive performance in aged does.

208. **Effect of Osmolality and Phosphate, 'TRIS', 'TES', 'MES', and 'HEPES' Hydrogen Ion Buffers on the Motility of Bull Spermatozoa Stored at 37 or 5°C.** R.C. Jones and R.H. Foote

Bull spermatozoa survived incubation, in unstoppered tubes at 5 and 37°C, better in solutions of 250 m-osmoles/kg than in solutions of higher or lower osmolalities regardless of the concentrations of sodium chloride or citrate in the solutions relative to the concentrations of phosphate, Tris, TES, MES, or HEPES. When buffer solutions contributed 120 mosmoles/kg to a total diluent strength of 250 mosmoles/kg, the ranking from best to worst on the basis of survival of spermatozoa was: MES, HEPES, TES, Tris, and phosphate. When 50 mM concentrations of the buffers were compared the same ranking resulted except that HEPES was as good as MES.

209. **Sperm Production, Output and Urinary Loss in the Rabbit.** W. Holtz and R.H. Foote

Daily sperm production (DSP) in sexually mature rabbits was estimated to be 40.5×10^6/g of testis parenchyma or 187×10^6/animal. The daily sperm output (DSO) during a collection regimen of 2x/48 hr was 90.2×10^6 sperm/buck, accounting for 48% of the DSP. Up to 70% of DSP was accounted for by ejaculated sperm plus sperm in the urine. When the same group of males was sexually rested for an extended period 82% of the DSP was accounted for in the urine. The sperm unaccounted for may be resorbed or in part result from small errors of estimation. During the period of sexual rest sperm were voided in the urine in a cyclical pattern. In other males sexually rested for 36 days only 10% of the DSP was found in urine, indicating that most sperm were resorbed. Thus, resorption of sperm in recently filled epididymides may be greater initially than after prolonged periods of sexual rest.

210. **Interaction of Bovine Spermatozoa and Steroid Hormone.** R.C. Wester, G.W. Salisbury and R.H. Foote

Recent studies show direct relationships between bovine spermatozoa and steroid hormones. The majority of the enzyme for conversion of 17β-estradiol to estrone was located in the flagellum. An inhibitor to the enzyme exists in the ejaculated cell. 17β-estradiol stimulated sperm O_2 uptake, while progesterone and testosterone were inhibitory. Testosterone enhanced motility and a significant interaction between testosterone and sperm concentration was found. Testosterone and ouabain had antagonistic effects on sperm motility, and have different binding sites. Thus it is likely that metabolism of sperm in the reproductive fluids of the male and female are affected by steroid hormones.

211. **Variance Components of Semen Criteria from Bulls Ejaculated Frequently and Their Use in Experimental Design.** G.E. Seidel, Jr. and R.H. Foote

Variance components for 16 characteristics of bull semen were computed from 1,536 ejaculates from 63 Holstein bulls ejaculated four times per week. Based on animal and error components of variance, number of bulls and ejaculates per bull required to detect treatment differences for two treatment experiments under specified conditions were calculated for continuous and reversal type experiments. In reversal design to have a 90% chance of detecting a mean treatment difference of 25% at 5% significance requires two ejaculates per bull treatment collected from 3 to 22 bulls, depending on the semen characteristic. For continuous experiments in which each bull receives only one treatment, usually two to three times as many ejaculates are needed for similar sensitivity. The optimal design may differ depending upon ages of bulls.

212. **The Effect of Progesterone on the Early Development of the Rabbit Embryo.** M.C. Allen and R.H. Foote

The effects of subcontraceptive levels of progesterone administered near the time of ovulation on (1) embryo development in vitro; (2) embryo development in the treated animals; and (3) following transfer to control recipients were studied. One group of does, designated as P_1, received 0.5 mg of progesterone on Day -2 and 1.0 mg on Days -1 and 0, considering Day 0 as the day an ovulating dose of

LH was given. Another group, P_2, received the same dose of progesterone starting on Day -1, and a third group served as controls. Approximately 2000 eggs and blastocysts were collected from 122 does, and examined, cultured, or transferred to recipients. Ovulation and fertilization rates were unaffected by treatment. Four days after giving LH, the percentages of embryos that had developed into blastocysts in the P_1, P_2, and control groups were 58.6, 84.8, and 82.8, respectively, indicating that the P_1 progesterone treatment tended to suppress development of zygotes into blastocysts. Four-day blastocysts from P_1, P_2, and control does transferred to control recipients yielded 4.1, 20.4, and 44.4% implants at Day 12 of pregnancy. Corresponding values for implantations in does without transfers were 8.6, 59.3, and 97.0%. Thus, there was a dramatic reduction in viable embryos between Days 4 and 12. Since this occurred regardless of whether or not the embryos continued to reside in the uteri of treated does, it is clear that the morphologically normal appearing blastocysts had been damaged prior to Day 4. Recovery data suggested that progesterone may have altered embryo transport and thereby led to asynchrony between the developing embryo and its environment. In all treatments embryos which implanted successfully developed normally and were equivalent to controls in postnatal development.

213. Reproductive Efficiency in Aged Female Rabbits Given Supplemental Progesterone and Oestradiol.

L.L. Larson, C.H. Spilman, H.O. Dunn and R.H. Foote

The reproductive efficiency of twenty-eight aged does, 49 to 72 months old, was compared with that of eighteen young does, 6 to 13 months old. Fertilization rate and development in vitro of fertilized ova from rabbits induced to superovulate were not influenced by the doe's age. Ovulation rates following natural mating were only slightly reduced with age. However, the number of embryos per doe was much greater in young than in old does at 12 and 24 days post coitum. All young does had viable embryos, whereas the percentages of aged does with detectable implantation sites and viable embryos were 80 and 40, respectively, at 12 days post coitum, and 77 and 44 at 24 days post coitum. Aged female rabbits were given supplemental exogenous progesterone and/or oestradiol benzoate in an effort to increase reproductive efficiency. Progesterone treatment had no effect on the total number of young kindled but did prolong the gestation period, increase the birth weight and result in fewer live young kindled/doe. When administered on Days 3 to 29 of pregnancy, 4 μg/day of oestradiol alone or in combination with 2 and 4 mg progesterone completely blocked pregnancy in all does. Starting on Day 5 of pregnancy, oestradiol levels of 1 μg/day, with or without progesterone, had no effect. Chromosomal analysis of fourteen embryos revealed eleven normal females (44,X), one normal male (44,XY), one abnormal embryo (45,XX) with an extra acrocentric chromosome and one embryo with a modal number of forty-two chromosomes in 35% of the metaphases. Since most of the embryonic wastage in aged rabbits occurred during the first 12 days post coitum, chromosome studies of embryos younger than 12 days post coitum are indicated. Most of the embryonic wastage could not be attributed to ovulation rate, fertilization rate, ovum potential, CL function, circulating levels of progesterone and oestrogen, or to chromosomal anomalies of the fetuses. It was concluded that uterine factors apparently limit reproductive performance in aged rabbits.

214. Progesterone, 20β-Hydroxypregn-4-en-3-one, and Luteinizing Hormone Levels in Superovulated Prepuberal and Postpuberal Cattle.

C.H. Spilman, G.E. Seidel, Jr., L.L. Larson, G.R. Vukman and R.H. Foote

Pituitary and plasma luteinizing hormone (LH) and luteal and plasma progesterone were measured in 47 prepuberal Holstein calves and in eight postpuberal heifers for 30 days after induced ovulation or superovulation. Mean pituitary LH in six of the eight heifers sacrificed 30-31 days after superovulation averaged 1.1 mg LH/g of pituitary tissue. Plasma LH rose to 7.2 ng/ml the day after PMSG injection and gradually declined to normal levels of about 2 ng/ml. Plasma progesterone peaked at 63 ng/ml 10 days after LH injection to induce superovulation and declined to nearly undetectable levels in three of the heifers by Day 24. These three heifers ovulated spontaneously, plasma progesterone rose to 6.3 ng/ml by Day 28, and each had a new active corpus luteum at slaughter. The others had not ovulated when

slaughtered on Day 30, and still had some old active luteal tissue. Mean pituitary LH in control calves was 5.0 mg/g of wet pituitary tissue and ranged from 0.7 to 1.6 mg/g during the 30 days after superovulation. Plasma LH in calves rose from 1.5 ng/ml before injection of pregnant mare serum gonadotropin (PMSG) to 5.5 ng/ml the day after PMSG and returned to the original level by 10 days after PMSG. Calf corpora lutea had a maximum average progesterone concentration of 43 μ/g of luteal tissue 10-11 days after ovulatory LH. This declined to a minimum on Day 26, but some calves had as much as 36 μg progesterone/g of luteal tissue on Day 30. Plasma progesterone rose to nearly 100 ng/ml 8-10 days after superovulation, and peak progesterone was correlated with the number of corpora lutea ($r = 0.76$, $P < 0.01$). Plasma progesterone declined after Day 15, but detectable levels were present throughout the experiment. Plasma progesterone and 20 β-hydroxypregn-4-en-3-one rose and fell simultaneously in eight superovulated calves. The major difference among the prepuberal and postpuberal animals was the fact that plasma progesterone in the prepuberal animals never declined to essentially undetectable levels and none ovulated spontaneously. Whether this reflects a difference between uteri of immature and mature cattle in regulating corpus luteum function has not been investigated.

215. **Phospholipid Composition of Bovine Sperm Fractions, Seminal Plasma and Cytoplasmic Droplets.**
E.D. Clegg and R.H. Foote

The phosphatidylcholine, phosphatidalcholine, phosphatidylethanolamine, phosphatidalethanolamine, sphingomyelin, cardiolipin, phosphatidylinositol and phosphatidylserine plus lysophosphatidylcholine were determined in sperm heads, tails and midpieces and in cytoplasmic droplets and seminal plasma. Several of these compounds were identified for the first time in semen, with significant differences in the different fractions of semen. Composition of all phospholipids in the different components of the sperm cell are reported.

216. **A.I. in Dogs, Cats and Furbearers.**
R.H. Foote

Semen quality and methods of semen collection in dogs, cats and a variety of fur bearers is described. Detailed methods of semen collection and evaluation and preserving media are given. One of the more common diluters used is composed of tris, sodium citrate dihydrate, glucose, glycerol (20% volume egg yolk), antibiotics and double distilled water. Preservation and insemination procedures are described.

217. **Equine Hypothyroidism: The Long Term Effects of Thyroidectomy on Metabolism and Growth in Mares and Stallions.** J.E. Lowe, B.H. Baldwin, R.H. Foote, R.B. Hillman and F.A. Kallfelz

Surgical thyroidectomies (Thx) were performed in 6 yearling grade horse colts, (3 males, 3 females). Five control colts (3 males, 2 females) were included and comparative studies were carried out for 67 weeks. The comparative measurements included rectal temperature, heart rate, feed consumption, packed cell volume, serum cholesterol, serum calcium, serum phosphorus, serum T_4, height, heart girth, body weight, epiphyseal plate closure and tooth eruption times. A thyroprotein supplement was fed to the Thx males during weeks 46 to 53. The Thx animals failed to grow in height, were sensitive to cold, shed their winter hair coats late and had dull coarse hair coats. The Thx animals were generally docile and lethargic, with a sleepy appearing expression. Edema of the rear limbs and a course thickened appearance of the face was evident. Rectal temperatures were significantly depressed and cholesterol levels were significantly elevated. Feed consumption and body weight gains were decreased compared to controls. This was particularly pronounced in females; Thx females consumed 46% less feed and gained 47% less weight than the controls. Serum calcium was unaffected; however, serum phosphorus was depressed, particularly in the females. Epiphyseal plate closure time was delayed as was eruption of incisor teeth. Thyroprotein supplementation

resulted in a rapid and dramatic reversal of the signs of hypothyroidism. Discontinuance of thyroprotein supplementation resulted in a return of hypothyroidism but not to the degree seen prior to supplementation, except the T_4 values fell again to non-detectable levels.

218. Cannulation and Recovery of Spermatozoa From the Rabbit Ductus Deferens.

W. Holtz and R.H. Foote

A cannula was designed that would permit flushing sperm regularly from the ductus deferens to evaluate characteristics of sperm as they left the testis, and to compare sperm output on the cannulated side compared with the cannulated side. More sperm were collected through the cannula than were ejaculated from the contralateral side. So, either sperm are lost regularly through the urethra or are absorbed in the epididymis, or both. The results point to the former. Teasing and collecting semen from bucks caused large numbers of sperm to appear in the cannula, whereas during periods of sexual rest, a more uniform flow of sperm into the cannula was observed. Overall, these results indicate that most sperm produced are removed by passage out of the urethra, but some absorption may occur, particularly in sexually rested animals.

219. The Economics of Selected Systems of Banking Semen vs. Maintaining Bulls.

G.H. Coulter and R.H. Foote

The banking of semen can be economically advantageous in today's A.I. industry. It may be applied profitably in small to medium sized studs where distribution and opportunity for the sale of large quantities of semen from one bull is unlikely. For example, using cost figures for the .5 ml straw, a banking system is economically advantageous for either 15,000 or 30,000 breeding units/low service volume bulls and 100,000 breeding units/high service volume bull at each of the four sperm levels/breeding unit (15, 20, 30 and 40 $\times$ 10^6). In addition, bank sizes of 15,000 or 30,000 and 200,000 breeding units/bull (systems III and IV) are profitable if 15 $\times$ 10^6 sperm/ breeding unit were acceptable. The ability to rank young bulls on their genetic merit and future demand would permit banking of up to 200,000 breeding units from top bulls economically (system V). All banking systems proposed for the low service volume breeds were economically advantageous. Semen banking could be made more advantageous economically (1) by using small packaging units (e.g., .25 ml or smaller volumes instead of .5 ml straws or larger volumes), (2) by using the minimum number of sperm required for maximum fertility (20 $\times$ 10^6 total sperm/ breeding unit instead of the 30-40 $\times$ 10^6 sperm now used frequently), (3) by selecting bulls with high sperm production potential and maximizing sperm output, (4) and by better ranking of young bulls on the basis of genetic merit, semen quality and fertility. No bull should be sampled with predictions of semen quality, fertility and transmitting ability that indicate the bull will be unsuitable for use following progeny testing.

220. Effects of Low Temperature Storage Upon Subsequent Energy Metabolism of Rabbit Embryos.

G.B. Anderson and R.H. Foote

The oxidation of pyruvate by two-cell rabbit embryos occurred at a very low rate at 10°C, but increased 10-15 times upon rewarming to 37°C. Pyruvate utilization of these embryos increased with continued development parallel to embryos not cooled to 10°C, but incubated directly at 37°C. Storage at 10°C did not affect the amount of glucose subsequently oxidized at 37°C, nor the shift from the pentose shunt to glycolysis and the Krebs cycle that normally occurs between the morula and blastocyst stage. Thus, the temporary inhibition of carbohydrate metabolism induced through temperature reduction did not impair the subsequent ability of the embryos to metabolize pyruvate and glucose.

221. Testicular Blood Flow Rates in Prepubertal and Adult Rabbits Measured by ^{85}Krypton.

L.L. Larson and R.H. Foote

Twenty male Dutch rabbits were studied. LH and FSH given to 12-week old rabbits for 17 days failed to alter spermatogenesis or blood flow. As testes grew in gonadotropin treated males, or in controls ranging in age from 12 weeks to 3 years, the increase in total blood flow corresponded to the

increase in testis size. Overall, the mean testicular blood flow rate for 80 measurements was 0.120 ml/g tissue/min.

222. **The Motility, Acrosomal Morphology and Oxygen Uptake of Bull Spermatozoa During Processing and After Freezing in Straws.**
G.H. Coulter and R.H. Foote

Since many procedures for processing liquid semen have been utilized, in part, for frozen semen without further testing, sperm motility, acrosome morphology and oxygen utilization were measured before cooling, after cooling to 5 C, before freezing and after freezing and thawing the semen from 18 Holstein bulls. Several conclusions which can be drawn from the data are as follows: (1) The ability of sperm to survive at body temperature for 6 hours following freezing and thawing decreases at each stage of semen processing. Acrosome damage also increases at each successive stage sampled; (2) the greatest damage to sperm occurs during freezing and thawing; (3) freezing rates in N_2 vapor may vary considerably with successive freezes unless special precautions are taken to provide a heat sink in the tank to reduce freezing damage; (4) a freezing rate of 8.4 C/min between -10 and -80 C gave better acrosomal protection than the 17.5 C/min freezing rate. The entire system of semen handling in straws should be tested in field trials in order to insure optimum fertility with this method of packaging.

223. **Physiological and Technical Aspects of Animal Reproduction**. R.H. Foote

This is a book based on lectures given during 2 weeks in Helsinki to scientists from all of the Scandinavian countries. The subjects included the physiology of the male, semen collection, evaluation, freezing and insemination. On the female side, the physiology of the cow, estrous detection and estrus cycle regulations, and pregnancy checking were among the topics in a 260-page book published by the Institute of Animal Breeding in Finland.

224. **Sexual Activity, Seminal Characteristics, and Reproductive Organs in Sexually Inexperienced Castrate Rabbits Following Testosterone Implantation.**
W. Greene, P. Johnson and R.H. Foote

Males castrated at 2.5 mo of age were supplied immediately with testosterone-filled implants (210.5 mm^2 vs 421 mm^2 inside surface area). Plasma testosterone levels, libido, and sexual development tended to parallel implant size. Other males castrated for 26 days before receiving testosterone implants were delayed in development, but otherwise responded similarly to testosterone therapy. Libido and semen volume were higher in control males than in other groups despite the fact that plasma testosterone in controls averaged 1.52 ng/ml compared to 2.41 ng/ml for bucks with 2-cm implants and 1.33 ng/ml for bucks with 1-cm implants. This result indicates that testicular products, in addition to testosterone in controls, were involved in producing normal sexual development and activity.

225. **Lipid Deficient Extender for Bovine Spermatozoa: Its Development and Use in Measuring Freezing-Induced Lipid Loss.**
G.H. Coulter and R.H. Foote

To facilitate the measurement of lipid losses from spermatozoa due to freezing, three low-lipid seminal extenders containing lactose, bovine serum albumin, or soybean protein were evaluated as potential cryoprotectants. All extenders were formulated to have an osmotic pressure within the range of 270 to 330 mosmol and a pH of 6.8 to 7.0. Soybean protein (Promine-D) maintained the highest post-thaw motility of spermatozoa with similar survival for spermatozoa frozen in ampules and straws. The extender derived from testing several components consisted of Tris (hydroxymethyl) aminomethane (245 mM), and citric acid monohydrate (78 mM), as the buffering compounds; and fructose (69 mM), glycerol (7% vol/vol), and Promine-D (1.5% wt/vol). Post-thaw sperm motility of approximately 40% was

not different from the Tris-egg yolk control. Fertility of fresh rabbit semen treated with the extender was normal. After freeze-thawing, protected spermatozoa contained more lipid (1.61 versus 1.20 μg/10^6 sperm) and lost less glutamic oxaloacetic transaminase enzyme (102 versus 108 Karmen units) than when Promine-D was not incorporated. However, even with protection by soybean protein, spermatozoa lipid content decreased from 2.43 to 1.61 μg/10^6 sperm after one freeze-thawing. The lipid status of spermatozoa frozen and thawed in conventional bull seminal extenders containing large amounts of lipid is unknown.

226. Estrus Detection and Estrus Detection Aids. R.H. Foote

There is no substitute for good management. Each animal should be identified carefully. The herdsman should know all signs of estrus or impending estrus. In addition, for visual detection of estrus to be highly effective, sufficient time must be taken at least twice a day to catch animals with a short estrus period. Presently there are two visual aids to estrus detection which provide potential 24-h surveillance. One aid is a pressure sensitive device mounted on the back of each cow which can be triggered when the cow stands for mounting. The second one is a marking device worn by sexually aggressive animals which will stripe with colored ink the back of estrous animals as the market animal mounts and dismounts. Both devices are effective when used properly. Other tests of changes in cervical mucus, vaginal characteristics, temperature, blood flow, and hormone changes in blood and milk are either not sufficiently reliable or else simple enough yet to be practical aids for routine detection of estrus in dairy cattle. Milk progesterone can assist in characterizing problem cows.

227. Effects of Low Temperature Upon Subsequent Nucleic Acid and Protein Synthesis or Rabbit Embryos. G.B. Anderson and R.H. Foote

Two-cell rabbit embryos, which were maintained without cleavage for 24 h at 10°C, and then allowed to develop into blastocysts in vivo or in a completely defined medium at 37°C, incorporated ^{3}H-uridine into RNA and ^{14}C-leucine into protein at rates comparable to blastocysts not previously exposed to 10°C temperature. Thus, temporary suppression of development did not impair nucleic acid and protein synthesis by embryos developing into blastocysts. Blastocysts which developed in vivo incorporated more tritiated-thymidine into DNA ^{3}H-uridine into RNA and ^{14} C-leucine into protein than did blastocysts which developed in vitro. These differences probably reflect not only differences in blastocyst size and number of cells, but also differences in endogenous precursor pools.

228. Effects of Hormones on the Maturation of Rabbit Oocytes Recovered from Follicles of Various Sizes. I.-H. Bae and R.H. Foote

Oocytes were obtained from follicles ≥ 1 mm in diameter. They were cultured in a simple medium containing 0.4% BSA, pyruvate, lactate and glucose. Progesterone stimulated oocytes to develop more rapidly in culture. The time-dependent effect was more pronounced on large preovulatory Graafian follicles than on small- and medium-sized follicles. Treatment with LH had no effect.

229. The Gametogenic Function of the Aging Ovary in the Mammal. R.H. Foote

The intent of this chapter is to focus attention on the changing gametogenic function of the aging ovary in the female. Origin and fate of the germ cells is reviewed for nine species, including human. With most organs one might limit the examination of a particular aging organ to changes in the aged female. However, considering the fact that the gametogenic function in the female is initiated as soon as the definitive gonad is formed early in fetal life, aging of certain ovarian components truly begins prenatally. The gametogenic function is influenced by endocrine secretions, but it is only necessary to call attention here to a few of the more important interactions. Although the body of endocrine information is proliferating rapidly, the nature of the interactions still is imprecisely understood. Throughout the chapter the terms 'gametogenic function' and 'oogenesis' will be used synonymously to include the formation, development, and maturation of the female

gamete. In the definitive female gonad, the ovary, the diploid germinal cells that undergo repeated mitotic divisions are called 'oogonia'. The last mitotic division gives rise to 'primary oocytes' that enter prophase I of meiosis. The term 'egg' will be used somewhat loosely in reference to ovarian oocytes. At the time of ovulation the egg is usually shed as a secondary oocyte in the metaphase stage of the second meiotic division. The term 'ovum' will be little used since this correctly applies to the post-ovulatory haploid female gamete from which both polar bodies have been extruded. This condition seldom is realized because sperm penetration usually precedes extrusion of the second polar bodies.

230. Utilization of Glutamine for Energy and Protein Synthesis by Cultured Rabbit Follicular Oocytes.

I.-H. Bae and R.H. Foote

Follicular oocytes developed in a balanced salt medium (Exp. Cell Res. 91:113-118. 1975), containing universally labeled ^{14}C-glutamine and bovine serum albumin, but no carbohydrates or other organic compounds. From the $^{14}CO_2$ produced and ^{14}C-TCA precipitable material isolated it is suggested that glutamine probably is utilized by oocytes and cumulus cells as a source of energy as well as for protein synthesis. The oocytes produced only about 32% as much $^{14}CO_2$ as did the associated cumulus cells, but produced 27% more protein.

231. Development of Rabbit Embryos After Storage at 10 C.

G.B. Anderson and R.H. Foote

Two-cell rabbit embryos were stored at 10 C for 24 hr in BSM II, a chemically defined medium, or BSM II with 4% gelatin, 4% Promine-D, or 3% egg yolk lipoprotein added for cryoprotection. Following storage and subsequent transfer to recipients, embryos were allowed to develop to term. The addition of 4% gelatin or 4% Promine-D to the storage medium was beneficial and resulted in a rate of development similar to embryos which were not stored at 10 C, but were transferred immediately after collection. Increasing the period embryos were stored in BSM II plus 4% Promine-D to 48, 72 and 96 hr resulted in a progressive decrease in viability. Regardless of the storage medium or the storage period, losses due to low temperature storage were shown to occur prior to day 12 of gestation. Postnatal development of treated embryos, as determined by 3-week weight gain, was equivalent to control embryos.

232. Fertility in New York Artificially Inseminated Holstein Herds in Dairy Herd Improvement.

R.W. Spalding, R.W. Everett and R.H. Foote

This was a field study to gain information on fertility of New York dairy herds and factors influencing it. Data were from 125 Dairy Herd Improvement Holstein herds with 9,750 cows. All breedings to milking cows were by artificial insemination. Herds were further selected with equal numbers of small and large herds and free-stall and conventional (stanchion) housing. Conception on first service averaged 50% and the 60 to 90 day nonreturns, 58%. Interval from calving to first service averaged 87 days and days open 116 days. Intervals between first and second service averaged 41 days and between second and third service 40 days. A total of 76 and 89% of all cows were pregnant on the first two and first three services. Conception declined markedly with increased production when age, herd size, and other variables in the model were not allowed to vary. Cows producing > 907 kg above herdmates were 20.5 percentage units in conception on first service lower than the base group of cows which were ≤ 907 kg below their herdmates in milk production. As age advanced beyond 4 yr, fertility declined for given milk production. As size of herds increased, reproductive efficiency, as indicated by conception rate, declined; however, milk produced per cow increased. Lengths of dry period were not influenced by production or herd size but did increase with age of cow.

233. Carbohydrate and Amino Acid Requirements and Ammonia Production of Rabbit Follicular Oocytes Matured in Vitro.

I.-H. Bae and R.H. Foote

Rabbit follicular oocytes were cultured at 37°C for 18-24 h in a basic salt medium containing 0.4% bovine serum albumin (BSA), carbohydrates and amino acids in various combinations (see details of

composition in the text). Osmolarity of the medium was maintained at 308 mOsm. The carbohydrates, pyruvate, lactate and glucose were all about equally beneficial, but not essential for rabbit oocyte maturation. Glutamine and proline, but not methionine or phenylalanine stimulated oocyte development. Glutamine stimulated more follicular oocytes to develop to prophase and metaphase II than did any of the three carbohydrates tested alone or in combination. Ammonia production after 24 h of culture was highest in media containing glutamine (15.2 μg/ml) but this was not inhibitory to maturation. Negligible amounts of ammonia were found with the other amino acids added. With 0, 0.08, 0.4, 2, 10 and 50 mM of glutamine in the basic salt medium, plus 0.4% BSA, but without carbohydrates, 30, 73, 70, 71, 59 and 45% of the follicular oocytes developed to the prophase or metaphase II stage. It is concluded that the optimum level of glutamine ranged from 0.08 to 2 mM and that no carbohydrate need be added to the medium for culturing oocytes when glutamine is included.

234. **Semen Quality From the Bull to the Freezer: An Assessment.** R.H. Foote

Frozen semen has provided the cattleman with the opportunity of using outstanding tested sires in a variety of breeding programs. However, to make the most effective use of these sires, high quality semen is essential to help achieve the good conception rates necessary. In view of the multitude of frozen semen producers, not all of whom subject bulls and processing semen to rigid quality control, and the difficulty of obtaining accurate nonreturn rates, the present status of frozen semen and research needs are assessed. Tests of semen quality are helpful in ranking bulls of unknown fertility, but they are not highly correlated with fertility of ejaculates within bulls. Therefore, further research is needed on the combination of tests which can be applied to maximize the prediction of ejaculate fertility. Furthermore, with direct semen sales and frozen semen tanks on farms, currently available nonreturn rates are not as accurate a reflection of pregnancy rates as in former times. Therefore, studies are needed to find ways of correcting biases in nonreturn rates, if possible. A major single source of variation in fertility is the bull, not the individual ejaculate. So if the true fertility of the bull can be established, and semen is carefully processed and stored to preserve this fertility, individual ejaculate selection becomes less critical. Testicular traits associated with semen quality and fertility are heritable. Therefore, more emphasis should be placed on pedigree selection of bulls for reproductive fitness initially, where the information is available, and in performance testing the bull at puberty (testis and semen evaluation) before putting it into service. Each batch of semen should be processed by the best procedure known in order to maximally preserve the fertility of the semen as it is produced by the bull. All semen suppliers should have, and buyers require, rigid quality control standards. Fertility and semen quality tend to decline with age of the bull. Liquid nitrogen storage technology could be used advantageously to preserve sperm cells from young sexually inactive bulls awaiting progeny test results. Herd management, particularly detection of estrus and insemination at the right time, presents another major challenge today if conception rates are to be maintained at a level that will attract cattlemen to use A.I. service from genetically superior bulls.

235. **Development of Rabbit Embryos In Vitro and In Vivo Following Storage of the Two-Cell Stage at 10°C.**
G.B. Anderson and R.H. Foote

This study involved four treatments of a total of 1602 embryos. Two-cell embryos were cooled to 10°C for 24 hours and cultured compared with *in vivo* grown uncooled controls and cultured embryos with and without cooling. The embryos produced *in vivo* were superior to the embryos grown *in vitro*. Those 2-cell embryos held at 10°C for 24 hours also developed more slowly. For 2-cell embryos cultured *in vitro* for 94 hours, 60% became blastocysts; 2-cell embryos held at 10°C for 24 hours before culture for 94 hours developed into 22% blastocysts; 2-cell embryos flushed immediately transferred and grown *in vivo* for 94 hours developed into 88% blastocysts; 2-cell embryos cooled to 10°C for 24 hours and then transferred and grown *in vivo* for 94 hours developed into 66% blastocysts. All means were different ($P < 0.05$).

236. Milk Progesterone in Postpartum and Pregnant Cows as a Monitor of Reproductive Status.

J.J. Schiavo, R.L. Matuszczak, E.B. Oltenacu and R.H. Foote

Milk samples were analyzed for progesterone content by a petroleum ether extraction and competitive protein binding assay validated for milk. In one experiment, 11 cows were sampled twice daily for 24 days beginning with an observed estrus 15 to 45 days postpartum, and again 19, 21, 23, and 25 days after breeding. Progesterone values during the estrous cycle paralleled those for blood plasma but were slightly higher at estrus (1.49 ng/ml milk) and maximum (9 ng/ml) on days 11 to 16 of the estrous cycle. After breeding, cows later diagnosed pregnant averaged 7.12 ng/ml while those later found to be nonpregnant averaged 2.36 ng/ml. All diagnoses of pregnancy were correct. In a separate experiment there was no difference between milk from front and rear quarters, but progesterone was highest in last milk, intermediate in composite milk, and lowest in first milk.

237. Effect of Age on Testicular Growth and Consistency of Holstein and Angus Bulls.

G.H. Coulter, L.L. Larson and R.H. Foote

A total of 5,909 scrotal circumference and 5,373 testicular consistency (tonometer) measurements were made on Holstein bulls in seven artificial insemination studs. Comparable measurements were made on 339 Angus bulls. The multiple regression equation, $\hat{Y} = -4.67 + 47.26 \log X - 11.74 (\log X^2)$, where $\hat{Y}$ is scrotal circumference in cm and X is bull age in months, described the high rate of testicular growth in young Holstein bulls, limited growth in mature bulls, and a slight decrease in the oldest bulls. Angus tended to have larger testes than Holsteins up to 3 years of age, but they were surpassed after that age by Holstein bulls. Regression of testicular consistency on age of Holstein bulls resulted in the equation $\hat{Y} = 20.83 - 9.06 \log X + 2.47 (\log X)^2$, where $\hat{Y}$ is the mean deflection averaged for weak and strong spring tonometers in mm and X is bull age in months. This equation describes the decrease in firmness of testes of bulls from 6 to 12 months of age up to 48 to 54 months of age. Then consistency became slightly firmer with age, but in the few oldest bulls measured there was a decline. Differences among bull studs existed for both traits. Differences in testis size among bulls of the same age were large. Selection of bulls with large testes could decrease the number required to produce enough sperm to inseminate a specified population.

238. Semen Characteristics in Thyroidectomized Stallions.

J.E. Lowe, B.H. Baldwin, R.H. Foote, R.B. Hillman and F.A. Kallfelz

Three Quarter-horse stallions were surgically thyroidectomized at 18 months of age. Weekly semen collections were made from these and three control stallions from 25 to 39 months of age. Although the induced state of hypothyroidism in young stallions has a depressing effect on libido and general animation and can be corrected by thyroprotein supplementation, semen characteristics, testicular histology and fertility are not affected by the operation.

239. Effect of Season and Year of Measurement on Testicular Growth and Consistency of Holstein Bulls.

G.H. Coulter and R.H. Foote

Seasonal effects were found for 635 testicular size and consistency measurements taken in early spring and 644 in late summer on Holstein bulls 6 to 180 months of age. Regression equations were calculated for each season to best describe the data. The equation for early spring measurements of scrotal circumference was $\hat{Y} = -9.54 + 53.93 \log X - 13.63 (\log X)^2$ where $\hat{Y}$ is scrotal circumference in centimeters and X is bull age in months. The corresponding equation for late summer was $\hat{Y} = .54 + 39.86 \log X - 9.29 (\log X)^2$. Tonometry measurements made in the early spring were described by the equation $\hat{Y} = 25.10 - 14.63 \log X + 4.24 (\log X)^2$, where Y is the average deflection in millimeters of weak and strong spring tonometers and X is bull age in months. The corresponding equation for late summer was $\hat{Y} = 22.53 - 10.33 \log X + 2.81 (\log X)^2$. Bulls 6 to 48 months of age were analyzed more extensively. An effect of season and/or artificial insemination center on both testicular characteristics

were found. Also, there was a significant year effect. Regression equations fitted to data for each of the 5 years were more accurate ($P < .01$) in describing the distribution of data than was a common equation for pooled data. These results emphasized that selection of bulls for these traits can best be made within the same group, bull stud and year-season. As more data become available correction factors could be developed and indexes computed to make more general comparisons possible.

240. Spermagglutinin Titers and Their Relationship to Fertility in Isoimmunized Female Rabbits.

H.L. Kummerfeld, R.L. Hintz and R.H. Foote

The sera from 48 female rabbits immunized by a series of multiple intradermal injections of washed epididymal, washed ejaculated, and β-amylase-treated rabbit spermatozoa in complete adjuvant were examined for spermagglutinins by the Kibrick gel agglutination test and a slight modification of the Shulman capillary agglutination test. Control animals receiving the adjuvant or saline usually had no positive titers. All three antigenic preparations produced similar titers, positive at dilutions as high as 8192-fold with the Kibrick test and 256-fold with the Shulman test. Maximal titer development was reached 4 to 6 weeks after starting the immunization, and positive sera were obtained from some does for 25 weeks. The correlation coefficients between positive titers obtained by the two tests were $r = 0.91$ during the first 10 weeks and 0.41 at 15 to 25 weeks after immunization. Immunization essentially rendered the does sterile 6 weeks after antigen injection started, with fertility gradually returning to near control levels within 25 weeks. At 6 weeks the correlations between the percentage of eggs fertilized and the spermagglutinin titers by the Kibrick and Shulman assays were 0.93 and 0.88, respectively. During declining immunity, titers obtained by the Shulman test were more highly correlated with fertility than were results from the Kibrick test. Also, Shulman procedure is simpler, and so it appears to have an advantage in monitoring sperm-agglutinins in sera of immunized animals.

241. Infertility and Embryonic Mortality in Female Rabbits Immunized With Different Sperm Preparations.

H.L. Kummerfeld and R.H. Foote

Female rabbits were immunized by multiple intradermal injections with washed ejaculated, epididymal or β-amylase treated rabbit sperm in Freund's complete adjuvant emulsion. All treatments suppressed fertility, with only 2.3 percent of 364 eggs recovered from 17 immunized rabbits fertilized following intravaginal insemination, while the control fertilization rate was 95.7 percent. Reinsemination of females when immunity was declining showed that normal fertility was attained by about 25 weeks postimmunization. Intramuscular booster injections given to 5 does 61 weeks after initial immunization resulted in 47 percent of 19 eggs recovered being fertilized, compared to 96 percent fertilization in 26 eggs from 5 control animals. The very low fertilization rate in immunized females prevented tests for embryonic and fetal mortality when immunized animals were inseminated. In order to test whether the uterus of immunized rabbits was hostile to conceptuses, normal embryos were transferred to immunized females. The actual embryonic and fetal mortality appeared to be higher in immunized animals, but only the results for young born attained statistical significance ($P < 0.05$). Collectively these results suggest that the infertility effect of immunization was primarily expressed as a block to fertilization.

242. Relationship of Testicular Weight to Age and Scrotal Circumference of Holstein Bulls.

G.H. Coulter and R.H. Foote

Testicular weights were recorded for 250 Holstein bulls ranging in age from 19 to 189 mo. The multiple regression equation that best described the relationship between paired-testes weight and age of bull was $\hat{Y} = -368.8 + 952.2 \log X - 180.3 (\log X)^2$, where $\hat{Y}$ is paired-testes weight in g and X is bull age in mo. Correlations between these two variables were .86 for 24 bulls ≤ 60 mo of age and .60 for 23 > 60 mo of age. A linear regression equation for 47 bulls

was $\hat{Y}$ = -645.5 + 34.0 X, where $\hat{Y}$ is paired-testes weight in g and X is scrotal circumference in cm measured within 1 mo of slaughter. The correlation coefficient was .79. Differences in testicular weights of bulls of similar ages were large. These should be taken into account by measuring scrotal circumference and selecting young bulls with larger testes and superior spermatozoal producing capabilities.

243. Detection of Estrus in Dairy Cows by Electrical Measurements of Vaginal Mucus and by Milk Progesterone.

P. Gartland, J. Schiavo, C.E. Hall, R.H. Foote and N.R. Scott

Electrical resistance (ohms) of mucus were analyzed in 20 postpartum Holstein cows by use of a probe inserted into the anterior vagina every other day for 30 days. Composite milk samples were taken on the same day, and progesterone was determined by radioimmunoassay. Cows were observed twice daily for standing estrus and reproductive organs palpated weekly per rectum (rectal palpation). Fifteen cows which were cycling showed increasing progesterone 6 to 7 days after the onset of estrus with values of 8.1 to 10.0 ng progesterone/ml milk on days 10 to 17. Concentrations had declined rapidly 2 days before onset of the next estrus. Progesterone in milk was affected by cow and by day of the cycle. Electrical resistance followed a similar cyclical pattern, but variability was large and only cows differed. The correlation between milk progesterone and mucus resistance was .22. Progesterone concentrations for four cows with follicular cysts fluctuated randomly with a mean of 2.6 ng/ml. Mean resistance of vaginal mucus was 44 Ω for both cycling and cystic cows, indicating that a single measurement of electrical resistance every 2nd day was unreliable in distinguishing physiological states. One cow had high progesterone in milk on days 19 to 25 and was diagnosed pregnant by rectal palpation 3 wk later. Cows were not seen in estrus 28% of the time when milk progesterone and rectal palpation indicated they were in the follicular phase of the estrous cycle and were cycling.

244. Heritability of Testicular Size and Consistency in Holstein Bulls.

G.H. Coulter, T.R. Rounsaville and R.H. Foote

During the period 1967 to 1973, 4,275 measurements of scrotal circumference and 3,859 measurements of testicular consistency were made on 1,521 Holstein sires in seven bull studs providing artificial insemination service. Heritability of scrotal circumference (a predictor of total sperm output), and three similar measures of testicular consistency as predictors of semen quality and fertilizing capacity were estimated from paternal half-sib correlations. These measurements were corrected for fixed effects of year-season and age of bull within 11 6-month age groupings on bulls ranging in age from 6 to 72 months. Heritability estimates, weighted according to the number of observations per group, averaged .67 for scrotal circumference, .30 and .22 for consistency measured with the weak and strong spring tonometers and .34 for the average of both tonometers. With these heritabilities, high repeatability, ease of taking the necessary measurements, their relationship to reproductive performance of bulls and the current low reproductive rates, it would seem desirable to place some emphasis on these traits in bull evaluation.

245. Antibacterial Agents for Bull Semen; Do They Help?

R.H. Foote and W.E. Berndtson

Extended semen may contain organisms from the bull or added by contamination during processing. Many organisms can survive in frozen semen. Considerable progress has been made over the years by major bull studs in eliminating various specific pathogens from their bulls. Under the best conditions addition of antibiotics to semen may not be helpful. However, the best of conditions don't always occur and antibiotics can provide some measure of insurance. A variety of antibiotic combinations that are potentially useful as additives to extenders for bull semen are available. Conditions may change, and the kind and quantity of antibiotic, time and temperature

of exposure of organisms to the antibiotics and composition of the extender should be considered in formulating a satisfactory procedure for controlling microorganisms in semen. The goal should always be to keep out the "bugs" in the first place! Data on sources of contamination and the testing of 17 antibiotics, mostly from unpublished data by the authors, is included. In addition to penicillin and streptomycin these include tetracycline, oxytetracline, chlortetracycline, chloramphenicol, neomycin, polymyxin B, erythromycin, kanamycin, tylosin, thiostrepton, nystatin, amphotericin B, colymycin, lincomycin, spectinomycin, epicillin and ampicillin.

246. **Monitoring Fertility of A.I. Programs: Can Non-Return Rate do the Job?**
E.A.B. Oltenacu and R.H. Foote

Extensive data sets are reported which indicate the value of nonreturn reports in assessing fertility. The nonreturn report can be improved in several ways to remove many sources of variation. Electronic calculation makes this task simple, provided items of importance are identified. The industry should move to adopt a standard 75-day report. Only first inseminations should be included in making critical comparisons between treatments, etc. Tests should not be conducted around holidays, and breedings at such times should be excluded when making ejaculate and other types of comparisons. Data obtained with heifers should be isolated from that obtained with cows. Fertility data obtained on ejaculates maintained in the field for long periods should not be included in making critical comparisons. Cows in DHIA herds should be identified and tabulated separately from others. The nonreturn report, adjusted for the factors noted, and others that may be detected in the future, can be a reliable tool in providing information vital to making decisions as outlined at the beginning of this report. With the large binomial variation inherent in cattle breeding, large numbers of observations are essential to estimate reliably the level of fertility associated with a particular bull, extender, procedure, or other component. Close scrutiny of bulls, considering especially testis size and consistency along with careful semen analysis, becomes increasingly important. These can be useful in ranking bulls, but no tests have been very reliable in predicting ejaculate fertility.

247. **Preparation of Teaser Bulls and Steroid-Implanted Steers and Their Effectiveness in Detecting Estrus.**
T.J. McDonald, R.H. Foote, M. Drost, L. Lu, M. Patrascu and C.E. Hall, Jr.

Pen-O-Blocks were easily installed in 15 bulls. Libido appeared to be unaffected. However, with the exception of one bull, infections occurred, or the penis bypassed the block, or the pin broke, and in 14 tests the Pen-O-Blocks were removed within 5 weeks of installation. Recovery from infection following removal of the device was rapid and uneventful. Eight bulls had their penes deviated between 2 weeks and 4 months of age. Four of these also were castrated and implanted with androgens at 12 months of age. Semen quality and libido in the deviated bulls were normal. Bulls and steers were equipped with Chin Ball mating devices. Bulls generally were more effective than steers in detecting estrus. When placed in pens 24 hours per day with 20 heifers each, one bull detected 97% and another bull detected 100% of the potential estruses during a period of two months. An observer watching for .5 hours each morning and each late afternoon detected 74%. While steers were less effective, they were more docile than the bulls. Thus, with selection of active animals and adjustment of hormone therapy, implanted steers may be successful detectors of estrus with minimal danger to the owner. Reproductive development in the androgen-treated steers was delayed but maturation of the penis had occurred in steers slaughtered at 31 months of age. Body weight gains of steers tended to be accelerated during periods when they had implants.

248. **Testosterone and Libido in Holstein Bulls of Various Ages.** R.H. Foote, N. Munkenbeck and W.A. Greene

Testosterone was measured by radioimmunoassay in the blood plasma collected from 197 and 195 Holstein bulls in the spring and fall seasons, respectively. Bulls ranged in age from 8 mo to 13.5 yr. The average value for spring was 8.0 ng/ml, higher than the value of 5.7 ng/ml in the fall. Also, there was a tendency for testosterone to increase with age of the bulls up to 6 to 7 yr. The concentration of circulating testosterone was unrelated to libido or semen quality.

249. **Fertility of Bull Semen Frozen with β-Amylase, β-Glucuronidase, and Catalase.** R.H. Foote

"Capacitase," a product combining β-amylase and β-glucuronidase, was compatible with survival of bull spermatozoa frozen in whole milk-glycerol extender at final concentrations per ml of 0, 5, 10, and 20 μg of β-amylase combined with 0, 75, 150, and 300 units of β-glucuronidase, respectively. Bull semen was frozen in whole milk-glycerol extender containing the three lower concentrations of enzymes tested in the previous trial and used to inseminate 9057 first-service cows within 4 mo of freezing. The 60- to 90-day percent nonreturns were 74.6, 75.6, and 75.0. The same treatments plus a fourth one containing 10 μg of catalase per ml were fertility tested in another trial. Insemination of 16,842 cows resulted in 75.6, 74.1, 74.6, and 74.2% nonreturns. In this trial semen was held immersed in liquid nitrogen and distributed for immediate use each mo for 6 mo. There was no change in fertility during 6 mo of continuous storage at -196 C. Under the conditions tested neither catalase nor β-amylase with β-glucuronidase enhanced fertility of frozen bull semen.

250. **Antibodies to Egg Yolk in Blood Serum of Rabbits and Cattle and Cervical Mucus of Cattle Inseminated Artificially.** G.H. Coulter, R.H. Foote, J.J. Schiavo and R.K. Braun

Serum titers to egg yolk were induced in 6 rabbits by intravaginal deposition of an egg-yolk citrate extender used for artificial insemination of cattle. There was no effect of the low serum titers to egg yolk on fertility of the inseminated rabbits. Titers to egg-yolk semen extender were found in 3% of 59 cows of normal fertility compared to 29% of 14 repeat breeder cows of low fertility, all previously inseminated with semen diluted with egg yolk-citrate extender. Four of 6 cervical mucus samples (67%) from the repeat breeder cows had high titers to egg yolk, but only one also had a positive titer in blood serum.

251. **Survival and Fertility of Antibiotic-Treated Bovine Spermatozoa.** W.E. Berndtson and R.H. Foote

Motility of spermatozoa stored at 5 C with up to 1000 units or μg of chloramphenicol, polymyxin, kanamycin, tylosin, ampicillin, lincomycin, spectinomycin, erythromycin, novabiocin, or terramycin per ml of extender was compared to extender with penicillin plus dihydrostreptomycin. Novabiocin and terramycin were toxic, but other antibiotic treatments had no effect. However, erythromycin and tylosin, as well as colymycin, depressed motility of frozen thawed spermatozoa. Spermatozoal motility was equivalent, following freezing in ampules or straws. All of the antibiotics which were nontoxic when added singly to frozen semen were also not harmful to frozen spermatozoa when as much as 2000 units or μg were added per ml of extender containing penicillin and dihydrostreptomycin. The addition of 1000 units or μg of ampicillin, chloramphenicol, kanamycin, lincomycin, polymyxin, or spectinomycin per ml of extender containing 750 units penicillin and 750 μg dihydrostreptomycin per ml did not influence the fertility of frozen spermatozoa in a field test involving 19,663 first inseminations.

252. **Seminal Quality, Spermatozoal Output, and Testicular Changes in Growing Holstein Bulls.** R.H. Foote, G.E. Seidel Jr., J. Hahn, W.E. Berndtson and G.H. Coulter

Nine Holstein bulls, initially between the ages of 17 and 22 mo. awaiting proofs in artificial breeding, were ejaculated each summer for 3 yr, and 8 and 7 survivors for 4 and 5 yr, respectively, to study changes in the same group of bulls and determine the predictive value of early measurements. Semen was collected twice a day, 2 days per wk, for 4 wk. Bulls differed in testicular size, consistency, ejaculate volume, total spermatozoal output, percentage of unstained spermatozoa and abnormal spermatozoa, and in several storage and freezing tests. The largest

yearly effects were on testis size and consistency, ejaculate volume, and total output of spermatozoa. The latter increased per bull from 28.3×10^9 per wk at 17 to 22 mo of age to 40.9×10^9 per week 4 yr later, representing a high output of spermatozoa with a total of only 20 min of intensive sexual preparation per wk. The correlation between testis size and spermatozoal output was .72. Testicular consistency was indicative of semen quality measured simultaneously as judged by correlations with % normal, % unstained and % motile ranging from .61 to .95. These characteristics are believed to be representative of those Holstein bulls in artificial breeding which are ejaculated frequently.

253. Embryo Mortality and Altered Uterine Luminal Proteins in Progesterone-treated Rabbits.

S.M. McCarthy, R.H. Foote and R.R. Maurer

This study was undertaken to elucidate the location, time, and nature of embryo mortality induced by preovulatory progesterone administration. Progesterone was injected into rabbits on days -2, -1, and 0 (the day of mating) at doses of 0.5, 1.0, and 1.0 mg, respectively. Normal fertilization rates resulted, but embryonic death occurred by day 4. Embryos residing in progesterone-treated does for up to 3 days survived normally when transferred to normal recipients, whereas day 4 embryos from treated does exhibited a reduced ability to implant. Uterine fluid (UF) protein pattern was examined on days 1 to 7 after mating. Total UF protein levels were significantly greater in treated animals on day 4 than in controls. Uteroglobin secretion was significantly advanced in the treated animals by day 3. Examination of the time of the arrival of embryos into the uterus revealed a delay in the progesterone-treated rabbits. This delay, coupled with the earlier secretion of uteroglobin in the treated rabbits, indicated a possible asynchrony of approximately 1 day between embryo arrival in the uterus and certain uterine proteins. To determine whether UF could be etiologically implicated in the progesterone-induced embryonic death, embryonic development in vitro and in vivo was examined after exposure to UF collected at different gestational stages. More normal day 3 morulae placed in UF from day 3 control and day 2 progesterone-treated rabbits developed than similar morulae placed in UF from day 2 controls and day 3 progesterone-treated does. Hence, partial physiologic synchrony was achieved. This was interpreted to mean that "asynchronous" UF can be embryotoxic. Infertility was transient. Ability of the does to produce young at a pregnancy immediately following a progesterone-treated pregnancy was not impaired.

254. Sex-chromosome Ratios in Cattle and Their Relationship to Reproductive Development in Freemartins.

W.A. Greene, H.O. Dunn and R.H. Foote

Nineteen "female" Holstein-Friesian calves born co-twin with a bull were purchased shortly after birth. One subsequently was judged to be a normal female with 0% XY (100% XX) karyotypes in cultures of blood lymphocytes. However, one typical freemartin also had 0% XY (100% XX) karyotypes in 300 cells when examined at three ages. Overall, 18 freemartins examined at 1, 24, and 52 weeks of age averaged 60.7%, 57.9%, and 55.5% XY cells, respectively ($P > 0.05$). No systematic relationship was found between the proportion of XY cells and the abnormalities of the reproductive organs, as judged by vaginal depth and clitoral development at 1, 24, and 52 weeks, and examination of the reproductive organs per rectum at 54 weeks of age. The clitoris of untreated freemartins and those treated with estrone or estradiol postnatally did not enlarge. Testosterone treatment caused enlargement. None of the hormones affected vaginal depth. These studies provide convincing evidence that the blood lymphocyte XX:XY karyotype ratio (1) is not related to the degree of masculinization and (2) is stabile in the postnatal freemartin. Thirdly, the freemartin reproductive organs do not respond to common sex-steroid hormones postnatally other than the clitoral response to testosterone.

255. Predictability of Testicular Traits in Young and Mature Holstein Bulls Reared Under Different Systems.

G.H. Coulter and R.H. Foote

Scrotal circumference and testicular consistency of Holstein bulls were measured in seven bull studs for 5 to 7 yr. Correlations between scrotal circumference of 160 young bulls (12 to 23 mo and 24 to 35 mo) and this trait at maturity (60 to 71 mo

of age) were .48 and .56 and for the combination of the two early measurements with those at maturity .58. Testicular consistency of 116 bulls measured at the same time intervals gave corresponding values of .38, .44, and .50. By introducing a quadratic component the latter was increased to .69. Measurements first made on additional groups of bulls when they were more than 35 mo of age also were correlated closely with the values for these traits when bulls were mature, but the information on young bulls is of greater operational significance for early decisions. Correlations between measurements at different time intervals on bulls reared individually versus those grouped by age and reared in loose housing were similar. Therefore, the difference in social interaction provided by the two systems apparently did not alter the pattern or degree of testicular development.

256. **Relationship of Body Weight to Testicular Size and Consistency in Growing Holstein Bulls.**
G.H. Coulter and R.H. Foote

The relationship between 1,203 measurements of scrotal circumference and testicular consistency, usually made at 6-month intervals, and body weight in 411 Holstein bulls, primarily 6 to 72 months of age, were analyzed. All bulls were weighed within 14 days of testicular measurement. The relationship between body weight in kg ($\hat{Y}$) and bull age in months (X) is described by the regression equation, $\hat{Y} = -65.18 + 42.14\ X - .39\ X^2$, ($r = .96$). The equation that describes the relationship between body weight in kg (X) and scrotal circumference in cm ($\hat{Y}$) is, $\hat{Y} = 22.53 + .032\ X - .000013\ X^2$, ($r = .81$). The linear regression equation relating average testicular tonometer deflections in mm ($\hat{Y}$) and bull weight in kilogram (X) is $\hat{Y} = 15.35 - .0028\ X$, ($r = -.45$). When age was held constant the partial correlation between scrotal circumference and body weight was .58 ($P < .01$). Thus, as expected, body and testis size were correlated, but reports of similar relationships often have ignored the common and considerable influence of age. There was no uniform relationship between body weight and testicular consistency, although the partial correlation of -.16 ($P < .01$) indicates that heavier bulls, and perhaps those which were fatter, tended to have softer testes. Bulls with large testis may be larger, partly because of an anabolic effect of androgens, but this is speculative because these data do not permit distinction between a causal and casual relationship.

257. **Genetic Correlations Between Testicular Traits in Holstein Bulls and Milk and Fat Production of Their Daughters.** G.H. Coulter, R.H. Foote and T.R. Rounsaville

Scrotal circumference of 585 Holstein sires and testicular consistency of 490 Holstein sires were available to compute genetic correlations between these measurements and the bull's predicted difference for milk and fat production by Northeast Artificial Insemination Sire Comparison. The genetic correlation coefficients and standard errors between scrotal circumference and predicted difference for milk and fat were -.19 + .12 and -.12 ± .12 while the corresponding coefficients for testicular consistency were -.08 ± .09 and -.05 ± .09. For USDA Herdmate Comparisons for production 730 sires with scrotal circumference measurements and 649 sires with testicular consistency measurements were available. The coefficient between scrotal circumference and predicted difference for both milk and fat production was .01 ± .01. In contrast the coefficients between testicular consistency and predicted difference for milk and fat production were -.31 ± .35 and -.24 ± .31. The genetic relationships between these traits were near zero, indicating that the traits essentially are inherited independently. Because the majority of the estimates are negative, the data also could be interpreted to indicate that selection resulting in gene combinations for higher milk or fat production may have slight deleterious effects on these testicular traits. Any such possible effect may be offset by culling bulls with inferior reproductive capabilities.

258. **Action of Androgen and Estrone Implants on Sexual Behavior and Reproductive Organs of Castrated Male Rabbits.** R.H. Foote, P.J. Draddy, M. Breite and E.A.B. Oltenacu

Sexually mature but inexperienced male rabbits were castrated, immediately implanted with either testosterone (T), estrone (E_1), dihydrotestosterone (DHT), T + E_1, or DHT + E_1, and tested for male sexual behavior. Other castrates were not

implanted, and testing was either begun immediately (Ca-I) or delayed for 4 weeks (Ca-D). Intact males served as controls (C). Latency to mount a teaser female and to ejaculate into an artificial vagina was tested twice in a morning three times per week for 8 weeks. Then, animals were sacrificed, and reproductive organs were measured. The Ca-I group responded slowly to sexual training and ceased nearly all sexual activity by 8 weeks, whereas the Ca-D males seldom displayed interest in the teaser female. Intact controls and the T and T + E_1 groups all responded to the teaser and mounted and ejaculated within a few seconds. DHT and E_1 individually maintained the sexual activity of castrates equivalent to that of C for 4-5 weeks, but the time required to mount and, particularly, to ejaculate increased thereafter. The results with DHT + E_1 were equivocal in that castrates with this hormone combination sustained sexual activity equivalent to that of the controls for 7 weeks, but one animal in particular became sexually inactive the last week of the experiment. Penis weight was at least partially maintained in all implanted castrates. Accessory sex gland weight was smallest in the DHT group and was significantly increased in the T + E_1 and DHT + E_1 groups. The largest ejaculates of fluid were obtained in the group receiving E_1 alone. These results may be interpreted to indicate a role of both androgen and estrogen centrally and peripherally in the rabbit.

259. Development of and Fluid Accumulation in Mammary Glands of Freemartins Administered Estradiol, Estrone, or Testosterone.

W.A. Greene and R.H. Foote

Eighteen Holstein freemartins acquired at 1 wk of age were assigned to a control and to four treatment groups, but only two of the groups received hormone the first 50 wk. Testosterone administered for the first 50 wk appeared to inhibit teat growth whereas estrone stimulated teat and udder length. At 50 wk of age, implants releasing daily approximately 12.9 μg of testosterone, 2.9 μg of estrone, or 2.6 μg of estradiol-17β per kg of body weight were implanted for 6 wk. One of three testosterone-treated and 9 of 11 estrogen-treated animals exhibited increased udder development associated with fluid accumulation. The 10 animals with fluid accumulation in the udders were milked once 39 days after implanting. The fluid contained 15.0% total solids, 4.1% fat, and 7.0% protein, values which are between those for normal milk and colostrum. These studies support the concept that administration of low concentrations of estrogens alone, released continuously from implants, were capable of stimulating fluid secretion in mammary glands of animals that had no detectable gonadal luteal tissue.

260. Effects of Package, Extender, and Light on Stored Frozen Bull Spermatozoa.

G.H. Coulter and R.H. Foote

Spermatozoa were analyzed when fresh, freshly frozen, and stored at -196 C for 6 and 18 mo after processing in egg yolk-citrate and Promine-D extender with varying exposure to light and packaging in straws or glass ampules. Over all treatments there were decreases with time in spermatozoal motility, oxygen uptake, and cellular glutamic oxaloacetic transaminase due to freezing-thawing. Percent progressive motility decreased from spermatozoa freshly frozen (36.5%) to those stored for 6 mo (33.6%). Oxygen uptake in freshly frozen samples was 8.4 μl per 10^8 cells per h. In frozen semen stored for 6 and 18 mo corresponding values were 12.2 and 8.3 μl. Packaging methods did not have a significant effect on oxygen uptake, but package interacted with storage. Egg yolk-citrate extender supported greater progressive spermatozoal motility than did Promine-D. Extender interacted with storage for all criteria. Visible light radiation reduced progressive spermatozoal motility from 40.2 to 38.0% and oxygen uptake from 11.8 to 10.8 μl and 10^8 cells per h. Light interacted with storage time. Correlations between all criteria and storage times were not important practically.

261. Sex Ratios in Dairy Cattle Under Various Conditions. R.H. Foote

Current calving information was obtained on 35,102 single births in 2254 dairy herds. The overall proportion of males to females was 50.8%. The 5 dairy breeds did not differ. Only 6 of 111 sires studied produced calves with a sex ratio different from breed average at $P \leq .05$. This is the number expected by chance alone. A slight bias seems to occur when reporting the sires of the cows according

to the sex of the cow's calf. The sex ratio deviated from expected in a small sample of repeat breeder cows, but when a new and larger sample of 2,084 such cows which calved was obtained, there was no change associated with service number. The time of insemination was recorded for 12,764 heifers and cows first seen in estrus in the morning and 4,799 animals first seen in estrus in the evening. There was no effect of time of insemination on sex ratio. Likewise there was no effect of age of cows or season of breeding on sex ratio at birth. Because the sex ratio for cows requiring one insemination per pregnancy was not different from repeat breeders it is suggested that the sex ratio at fertilization and birth may be similar.

262. **Maintenance of Fertility of Spermatozoa at -196°C**. R.H. Foote

Extensive data sets presented show that fertility of bull semen stored in liquid nitrogen under routine use in the field is influenced potentially and practically by intermittent exposure to undesirable storage conditions. Thus, such data are unsuitable for estimating true age effects on fertility of spermatozoa attributable to storage per se. Experiments designed to keep bull semen continuously protected at -196°C for up to 5 yr consistently and conclusively reveal no decline in fertility, as measured by nonreturn rate. The biochemical changes reported during storage, and apparent slight loss of motile spermatozoa during storage without a concomitant reduction in fertility, is a scientific puzzle. These results have led the author to suggest that some of the changes monitored may have little significance for fertilization or the developing embryo. Secondly, and perhaps more importantly, it is postulated that any cells damaged by the storage process may not participate in fertilization. It is conceivable that the female tract acts as a selective filter, and such spermatozoa may not be transported to the vicinity of the egg. Any cells that are transported may be at a competitive disadvantage in penetrating the investments of the egg, at least in cattle. Thereby, fertility of stored bull spermatozoa would be unimpaired, given a sufficient number of spermatozoa initially. These conclusions are supported by controlled field trials with bull spermatozoa stored for 5 yr. Extrapolation always is dangerous, but these data give no hint of any precipitous decline for extended periods of storage. Also, sporadic surveys and monitoring of births by cattle inseminating organizations have not uncovered any increase in "birth defects" resulting from the use of frozen semen.

263. **Sources of Variation of Semen Output.**
R.W. Everett, B. Bean and R.H. Foote

Data from 5033 ejaculates during 1 yr from 55 mature Holstein bulls were analyzed to determine the effects of ejaculate number, days between ejaculates, month of the year, solunar periods, and sires on spermatozoal output and seminal characteristics. First ejaculates contained 49.7% more sperm and 40.8% more sperm per ml than second ejaculates. Increasing the interval between collections from 2 to 6+ days resulted in 45.9% more sperm per ejaculate, 31.0% more sperm per ml, and 14.0% greater volume. However, daily sperm output was highest when bulls were ejaculated most frequently. Lowest semen output was in February and March, associated with a decrease in sperm per ml. Highest semen output was in April and June, associated with the highest concentration of sperm per ml. Solunar periods had no effect on any measure of semen output. The concentration of sperm per ml varied more than the volume per ejaculate and had the largest influence on variation in total sperm harvested per ejaculate. Frequency of ejaculation was a major factor in total sperm harvested per bull per year.

264. **Composition of Rabbit Semen and the Origin of Several Constituents.**
W. Holtz and R.H. Foote

Semen was collected twice a day every other day (2x/48 h) for 36 days from 12 Dutch-belted rabbits. Sex drive, ejaculate volume, microscopic appearance of the semen and sperm counts were recorded. Seminal plasma was analyzed for its content of fructose, citric acid, glycerylphosphorylcholine (GPC) and the minerals Na, K, P, Mg, Ca and Zn. Six males were exsanguinated 24 h after the last semen collection and 6 were exsanguinated 5 weeks later. The organs of the reproductive tract were weighed and analyzed for their contents of spermatozoa, fructose, citric acid and GPC. Rabbits differed in libido and several characteristics of semen. Second ejaculates had lower concentrations

of fructose, citric acid, K and Zn than did the first ejaculates. Fructose was found chiefly in the different portions of the prostate gland and major concentrations of citric acid were found in the ampulla of the ductus deferens and in the vesicular gland. GPC concentrations were highest in the caput and cauda epididymides. Correlations among different constituents were calculated and their significance is discussed.

265. Reproductive Performance and Problems in New York Dairy Herds.

R.H. Foote

A study of reproductive performance was conducted in 1951-52 in 2254 member herds of the New York Artificial Breeders' Cooperative, Inc. Herd owners were contacted frequently and assisted in record keeping so that accurate information was obtained on a wide cross-section of herds and was not limited to the conventional studies of experiment station herds or those enrolled in DHIA. Cows in DHIA comprised 43 percent of the total sample, which consisted of 30,280 Holsteins, 7399 Guernseys, 3545 Jerseys, 2100 Ayrshires, and 503 Brown Swiss cows. A higher proportion of the herds were enrolled in production testing and several other recommended management programs than the average for New York State. However, only 6.2 percent had routine veterinary herd health service. The percentages of cows conceiving to either 1, 2, 3, 4, 5, 6, 7 to 13 inseminations were respectively 66, 59, 54, 47, 34, 33, and 21. The 150-180 -day nonreturns (cows not returning for subsequent insemination within 5 to 6 months) were 3.3 percent lower than 60-90-day nonreturns, and 2.6 percent higher than conception rates on first service, but thereafter were progressively inflated. The non-return and conception rates for grade cows were higher than for registered cows. But registered cows were retained to be inseminated more times. The conception rate of Guernsey cattle at first service (62 percent) was about 4 percent units below the other dairy breeds. Of the 15.5 percent of the cows inseminated at least once and then sold during the year, 61 percent were believed to be pregnant. The proportion sold without breeding is unknown. Sterility ranked third as the cause of culling. Gestation lengths were 271, 272, 269, 276 and 280 days for Holsteins, Guernseys, Ayrshires, and Brown Swiss, respectively. Holsteins had 3 percent multiple births, higher than other breeds. Bull calves were carried 1 day longer than heifers, and twins averaged 8 days less in utero. Sires of the cows included in this survey, as well as mates (semen donors), tended to influence gestation length. Calves born 10 days or more prematurely had a substantially higher death rate. The proportion of abnormal calves at birth was negligible, and was not correlated with the extent of radioactive minerals in this area, as had been postulated for the incidence of human birth defects. The overall sex ratio was 50.8 percent males to 49.2 percent females. Breed, month and season of the year, weather conditions, mate and sire of the cow, herd, and time of breeding did not affect sex ratios. The 6 out of 111 bulls studied with sex ratios among their progeny deviating from the expected ($P<.05$) was about what one would expect at this level of probability. First-calf heifers differed from older cows mainly in having fewer multiple births and one day less of gestation. Heritability of services per conception was not different from zero. Repeatability of services per conception was 0.10. Seventy-two percent of the cows were first seen in estrus in the morning. The highest fertility resulted when these were inseminated during the afternoon of the same day. For cows in estrus in the afternoon, insemination on the morning of the following day gave best results. Nearly 14 percent of the cows were inseminated <40 days after calving with about 20% lower fertility than those inseminated more than 60 days after calving. At the same time 20 percent of the cows were not inseminated until after 90 days postpartum, and the interval to subsequent insemination averaged 35 days. Thus, there was a considerable range in average days open from 342 to 434. The range for individual cows was greater.

266. Behavioral Characteristics of Freemartins Administered Estradiol, Estrone, Testosterone, and Dihydrotestosterone.

W.A. Greene, L. Mogil and R.H. Foote

Eighteen genetic females born co-twin with males and diagnosed as being sterile intersexes (freemartins) were studied from birth to 79 weeks of age. Testosterone (T) and estrone (EI) were administered in Silastic capsules to two groups from birth to 50 weeks of age and other animals were left untreated. At 50 weeks the two treated groups had

larger implants installed and the untreated animals were assigned to a new estrone (EII) and estradiol (E_2) treatment. Later a dihydrotestosterone (DHT) group was formed in comparison with new E_2 and testosterone propionate-enanthate (TP-TE) groups, plus untreated controls. Vulvar interest, Flehmen lip curl, mounting, and agonistic behavior were recorded daily for 30 min while animals were allowed social interaction. Agonistic behavior, interest in the genital area, and mounting were induced or stimulated by T, TP-TE, and E_2, but not by DHT or estrone (EI or EII). Also, only animals in the T, TP-TE, and E_2 groups induced to mount displayed the standing type of behavioral estrus. Flehmen lip curl was stimulated only by T or TP-TE. The evidence is interpreted to indicate that T, per se, evokes the lip curl, but it probably stimulates other responses at the neural level by conversion to E_2. Also, the freemartin response, the response of castrates to steroid hormones, and current knowledge of circulating steroid hormones in male and female cattle could be interpreted to indicate that the neural tissue responsible for sexual behavior in both sexes of this species may respond similarly in several respects.

267. Detection of Estrus and Quality of Semen Produced by Rams With Deviated Penises.

P.J.H. Ball, T.F. Wilson and R.H. Foote

Nine rams had their penises deviated and three others were vasectomized. Six additional intact rams served as controls. Three of the penis-deviated rams were raddled and placed in small flocks of ewes where they proved to be satisfactory detectors of estrus. Vasectomized and penis-deviated rams each were exposed to an estrous ewe for 5 minutes. The number of mounts (mean ± SE) for vasectomized rams was 4.8 ± .5 and for penis-deviated rams was 4.3 ± .5 ($P>.05$). When the rams had become experienced there were very few failures to mount at least once during the 5-minute period in any of the groups. Semen collection was facilitated in penis-deviated rams. There were no significant differences ($P > .05$) between penis-deviated and control rams with respect to volume of the ejaculate, or motility, concentration or presence of abnormal spermatozoa. Rams with deviated penises could be useful for detecting estrus and supplying spermatozoa for experimental study or use in artificial insemination.

268. Controlled Superovulation in Dairy Heifers Using Prostaglandin $F_{2\alpha}$ and Pregnant Mare Serum Gonadotropin.

D.R. Gengenbach, N. Butendieck, P.M. Riek, R.L. Scipioni, E.B. Oltenacu and R.H. Foote

Twenty-four sexually mature dairy heifers in six groups of four each received 25 mg of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) between days 7 and 14 of the estrous cycle. The control heifers received only this hormone; the other groups of heifers received 500, 1,200 or 2,000 IU of pregnant mare serum gonadotropin (PMSG). Half of the animals in the two latter groups received the PMSG simultaneously with the $PGF_2\alpha$ and half were given the hormone 12 hr later to determine if the ovarian response would be affected by reduced progesterone following $PGF_2\alpha$-induced luteal regression. Ovulation rate was not affected by changes in progesterone prior to PMSG. Each of the eight animals receiving only $PGF_2\alpha$ or $PGF_2\alpha$ plus 500 IU of PMSG had one ovulation. The average ovulation rate for eight animals receiving 1,200 IU of PMSG was 3.2 (range 1 to 6) and for eight receiving 2,000 IU of PMSG it was 5.6 (range 1 to 19). Duration of increased plasma progesterone concentrations seemed to be more important in determining ovarian response than changes in progesterone prior to PMSG treatment. However, plasma concentrations of estradiol, progesterone and LH did not differ significantly among groups of heifers. Systemic concentrations of plasma progesterone were correlated with the number of corpora lutea 13 days after treatment ($r = .99$, $P < .001$).

269. Monitoring of Plasma and Milk Progesterone for Evaluation of Postpartum Estrous Cycles and Early Pregnancy in Mares.

B. Hunt, D.H. Lein and R.H. Foote

Plasma and milk progesterone concentrations in 13 mares were determined 3 times a week for 5 months, beginning at parturition. The estrous cycle was divided into 2 phases. Estrus was considered to occur when the plasma progesterone concentration was < 1 ng/ml, with diestrus occurring when plasma progesterone content was ≥ 1 ng/ml. Based on this classification, the period of estrus averaged 8.9 days,

diestrus averaged 13.9 days, and the estrous cycle averaged 22.8 days. During estrus, the progesterone concentration in plasma averaged 0.4 ng/ml and in milk averaged 2.2 ng/ml. During diestrus, corresponding values were 6.8 and 6.9 ng/ml. In the early pregnant mare, the average progesterone concentration was 5.4 ng/ml for plasma and 4.7 ng/ml for milk. The progesterone pattern was similar in milk and in blood plasma, and the correlation between 362 paired values was 0.7 ($P < 0.01$). It was concluded that the estrous cycle of the mare can be mapped accurately by measuring plasma or milk progesterone content at 2- to 3-day intervals. A breeding program based on monitoring blood progesterone was outlined.

270. Symposium on Freezing of Gametes and Spermatozoa -- Synopsis Papers.

R.H. Foote

Protection of sperm against thermal and osmotic effects during freezing and thawing is a challenge. The lipids of egg yolk and milk have been widely used successfully, when combined with glycerol, to protect sperm. Optimal cooling to 5°C and freezing rates, especially through the critical range to about -40°C, are important. They may differ for different extenders. Constant storage in liquid nitrogen at -196°C is critical. As the semen collector, evaluator, processor and inseminator are all interposed between the bull and the cow, as compared with natural service, every step must be performed well to prevent accumulated damage.

271. Release Rate of Testosterone and Estrogens from Polydimethylsiloxane Implants for Extended Periods in Vivo Compared with Loss in Vitro.

W.A. Greene and R.H. Foote

Release rates of testosterone, estrone, and estradiol placed in chambers made from polydimethylsiloxane (PDS) tubing (Dow Corning "Silastic," 3.35 mm ID × 4.65 mm OD) were studied in 14 freemartin cattle with minimal or non-detectable endogenous hormone secretion, and in 0.9% saline:methanol (1:1) baths shaken at 38°C. Eighty-seven implants, varying in length from 2 to 10 cm, were placed in 14 animals for 27 to 235 days. The average release rates ± standard errors, in μg/cm/day, were testosterone, 55.9 ± 2.4, estrone, 12.6 ± 1.8, and estradiol, 11.1 ± 1.1. A relatively constant release rate was found over the period of time studied and sufficient steroid remained for potential release over periods exceeding 1 year. The dose of hormone delivered was sufficient to increase mounting activity in testosterone-treated animals and estrual activity in those receiving estrogens. Corresponding release rates in vitro for four 10-cm implants containing either testosterone, estrone, or estradiol were 94.3 ± 1.9, 15.5 ± 0.7, and 12.7 ± 0.6 μg/cm/day, respectively. The general magnitude of release rate in animals could be predicted from laboratory tests.

272. Extenders and Extension of Unfrozen Semen. R.H. Foote

This chapter with 213 references documents the many concepts and principles to follow to preserve sperm properly. Different types of semen extenders, their composition and fertility obtained with their use are included. Ambient temperature storage in appropriate extenders vs 5°C storage is discussed. Microorganisms in semen and agents for their control is covered extensively. The extension and cooling procedures and number of sperm per breeding unit, with extensive fertility information is included. Data not previously published on semen extenders and on sperm survival is included.

273. Principles and Procedures for Photometric Measurement of Sperm Cell Concentration.

R.H. Foote, J. Arriola and R.J. Wall

There are four essential and very important steps which must be followed to put the desired number of spermatozoa in each straw, ampule or other breeding unit. Firstly, the accurate measurement of sperm cell concentration per ml (so-called "sperm count") is extremely important! This tells you how many spermatozoa you have per unit volume to dilute so that you will have the right number of sperm cells in the extended semen. Secondly, the dilution itself must be done accurately. Thirdly, the extended semen must be mixed gently but thoroughly to insure that a uniform suspension of spermatozoa remains during filling of straws or ampules. Fourthly,

all packages should receive the same volume. These are essentials for maximizing "bull power" in artificial breeding. If these steps are taken, along with proper semen processing and quality control, top fertility can be achieved. The sperm concentration in the ejaculate is the most variable characteristic. It should be measured accurately. The procedures for instrument calibration and accurate determination of sperm concentration are described in detail.

274. **General Principles and Basic Techniques Involved in Synchronization of Estrus in Cattle.** R.H. Foote

1) Cows should be in postpartum condition so that they are cycling and heifers should be large enough so they are cycling. 2) Nutritional supplements decrease the frequency of anestrus. 3) A short progestagen treatment initiates cycling in some anestrous females. 4) Long progestagen administration decreases fertility, but it does not cause abortion. PG injections will abort pregnant cattle. 5) Prostaglandins injected 2X about 11 days apart synchronize cattle effectively. The 1X PG treatment is the simplest, but is less effective. 6) Progestagen combinations with or without $PGF_2\alpha$ or a PG analog can results in the following: a) well-synchronized ovulations; b) fertility equivalent to controls with some more cows pregnant early in the season; c) two fixed time inseminations result in more cows pregnant than one fixed time insemination or insemination at detected estrus. (Some cows ovulate without showing estrus). 7) Calf removal in suckling cows improves ovulatory response and conception rates. 8) Semen used should be highly fertile and from bulls of superior genetic quality. 9) Inseminator skill is of obvious importance. 10) Of the many ingredients in the procedures, manager skill is one of the most important. 11) The benefits of controlled breeding should more than offset costs when properly applied. 12) There are many products for estrous cycle regulation which appear to be effective and safe, but some are not approved for use in the U.S.A.

275. **The Anatomy of the Reproductive System in Male Dutch Rabbits (*Oryctolagus cuniculus*) with Special Emphasis on the Accessory Sex Glands.** W. Holtz and R.H. Foote

The morphology and microscopic anatomy of the reproductive organs and colliculus seminalis of the male rabbit are described and illustrated. Special attention is given to the accessory sex glands, because not all have been accurately identified previously. The terminology suggested for the accessory glands (with other terms commonly used in the literature in parentheses) follows: glandula vesicularis (glandula seminalis, vesicula seminalis), proprostata (glandula vesicularis, coagulating gland, prostata), prostata, paraprostata (glandula Cowperi superior), glandula bulbourethralis (glandula Cowperi inferior). The English equivalents are vesicular, proprostate, prostate, paraprostate and bulbourethral glands. Organs were obtained from 39 adult Dutch-belted rabbits that averaged 2,023 g in bodyweight. Organ weights (means and standard deviations) were as follows: testis, 2.035 ± 0.529 g; caput epididymidis, 0.264 ± 0.087 g; corpus epididymidis, 0.046 ± 0.019 g; cauda epididymidis, 0.398 ± 0.123 g; proximal part of the ductus deferens, 0.098 ± 0.026 g; ampulla of the ductus deferens, 0.177 ± 0.069 g; vesicular gland, 0.529 ± 1.169 g; proprostate gland, 0.633 ± 0.304 g; prostate gland, 0.411 ± 0.181 g; paraprostate gland, 0.040 ± 0.019 g; bulbourethral gland, 0.390 ± 0.133 g; and epididymal fat pad, 0.545 ± 0.339 g. Correlations among organ weights were calculated and interrelationships among them and bodyweight are discussed.

276. **Progesterone-Induced Asynchrony and Embryo Mortality in Rabbits.** C.J. Schacht and R.H. Foote

Preovulatory administration of low doses of progesterone permits normal ovulation and fertilization, but results in an embryotoxic condition in the uterus of treated rabbits by Days 3 and 4, where Day 0 is the day of breeding. Normal embryos were

transferred on Days 2, 3, and 4 to control and treated temporary recipients for 24 h and then retransferred to untreated chronologically synchronized final recipients. Day 2 embryos so transferred to the oviducts of treated rabbits were not adversely affected, but normal 3- and 4-day-old embryos exposed for 24 h to the uterus of treated Day 3 and Day 4 rabbits, respectively, were irreversibly damaged. Following retransfer, implantation rates for these embryos were 5 and 4% compared with 40 and 33% for corresponding embryos exposed to untreated rabbits ($P < 0.01$). The possibility that progesterone-induced embryo-uterine asynchrony was responsible for the toxic effect was tested by transferring Day 4 normal embryos to Day 3 treated temporary recipients and to Day 3 and 4 untreated temporary recipients. Following retransfer, implantation rates were 37, 37 and 36%, providing evidence that the harmful effect could be overcome by adjustment for the induced asynchrony. Exposing Day 3 embryos for 24 h to uteri of does pseudopregnant for 3, 4, 5, 6, and 8 days resulted in 45, 53, 44, 44 and 29% implantations, respectively ($P > 0.05$). The toxicity of the early asynchrony was not mimicked, indicating that the uterine protein pattern or other uterine factors induced by the early progesterone treatment were uniquely harmful.

277. **Embryonic Mortality in Dairy Cows Estimated by Nonreturns to Service, Estrus, and Cyclic Milk Progesterone Patterns**. H.L. Kummerfeld, E.A.B. Oltenacu and R.H. Foote

Cyclic patterns of progesterone in milk were studied in 262 Holstein cows. Milk progesterone data were obtained from 153 cows for at least 75 days after conception as indicated by continuous high progesterone concentrations in milk for 28 days or more following artificial insemination. Cycling was reinitiated in 11 of these cows between 28 and 75 days after breeding, for an estimated 7.2% rate of embryo-fetal mortality. This was considerably less than a corresponding rate of 22.7% estimated for this herd by the delayed returns to estrus (28 to 75 days). In 350,180 cows inseminated with semen from Holstein bulls the estimated embryo-fetal mortality by the same delayed return to service method was 12.5%. Biases in this latter method of estimation are discussed.

278. **Factors Influencing the Quantity and Quality of Semen Harvested From Bulls, Rams, Boars and Stallions**. R.H. Foote

Several inherited conditions associated with testicular defects, abnormal spermatogenesis and morphologically abnormal sperm have been found. These usually are controlled by single gene pairs. A notable exception is testicular size, with heritability in young bulls ranging from .42 to .88. Testicular size directly affects sperm output potential. The major contributor to variation in semen quality is the environment. Environmental effects may be temporary or permanent. Permanent effects occurring during prenatal and prepubertal periods and temporary or permanent factors acting after spermatogenesis is initiated can alter semen quality. Semen quality improves during the first few months after puberty and declines in old age. Malnutrition and the ingestion of toxic materials can have a major effect on testicular development and spermatogenesis, but the reproductive system has considerable regenerative capacity unless the dietary deficiencies are severe and prolonged. Elevated testicular temperatures resulting from incomplete descent of the testes (cryptorchidism), high environmental temperatures or inflammation are detrimental to spermatogenesis in all scrotal mammals. Cold temperatures appear to be innocuous unless actual freezing of tissue occurs. During periods of decreasing daylight semen quality declines in stallions and improves in seasonally breeding sheep. The time required to form and transport sperm in bulls, rams, boars and stallions is about 64, 56, 47 and 59 days. Therefore, a considerable potential lag may exist between a testicular event responsible for a change in semen quality and the time that this change is evident in ejaculated semen. Conditions imposed at the time of semen collection, such as frequency of ejaculation, degree of sexual preparation and type of semen collection may influence the quality of semen harvested. Finally, certain semen characteristics are more variable than others and investigators should utilize suitable existing data to design the most effective least-cost experiments.

279. **Estrous Cycle Patterns in Cattle Monitored by Electrical Resistance and Milk Progesterone.** G.S. Heckman, L.S. Katz, R.H. Foote, E.A.B. Oltenacu, N.R. Scott and R.A. Marshall

An electronic probe with parallel stainless steel electrodes was designed to measure changes in electrical resistance in the anterior vagina associated with increased cervical mucus secretion at estrus. Three groups of Holsteins were probed, and a sample of residual milk after milking was taken for progesterone assay every 2nd day for 28 to 40 days. Group I consisted of 29 cycling virgin heifers, Group II consisted of 24 lactating cows in the Cornell herd, and Group III consisted of 62 lactating cows in three commercial herds. Lactating cows were 26 or more days postpartum when sampling began. In Group I, 77% of the measurements at the ventral surface of the anterior vagina were lowest on the day of estrus and correlated well with days KaMaR Heatmount Detectors were triggered or chalk was erased. In Groups II and III, average electrical resistance also was minimal at estrus and was correlated .92 to .99 with average milk progesterone during the 4 days preceding and including estrus. The electronic probe appears to enable one to detect which cows are cycling normally and to aid in determining when to expect estrus.

280. **Pregnancy Rate in Dairy Cows Inseminated on the Basis of Electronic Probe Measurements.** R.H. Foote, E.A.B. Oltenacu, J. Mellinger, N.R. Scott and R.A. Marshall

A new vaginal probe with two pairs of parallel electrodes 90° apart was designed with a switching arrangement so that electrical resistance (ohms) of the dorsal and the ventral areas of the anterior vagina could be monitored separately during the estrous cycle. Three inseminators probed cows during February-April, 1977, in nine herds in stanchion barn housing. Cows not seen in estrus by 40 to 60 days after calving were probed every 3rd day. At each dairyman's option normal cows with a low probe reading were inseminated. Controls consisted of cows seen in estrus by the dairyman and reported for insemination in the routine manner. The diagnosed pregnancy rate for 86 first service controls was 49% versus 52% for 58 cows not seen in estrus, but identified by low probe readings. Cows varied in electrical resistance at breeding, but the probe was as effective in identifying cows suitable for insemination as was visual observation of estrus by dairymen.

281. **Time of Artificial Insemination and Fertility in Dairy Cattle.** R.H. Foote

Data were from 44,707 cows and first-calf heifers in a commercial artificial insemination program. The percentage of heifers first seen in estrus in the morning was 73.2, not different from 72.6 for cows. Unfrozen semen was used for insemination. The 150- to 180-day percent nonreturns for cows inseminated before noon the same day (67.1) was not different from those inseminated between noon and 1800 h (69.9) and after 1800 h (68.9) the same day. However, insemination the following morning was too late for best results (62.7%). Cows first seen in estrus in the evening should be inseminated by noon the next day, as the nonreturn rate was 69.9%. The non-return rate for cows inseminated after 1400 h the next day resulted in 63.8% 150- to 180-day nonreturns. A single mid-morning insemination for all cows first seen in estrus the night before or the same morning should give near maximum conception.

282. **Bovine Testicular Measurements as Indicators of Reproductive Performance and Their Relationship to Productive Traits in Cattle: A Review.** G.H. Coulter and R.H. Foote

Scrotal circumference is an easily measured trait and measurements are highly repeatable among technicians. The trait is inherited and it provides a substantial amount of information on the reproductive capacity of bulls. Several reports of clinical findings demonstrate that bulls deficient in testicular development have a much higher probability of being unsatisfactory breeders. Large testis size has been reported to be correlated with younger age at puberty and higher conception rates in related females. Testicular consistency (TC) can be measured objectively by a tonometer. Measurements are repeatable, heritable, and related to seminal quality and fertility.

Borderline individuals can be identified for a more thorough examination for breeding soundness. Factors affecting the measurement of testicular traits include breed, age, body weight, and the season of year. Additional research is required to refine estimates of the effects of these factors on testicular measurements. An indexing system could then be developed to adjust for all factors known to influence testicular development. The high heritability of SC and TC testicular traits, particularly SC, combined with a high degree of predictability, allow bulls to be selected initially by pedigree and then evaluated and further selected for potential reproductive performance at a relatively young age. Seminal characteristics and libido also should be considered to prevent bulls with low indexes for reproductive performance from entering breeding programs. Testicular size appears to be positively related to both body growth and reproductive performance in closely related females. Further studies are necessary to accurately assess these relationships, but these recent findings should provide incentive to give even greater consideration to the testicular development of herd sires and those selected for artificial breeding.

283. Growth and Reproductive Development in Freemartins Hormonally Treated From 1 to 79 Weeks of Age.

W.A. Greene, L.G. Mogil, D.H. Lein, A.D. McCauley and R.H. Foote

Postnatal growth, steroid hormone profiles and response to steroid hormone treatment were studied in 18 freemartins and one normal female born cotwin with a bull. They were either treated postnatally with testosterone or estrone at one week of age, or left untreated until 50 weeks of age when they received silastic implants calculated to release either 12.9 μg of estrone or 2.6 μg of estradiol per day per kg of body weight. Later a dihydrotestosterone-treated group was added. Reproductive development was studied by palpation per rectum and by examination when animals were slaughtered at 79 weeks of age. Treated animals grew slightly faster than untreated animals. Testosterone in untreated freemartins averaged 76 and 87 pg/ml of blood plasma during weeks 1 to 48 and 52 to 56. Corresponding values for those animals with small testosterone implants (weeks 1 to 48) and with larger implants (weeks 52 to 56) were 130 and 272 pg/ml. Estrone and estradiol values appeared to fluctuate between 10 and 50 pg/ml but values are uncertain because they were below the sensitivity of the assay then available. Thus, circulating steroid hormone concentrations were similar to those reported for castrates. Testosterone stimulated clitoral development prenatally and postnatally. None of the treatments influenced vaginal depth, which averaged 4.0, 9.0 and 10.9 cm at 1, 24 and 52 weeks of age. Vaginal depth at birth was not a reliable indicator of freemartinism. Androgen may have inhibited udder development, whereas estrogen appeared to be stimulatory. The reproductive organs of the freemartin were characterized by differences in underdevelopment and the general presence of seminal vesicles. The latter structures, plus clitoral development at birth in 3 animals and postnatal response of the clitoris to testosterone is interpreted to indicate that the presence of androgens is one factor in abnormal development. Otherwise, gross morphology of the reproductive tract was not related to hormone treatment, postnatal gonadal histology, endocrinology or lymphocyte chromosomal karyotypes.

284. Hormones in Milk That May Reflect Reproductive Changes.

R.H. Foote

Several hormones, particularly progesterone (P_4), found in milk of the dairy cow reflect, changes in reproductive status. The major advantage of using milk samples over blood samples is the ease with which large numbers of samples can be obtained at the regular time of milking under research and field conditions without disturbing the cow. Progesterone concentration in whole milk is several times that in peripheral blood. About 80% of milk P_4 is associated with the lipid fraction of milk. Thus, the milk concentration of P_4 is affected considerably by sampling technique (first milk, composite milk, or the last strippings). Milk P_4 analyses have revealed that most cows are cycling and that major causes of long calving intervals are "missed heats" and reporting of cows for insemination when they were not in estrus (about 20%), with lowered conception rates. Milk P_4 values provide a reliable early nonpregnancy test and aid in diagnosing whether ovarian cysts are primarily follicular or luteal. Thus, milk P_4 profiles can be a powerful tool in evaluating and improving many aspects of reproductive management programs for

dairy cattle. Estrogens are mainly in the lipid fraction of milk, and concentrations are generally lower than in blood. Estrogens in milk increase significantly at estrus, but do not peak sharply. Estrone, estradiol-17β, and total estrogens in milk are correlated with blood plasma concentrations, (r = .38, .25, and .48) on a within-cow basis. Glucocorticoids are more polar than ovarian steroid hormones, and they are found chiefly in the non-fat portion. The concentration in milk is usually less than 5% of that in blood, and the two are not correlated except when large changes are induced experimentally. Prolactin is present in milk at higher concentrations than in blood. Milk prolactin may be useful in predicting circulating hormone concentrations without disturbing the animal, with application to lactation rather than fertility. Little is known about gonadotropic hormones in milk. the polypeptide releasing hormone for gonadotropins (GnRH) has been reported to be present in cows' milk. Progesterone patterns in mares' milk and blood plasma are correlated (0.7) during the estrous cycle and pregnancy. Goat milk has been used for pregnancy testing. The information available indicates that patterns discussed for cattle apply partially to sheep and goats.

285. **Electrical Conductivity Probes for Detection of Estrus in Cattle**.
R. Marshall, N.R. Scott, M. Barta and R.H. Foote

The basic principles and design of an electrical conductivity probe to detect changes in volume and electrical conductivity of vaginal mucus associated with estrus in dairy cattle are presented. An optimal probe design with axially parallel electrodes is recommended. Data from laboratory and field experiments suggest that a probe of this design may prove to be a useful aid in detecting estrus in cattle, and fertility results obtained with its use are equivalent to those obtained with visual detection of behavioral estrus.

286. **Milk Progesterone as a Diagnostic Aid**. R.H. Foote, E.A.B. Oltenacu, H.L. Kummerfeld, R.D. Smith, P.M. Riek and R.K. Braun

Methods of obtaining, storing and assaying milk samples have been studied. Under field conditions last milk has been preferable, with either whole milk or butter-fat used in the RIA. Progesterone is very stable in storage. Herds using artificial insemination, and free from known diseases, have tended to have lower fertility as herd size and level of milk production increased. Milk progesterone analysis revealed that most cows were cycling normally by 50 days post partum, but frequently were not seen in oestrus or oestrus was inaccurately detected. Cows on a high, medium or low energy ration had their first post-partum milk progesterone cycle start on average 24, 24 and 31 days after calving respectively. Observed oestrus occurred later and was more variable. With the selected use of heat mount detectors, chalk, electronic probes and computerized reproduction management guides, the problem of missed oestrus can largely be overcome. Milk progesterone can serve as a non-pregnancy test with 98% accuracy about three weeks after insemination. This focuses attention on cows requiring reinsemination and frequently results in observed oestrus and insemination without missing a cycle. Milk progesterone also was useful in distinguishing between cows with follicular cysts, luteinized follicles and persistent corpora lutea, and in evaluating experimental and clinical trials using GnRH. Palpation of the reproductive organs per rectum at about six weeks is recommended as a final check of pregnancy. Embryo mortality between 28 and 75 days was estimated by progesterone analyses to be 7.2%.

287. **Effects of Heat Detection, Conception Rate, and Culling Policy on Reproductive Performance in Dairy Herds**. T.R. Rounsaville, P.A. Oltenacu, R.A. Milligan and R.H. Foote

A simulation model of the reproductive process in dairy herds was developed to examine the effects of heat detection, conception rate, and culling policy on measures of reproductive performance. Twenty-one 65-cow herds were simulated to quantify over 10 yr changes in days to first service, days open, calving interval, number of services required annually, and annual culling rate attributed to strategies of reproductive management. Increasing heat detection shortened first service interval, days open, and calving interval. Improved conception rate and earlier culling for reproductive failure decreased number of annual services required by herds and

increased annual culling rate. Costs of implementing such management strategies were defrayed by savings in annual insemination and replacement costs alone. Although the model was insufficient to determine optimum reproductive management strategies, it did indicate relative merits of improvement to current typical dairy herd management policies.

288. Effect of Cannulae in the Ampulla of the Oviduct and in the Tubo-Uterine Junction on Reproductive Phenomena in Sheep.

P.J.H. Ball, M.E. Simkin and R.H. Foote

Permanent cannulation systems designed for injecting spermatozoa or for collecting eggs and fluids at the tubo-uterine junction and at the ampulla near its junction with the isthmus of the oviduct are described. In a preliminary experiment three ewes bilaterally cannulated in the tubo-uterine junction had spermatozoa injected through the cannulae and 4 of 8 eggs recovered were fertilized. Six more ewes were bilaterally cannulated. They continued to cycle and few complications were experienced. But cannulation appeared to reduce superovulatory response to exogenous gonadotropins. This also occurred in a confirmatory study with 16 rabbits in which superovulated controls averaged 25.6 ovulations versus 8.6 in those with cannulae. When cannulae were installed near the ampullar-isthmic junction no eggs were recovered surgically from 4 controls. Thus, these cannulae appeared to interfere with ampullary transport of eggs. Blood plasma concentrations of LH and progesterone were not affected by cannulae. As cycle length also was normal it appeared that these devices were not luteolytic. The cannulae were still functioning in the animals euthanized more than six months after installation. Thus, they offer a non-surgical means of examining several aspects of reproductive function. The observations on altered egg transport and ovarian response to gonadotropins may be of significance in cases of infertility.

289. Measurement of Bull Sperm Motility and Velocity.

R.J. Wall, D.R. Hagen and R.H. Foote

A Bausch and Lomb Spectronic 20 instrument equipped with a flow cell was used as a simple, relatively inexpensive, and objective method of estimating the motility of bull spermatozoa. Wavelength, type of flow cell, flow rate, sperm concentration, and temperature were studied as components which might contribute to formulation of an optimum procedure. A wavelength of 550 nm was found to provide near-maximal sensitivity and is one used commonly for sperm concentration measurements. Using a flow cell with a 10-mm light path, flow rates of 2, 4, 6, and 8 ml/min, and sperm concentrations of 14.5, 29, and 51 $\times$ 10^6 per ml were compared. The optima were a flow rate of 4 ml/min and a sperm concentration of 29 $\times$ 10^6 sperm/ml. The motility index was based on a change in absorbency during a period of 60 sec after the flow was stopped. The correlation between MI and subjective estimates of the percent of progressively motile cells was 0.87. Several flow cells were compared. Those permitting flow parallel to the light path were superior to those with the flow perpendicular to the light path. Velocity of spermatozoa in six samples of bull semen was measured at 26, 29, 32, and 35°C by recording the sperm movement on videotape and viewing the cells on a TV monitor equipped with a grid. The MI also was measured. Both increased with temperature, but MI increased only up to 32°C. Velocity was found to be a complex component of the MI.

290. Maturation of Rabbit Follicular Oocytes in a Defined Medium of Varied Osmolality.

I.-H. Bae and R.H. Foote

Rabbit oocytes from follicles $\geq$1 mm in diameter were cultured for 18 h in a defined medium with osmolality adjusted in 20 mosmol increments from 230 to 350 mosmol by altering only the NaCl concentration. The defined medium contained the salts of K, Ca, Na and Mg with $NaHCO_3$, glutamine and bovine serum albumin. The pH was 7.2. Adjustment, based upon determination of the osmolality of the medium, was necessary because a difference existed between calculated and achieved osmolality in this complex solution. The proportions of oocytes which matured to meiosis II with polar body formation were 64, 68, 64 and 65% in media of 250, 270, 290 and 310 mosmol, respectively. However, development was retarded in media measuring 230, 330 and 350 mosmols.

291. **Milk Progesterone Assays as Part of a Reproductive Management Program for Dairy Cattle**. R.H. Foote, R.D. Smith, E.A.B. Oltenacu, R.K. Braun and T.J. Reimers

Current calving intervals appear to be too long for optimal milk production and progeny born per year. These long intervals may be associated with a lack of cyclic ovarian activity, but the real problem may be failure to detect estrus. A research program was established to develop methodology for assaying progesterone in milk, and to assess the relative importance of ovarian inactivity versus failure to detect estrus as causes of long calving intervals. Detection of estrus was the major problem. Animals with normal cyclic progesterone patterns were not seen in estrus 5 to 50% of the time. Some cows were reported for insemination when milk progesterone concentration was high. Only 8% of these cows conceived. Pregnancy status of cows predicted from milk samples taken 21 to 24 days after breeding was 98% accurate for cows not pregnant and 80% accurate for those which were pregnant. Milk progesterone data were useful in evaluating the accuracy of electronic cervical probes and other devices designed to aid dairy farmers in detecting estrus. Also, this information assisted veterinarians in diagnosing ovarian dysfunction and monitoring response to veterinary treatment. A diagnostic service to provide multiple aids for dairy farmers, veterinarians and others dealing with reproductive management of the dairy herd was established following an extensive educational program. The service is offered through the combined efforts of the New Dairy Herd Improvement Coop., and the Diagnostic Laboratory at the New York State College of Veterinary Medicine, thus providing a strong field service backed by a highly competent technical laboratory.

292. **Modelling Reproduction in a Herd of Dairy Cattle**. P.A. Oltenacu, R.A. Milligan, T.R. Rounsaville and R.H. Foote

A dynamic stochastic model to simulate the reproductive process in a herd of dairy cattle has been constructed. The next event scheduling approach was utilised with four events being specified: parturition, ovulation, embryonic loss and replacement. The control variables included in the model are first breeding policy (1BDG), reproductive culling policy (RCLG), heat detection program (HDPM), breeding program (BDPM) and service sires selection program (SSPM). In addition, the model uses numerous endogenous variables such as parturition type (PRTY), oestrus behaviour (EBHV), cow's fertility (CFTY), embryonic mortality (EBLS), non-reproductive culling rate (NRCL) and others. The model was evaluated against independent experimental results. The simulated result closely approximates the average reproductive performance and variability of reproductive performance in a dairy herd.

293. **Effects of Two Simulated Semen Culling Programs on Predicted Fertility in an Artificially Inseminated Cow Population**. E.A.B. Oltenacu, R.H. Foote and B. Bean

Two semen culling programs designed to improve fertility of semen used in an artificial insemination program were compared. First inseminations and return service data on cows inseminated with semen from 21 Holstein bulls during 1 yr were studied. The culling of less fertile ejaculates within bulls, based on any available semen quality test or combination of tests, would bring about less than a 1% increase in nonreturn rate if 40 to 50% of ejaculates were culled. If all semen from the 19% low fertility bulls were culled, this would entail culling 22% of the semen used to inseminate the population and would achieve an improvement of 1% in overall fertility. This could be done with no loss in predicted difference for milk yield of bulls used. Culling of bulls is a functionally simpler and less expensive program than is testing and culling ejaculates within bulls, but this requires that fertility of bulls be known and that it will not change substantially from predicted.

294. **Artificial Insemination**. R.H. Foote

The 4th edition is a revision of previous editions to which the author contributed. This chapter includes a discussion of artificial insemination of all species of common farm animals. The steps from semen collection, evaluation, processing, distribution, insemination and field results are described. Equipment used and general procedures to follow, along with diagrams and photographs are included.

295. **Increasing Fertility in Artificial Insemination by Culling Bulls or Ejaculates Within Bulls.**
R.H. Foote and E.A.B. Oltenacu

There are many factors which affect conception rates, and one important one is the fertility of spermatozoa used for insemination. This is dependent upon the fertility of the bulls used in A.I. and the fertility of individual ejaculates of semen processed for insemination. Because nonreturn rate is a heritable trait some selection for this trait when choosing sires to produce sons is advised. Secondly, testicular size and consistency is inherited. These traits are related to sperm production and fertility. Thus, sires selected to produce sons, and sires selected for performance and progeny testing should be scrutinized carefully for testicular development. This selection will result in some desirable permanent effects of gradually changing the gene pool in the population. Further improvement in fertility can be made by culling bulls with low nonreturn rates or ejaculates within bulls. Bull variance is greater than ejaculate variance, and more improvement in fertility can be made by culling bulls as opposed to culling ejaculates from various bulls. Eliminating some bulls does reduce cost, but does provide less reserve sire power. Also, traits other than fertility can be important to consider along with selection for high fertility. Any selection program must be predicted on having sufficient sperm cell producing power to allow for the desired culling rate while meeting semen needs. The requirements for spermatozoa can be met by a combination of procedures. These are judicious selection of bulls, intense sexual preparation and frequent semen collection of bulls, and especially processing semen so that the minimum number of spermatozoa consistent with overall maximal or optimal fertility of the A.I. organization is achieved.

296. **Bull Spermatozoa -- Temperature Interactions: The Influence of Semen Collection Equipment.**
R.J. Wall, M.E. Simkin and R.H. Foote

Since the advent of frozen semen little attention has been devoted to studies of semen cooling from the time of collection until freezing. During the freezing process a large percentage of the sperm cells are damaged and until our fundamental understanding of cryoprotection is improved these losses will continue to be experienced. Methods that can improve sperm cell survival up to the time of freezing would be beneficial to the overall process. In this report we characterized the cooling rates of semen collected at a commercial stud and compared them with rates achieved using an experimental low heat capacity collection assembly. The low heat capacity collection assembly influences the semen temperature less than the conventionally used high heat capacity collection assembly. Lightweight plastic, which has little mass and low thermal conductivity, was compared with conventional glass semen collection tubes. Thermistors were installed in both types of semen collection equipment to accurately record the temperature. The plastic provides greater control over potential changes in semen at the time of collection, when environmental temperatures differ considerably from body temperature. The plastic tube-foam jacketed collection assembly also offers the advantages of a larger collection tube volume, low cost and toughness with flexibility. Minor changes in cooling semen following collection and pre-extension were tested in a field trial. The optimal conditions were not necessarily established but fertility was equivalent to the control.

297. **Impact of Applied Genetics: Animal Breeding.** B.G. Brackett, G.E. Seidel, Jr., S.M. Seidel, H.W. Clark, R.H. Foote, K. Inskeep, T.J. Sexton and L.D. VanVleck

This is an extensive report prepared for the Office of Technology Assessment, a technical group with the main function of preparing reports for Congress on important emerging technologies. The report deals with artificial insemination, embryo transfer, sex selection, cloning and related topics in domestic animals, poultry and fish. Later much of this was revised and published in a book "New Technologies in Animal Breeding," edited by B. G. Brackett, G. E. Seidel, Jr. and S. M. Seidel, Academic Press, N.Y. 1981.

298. **Milk Progesterone Testing to Determine Reproductive Status of Cows.**
T.J. Reimers, R.D. Smith and R.H. Foote

Dairy cows should calve at 12-12.5 month intervals for maximum profit and production. One reproductive management tool which helps attain this goal is quantification of milk progesterone (MP_4). Reproductive status of 254 cows in 12 commercial dairy herds was determined by examining changes in MP_4 during the 35-60 day postpartum period. In this study, 82% of the cows had normal estrous cycles by 60 days postpartum. Fourteen percent had consistently low and 4% had consistently high MP_4 levels. Accuracy of the MP_4 test 21-23 days after insemination for predicting pregnancy and non-pregnancy was 80% and 98%, respectively. Because of this and other field studies, the New York Dairy Herd Improvement Cooperative and the College of Veterinary Medicine began a MP_4 testing service for farmers and veterinarians. A new, rapid radioimmunoassay using assay tubes treated with anti-progesterone serum (Micromedic Systems) was validated for this service. Accuracy for predicting reproductive status was indicated by the fact that 0% (n=16), 50% (n=10) and 83% (n=80) of tested cows were pregnant (determined by palpation per rectum and non-return to estrus) when MP_4 concentrations were <1.0, 1.0-3.0 and >3.0 ng/ml, respectively, 21-24 days after breeding. Farmers, responding to a questionnaire, indicated that the test was useful for getting cows bred, identifying non-pregnant cows early, checking estrus and determining ovarian function in problem breeders. Veterinarians found the test valuable for confirming presence of follicular or luteal cysts and determining response to therapy.

299. **The Behavioral Responses in Ovariectomized Cattle to either Estradiol, Testosterone, Androstenedione, or Dihydrotestosterone.**
L.S. Katz, E.A.B. Oltenacu and R.H. Foote

Steroid hormone effects on sexual behavior were measured in 15 sexually mature nulliparous cattle which were bilaterally ovariectomized. They were allotted at random to five groups of three animals each (sesame oil vehicle control, estradiol, testosterone, androstenedione, and dihydrotestosterone) in the fall of the year and reassigned at random to replicate the study the following spring. Each experiment was divided into three weekly trials. Animals within treated groups were reassigned each week to receive in random order one of three levels of a particular hormone (200, 400, and 800 μg of estradiol and up to 1000 times these doses of androgens). Estradiol, and to a lesser extent, testosterone were capable of increasing the frequencies of occurrence of most behavioral parameters studied. These were: (1) elicitation of vulval interest; (2) vulval sniffing; (3) agonistic interactions; (4) giving chin rests; (5) receiving chin rests; (6) attempted mounts; (7) successful mounts; and (8) standing when mounted. The mean interval from treatment to first standing to be mounted was 25.4 ± 0.8 and 33.3 ± 5.2 hr for the estradiol-treated and testosterone-treated heifers, respectively. Peak activity generally occurred the second day after initiation of hormone treatment. Flehmen lip curl and bellowing were not stimulated by either hormone. Neither androstenedione nor dihydrotestosterone was capable of stimulating sexual behavior in these heifers, as measured by any of the parameters studied.

300. **Reproduction and Artificial Insemination**. R.H. Foote

This conference consisted primarily of a series of lectures by the author on all aspects of artificial insemination. This included selection of sons and sires with large testes as well as on milk production information. Libido of bulls and various methods of preparing bulls for semen collection were discussed. Methods of measuring semen quality and freezing procedures, along with detection of estrus and breeding recommendations were included. These lectures were accompanied by mimeographed notes that were collected and published.

301. **Influence of Bovine Cervical Mucus Samples and Storage Conditions on Sperm Migration In Vitro.**
H.L. Kummerfeld, J.K. Vosburgh, S.P. Lorton and R.H. Foote

In vitro sperm migration assays were performed using bovine spermatozoa and cervical

mucus. Experiments were designed to test the effects of storage temperature, method of storage, duration of storage, and source of cervical mucus. Significant variation in migration of spermatozoa was due both to differences in mucus samples and to short-term mucus storage at temperatures ranging from ambient to -196°C. The parallel-orienting effect of cervical mucus on migrating sperm was shown to be a major factor in quantitative assays based upon migration distance. Thus, comparisons of migration among different specimens of semen likely will be biased unless the tests are run simultaneously. Implications of these results are discussed relative to the performance of quantitative sperm migration assays in the clinical or research laboratory.

302. **Polyacrylamide as a Substitute for Cervical Mucus in Sperm Migration Tests**. S.P. Lorton, H.L. Kummerfeld and R.H. Foote

A synthetic migration medium for capillary sperm migration studies was developed. Parallel sperm migration in 1.8% polyacrylamide cross-linked with 0.042% N,N'-methylene bis acrylamide was similar to sperm migration in bovine cervical mucus. Bull spermatozoa varying widely in migration distances in bovine cervical mucus maintained similar relative migration distances in this synthetic medium. The advantages of the synthetic medium are its availability in large quantities, its uniformity, and its stability. There was no change in parallel sperm migration distances in the synthetic medium stored at 4°C for up to 4 months. Use of this synthetic medium for human or bovine sperm migration studies would appear to overcome problems associated with the variability of cervical mucus.

303. **Relationship of Spermatozoal Migration in Cervical Mucus to Bovine Fertility**. H.L. Kummerfeld, S.P. Lorton and R.H. Foote

Frozen bovine semen from males of well-established, generally high fertility levels was thawed and used in three experiments for in vitro capillary tube migration through cervical mucus. Bovine cervical mucus from individual heifers or a group of heifers was evaluated in these assays. Correlations between spermatozoal migration and bull fertility were small and not significant. While generally positive four different estimates ranged from -0.21 to 0.46. Significant differences in spermatozoal migration distances were due to bulls, source of mucus, and storage time of mucus before use in the assay. These differences did not appear to affect the correlations obtained, but they require that the sperm migration test be standardized relative to sources of mucus and storage time if different seminal specimens are to be compared.

304. **Factors Affecting Gestation Length in Dairy Cattle**. R.H. Foote

Data were collected through a special record keeping system for 2,254 herds that used artificial insemination, 37% of which were enrolled in Dairy Herd Improvement Associations. Information was available on a total of 35,162 gestations, consisting of 24,367 Holstein, 5,849 Guernsey, 2,872 Jersey, 1,667 Ayrshire and 407 Brown Swiss pregnancies resulting in single births plus 930 multiple births. Breeds, twinning, sex of calf, parity of the cow and time of day on which estrus occurred were associated with differences in gestation length. Month of insemination had no effect. Mates and sires of the cows both affected gestation length slightly, but significantly. Selection of sires for high milk production of their daughters over a 23-year period appears to have had little, of any, effect on gestation length, as the mean gestation length has increased only one day, from 278 to 279 days for Holsteins during that time.

305. **Systems Analysis for Designing Reproductive Management Programs to Increase Production and Profit in Dairy Herds**. P.A. Oltenacu, T.R. Rounsaville, R.A. Milligan and R.H. Foote

Relative economic merits of three heat detection rates and three conception rates were evaluated by mathematical modeling and dynamic simulation. Three heat detection programs evaluated were: a) poor, with no specific time set aside for detection and detection rate of .35; b) average, with two 45-min observations each day and detection rate of .55; c) good, with three 45-min observations each day and detection rate of .75. Changing the heat detection

program from poor to average and average to good decreased days open from 136 to 119 and to 105. Corresponding increases in net return per cow per year were $60 and $4. Three breeding programs evaluated were: a) poor, direct service by an inexperienced inseminator and conception rate of .42; b) average, professional artificial inseminator servicing the cows with a single insemination at each service and conception rate of .50; c) good, professional artificial inseminator using two inseminations during each service period and conception rate of .58. Changing breeding program from poor to average and average to good decreased days open from 123 to 119 and to 115. Corresponding changes in net return per cow per year were an increase of $39 and a decrease of $7.

306. **The Artificial Insemination Industry.** R.H. Foote

This chapter focuses on artificial insemination (A.I.) in dairy cattle with information on other classes of livestock and poultry. Subjects covered on A.I., include the growth of the AI industry, and (b) major milestones in collecting, processing and use of semen. The impact of A.I. on milk production and disease control is reviewed. Future likely developments and costs of A.I. and new biotechnologies are discussed. Key references are included.

307. **Decreased Motility of Bull Spermatozoa Caused by *Mycoplasma bovigenitalium*.** V.S. Panangala, A.J. Winter, A. Wijesinha and R.H. Foote

Mycoplasma bovigenitalium mixed with bull semen in egg yolk-citrate buffer and held at 5°C caused a highly significant time- and dose-dependent depression in sperm motility. The association of mycoplasma with sperm was examined by immunofluorescence microscopy and transmission electron microscopy. Mycoplasma adhered to a majority of spermatozoa, principally to the acrosome, but also to the midpiece and tail. This may reflect the basis for a naturally observed condition in young bulls in which genital mycoplasmosis is associated with low sperm motility.

308. **Cryopreservation of Spermatozoa and Artificial Insemination: Past, Present, and Future.** R.H. Foote

This review article is intended to provide an in-depth analysis of the principles and programs established, as well as problems overcome, that have led to a successful artificial insemination (AI) program using cryopreserved semen in cattle. The components of such a program considered are: semen production, evaluation, processing, storage, and insemination. Advances with cattle and other species of domestic animals may provide clues to mechanisms involved which could lead to methods of improving the cryopreservation of human spermatozoa. Several current developments give hope for substantial improvements in the future.

309. **Migration of Fresh and Cryopreserved Human Spermatozoa in Polyacrylamide Gel.** M.C. Goldstein, L.S. Wix, R.H. Foote, R. Feldschuh, and J. Feldschuh

The ability of freshly collected and frozen human spermatozoa to migrate in round capillary tubes containing specially formulated polyacrylamide gel was investigated, using 33 ejaculates from 27 donors. Each semen sample was divided; one portion was left undiluted, and the other portion was diluted to 50×10^6 sperm/ml. Glycerol was used as the cryoprotectant. The percentage of motile sperm cells was determined before and after freezing. Fresh semen contained a higher percentage of motile cells, which migrated farther than those of cryopreserved-thawed semen. Various correlations between the percentage of motile sperm and migration distance ranged from 0.57 to 0.62. There was a low positive correlation of migration distance with sperm cell concentration per milliliter, $r = 0.25$ to 0.34; and thus adjusting semen samples to a standard sperm concentration improved the accuracy of the test only slightly. The regression coefficient of migration distance on the percentage of motile sperm in fresh semen was 0.65, indicating that for each 10% increase in sperm motility, migration distance is predicted to increase 6.5 mm. Five batches of polyacrylamide gel gave uniform results.

310. **Effects of Amikacin Sulfate on the Motility of Stallion and Bull Spermatozoa at Different Temperatures and Intervals of Storage**. J. Arriola and R.H. Foote

Because microfloral content of stallion semen tends to be high, and strains may be resistant to commonly used antibiotics, amikacin was tested with stallion semen and compared with bull semen. Nine ejaculates of stallion semen were incubated at 37 C in egg yolk-tris extender for 0, 2, 4, 6, 8 and 10 h in the presence of amikacin concentrations of 0, 50, 100, 250, 500, 1,000 and 10,000 μg/ml, with penicillin and penicillin-streptomycin as controls. Averaged over all incubations, spermatozoal motility was 44, 48, 49, 46, 45, 45 and 19%, for increasing concentrations of amikacin, compared with 52 and 47% for penicillin and penicillin-streptomycin controls. The 10,000 μg/ml concentration of amikacin was the only treatment that suppressed sperm motility ($P<.01$). Amikacin (0, 50, 100, 250, 500, 1,000, 2,500, 5,000 and 10,000 μg/ml) and 1,000 IU of penicillin G plus 1,000 μg of streptomycin/ml or 10,000 IU of penicillin G/ml were added to nine ejaculates of bull semen stored at 4 C in egg yolk-tris extender, and evaluated after 0, 1, 3, 5 and 7 d. The percentage of motile spermatozoa, with increasing levels of amikacin, was 66, 67, 66, 64, 67, 68, 74, 68 and 53%, respectively. Amikacin, at 2,500 μg/ml, resulted in the highest ($P<.01$) motility compared to the other levels of antibiotics after 7 d storage. Both 10,000 μg of amikacin and 10,000 IU of penicillin G/ml depressed ($P<.01$) the mean percentage of motile bull spermatozoa. These studies demonstrate that high concentrations of amikacin can be added to stallion and bull semen without depressing motility of spermatozoa.

311. **Separation of Bovine Spermatozoa by Density on Water Insoluble Newtonian Gels and Their Use for Insemination.**
A.A. Luderer, W.W. Dean, A.R. Zine, D.M. Hess, R.H. Foote and R.J. Wall

Bovine spermatozoa were separated into different density subpopulations utilizing water insoluble hydrocarbon and silicone gels of defined specific gravity. Sperm density profiles were generated for 13 bulls. The separations were found to be repeatable and characteristic of the bull examined. Considerable density variation among animals was demonstrated. Analysis of the separated spermatozoa, before and after freezing, demonstrated that good motility and acrosomal integrity of spermatozoa were maintained. When the least dense fraction of spermatozoa was used for insemination, conception rates were similar to those obtained routinely by artificial insemination with unfractionated spermatozoa. Therefore, this system may be useful in separating spermatozoa of various densities and for removing extraneous matter from semen. However, the sex ratio, among 51 60-day-old fetuses recovered from heifers inseminated with the lowest density fraction of spermatozoa, was 26 males:25 females.

312. **Electronic Probe Measurements of Cervico-Vaginal Mucus for Detection of Ovulation in Dairy Cows: Sanitation, Clinical Observations and Microflora.**
R.L. Scipioni, R.H. Foote, S.V. Lamb, C.E. Hall, D.H. Lein and S.J. Shin

An electronic vaginal probe with a sanitizing carrier unit has been designed to measure changes in the electrical resistance of cervico-vaginal mucus in the cow. Over 400 cows and heifers have been probed during the period prior to breeding. When 33 heifers and cows were probed 2-3× per day for 30 days, mild irritation of the vaginal mucusa developed during the luteal phase. In all other studies no grossly detectable lesions or other complications resulting from probing were observed either in experimental herds or in farmer herds where breeding tests were conducted. Fertility of probed cows was equal to cows inseminated when estrus was detected visually. Weekly culture of microorganisms from cervico-vaginal mucus collected from three cows probed 3× per week for 22 days resulted in no detectable change in the population of microorganisms. The number of isolates each successive week was 11, 8, 8 and 5, respectively. The sanitary procedures followed appeared to prevent introduction of organisms of any consequence, as judged by the decreasing number of isolates during the probing sequence.

313. **Functional Differences Between Sperm Bearing the X- or Y-chromosome**. R.H. Foote

Functional tests used in an attempt to separate X- and Y-bearing sperm of several species include sedimentation (natural gravity), centrifugation, galvanic and variations of electric charge and Sephadex columns. The characteristics of sperm which would allow for separation by these techniques do not appear to be uniquely associated with X- and Y-chromosomes. Separation by these techniques is likely to be incomplete. Consequently, only a small but useable yield of spermatozoa with substantial deviations from the normal sex ratio should be expected. The design of some experiments could be improved by controlling the spermatozoal preparation and the environment surrounding the cells during separation, so as to minimize the effects of extraneous variables. Many experiments are too small in scope and have inadequate controls. This is a special problem when obtaining data for humans. Large scale experiments with cattle have not resulted in significant alterations of the sex ratio. Studies are proceeding with humans and cattle. Of special significance is research with cell flow cytometry. Differences consistent with those due to X- and Y-chromosomes are being measured. Presently this method of separating cells is destructive and somewhat slow for commercial use. However, this need not be a deterrent to advancing the potential of mass sperm cell separation. Low yields of highly separated spermatozoa, for example, might permit monoclonal antibody production against reachable antigenic sites. Such a procedure would be highly effective, inexpensive and rapid for large batch processing of spermatozoa for sex control.

314. **Testing for Altered Sex Ratios: Statistical Considerations**.

R.L. Quaas and R.H. Foote

An experimenter attempting to alter the sex ratio must deal with data that are discrete: an animal is either male or female. We have stressed the importance of the size of the experiment (N) when faced with the random variation inherent in such data due to binomial sampling. If N is not large enough, the experimenter is almost guaranteed to make one of two kinds of errors; claiming an efficacious treatment when the results were due to chance or failing to detect a true treatment effect that exists. One must conduct the experiment with both treated and control groups. An example of this is included in the following table.

Number of observations needed in treated and control groups to detect a significant shift in sex ratio.

Probability of detecting a true treatment difference (β)	Degree of true sperm separation			
	60:40	70:30	80:20	90:10
.80	304	72	29	14
.90	422	99	40	19
.95	530	125	50	23

[a]Calculated with the type I error (α) = .05 and the type II error (β) = .80, .90 or .95 and using a one-tailed test as outlined by Snedecor and Cochran (1967). Note, that an experiment containing the designated number of animals in each group will establish that there is a significant difference in sex ratio, but the confidence interval for the observed sex ratio would have to be calculated.

315. **Considerations in Evaluating the Risk to Male Reproduction**.

M.J. Bedford, R.P. Amann and R.H. Foote

The literature is reviewed for mouse, rat, rabbit, dog, monkey and man, relative to criteria that might be useful in evaluating reproductive function in models for man when exposed to possible noxious agents. The characteristics of spermatogenesis are reviewed. Mice and rats are the least expensive animal models that are well-characterized. However, the rabbit and dog are models which have the possibility of monitoring semen quality regularly. The rabbit is less expensive and vastly superior when fertility tests are planned. So, the major criteria useful in evaluating risks to reproduction in the male, besides general health are 1) semen quality, 2) fertility testing using graded doses of sperm with artificial insemination (rabbits), 3) effects on the testis and accessory sex glands and 4) endocrine

changes. Among in vitro tests of sperm function, in vitro fertilization is included.

316. **Milk Progesterone Concentration and Production in Superovulated Holstein Cows.**
R.H. Foote, P.C. Ladd, N.A. Lafaunce, A.D. McCauley and J.F. Hasler

Six Holstein cows in a commercial herd (three superovulated and three controls) and eight Holstein cows superovulated a total of 12 times in an experimental herd were studied. Superovulation was induced primarily by treatment with follicle stimulating hormone and prostaglandin $F_2\alpha$. Milk was weighed twice daily for 30 days following treatment in the commercial herd with no effect on production. Milk samples were saved on Monday, Wednesday, and Friday to determine progesterone content. Last milk at mid-cycle averaged 8.2 ng/ml of progesterone for the cows in the experimental herd, and 7 days after superovulation they averaged 52.6 ± 10.2 ng/ml (mean ± standard error) of progesterone. The correlation with number of embryos recovered was .86. Therefore, milk progesterone may be useful in monitoring superovulatory response. Also, injection of prostaglandin $F_2\alpha$ into superovulated cows 9 days after a previous injection did not initiate a new estrous cycle, a fact accurately monitored by milk progesterone determinations.

317. **Behavioral Characteristics of Beef Steers Administered Estradiol, Testosterone and Dihydrotestosterone.**
D.A. Dykeman, L.S. Katz and R.H. Foote

Twelve beef steers ranging in age from 6 to 8 mo were randomly assigned to one of four steroid treatment groups so the ability of each hormone to stimulate sexual and (or) agonistic behavior could be assessed. The steers received im injections of either 200 mg of testosterone (T), 200 μg of estradiol-17β (E_2), 200 mg of dihydrotestosterone (DHT) or sesame oil vehicle as control (C), every other day for 10 d. During these 10 d, behavior was studied for 60 min at 0600, 1200, 1800 and 2400 h. Peak activity occurred during the first half of the experiment with maximum behavioral expression observed at the 2400-h observation period. Estradiol was most effective in stimulating eight of the 13 behaviors studied. These included: sniffs received, sniffs given, successful mounts, chin rests given, chin rests received, stands to be mounted, refusals to stand and head butts received. Testosterone was most effective in stimulating Flehmen lip curls, attempted mounts and head butts given. Testosterone significantly increased activity in seven other categories over that of controls. Dihydrotestosterone was never the most effective steroid. It was capable of stimulating several of the behaviors studied above that of controls, but reduced mounting activity of steers. In view of the major responses observed with E_2 and the aromatizable androgen, T, these data are interpreted as an indication that E_2 plays a significant direct role in sexual behavior in the bull.

318. **Superovulatory Responses of Holstein Cows.** J.F. Hasler, A.D. McCauley, E.C. Schermerhorn and R.H. Foote

Approximately 1000 registered cows and heifers were superovulated one to 10 times. Nonsurgical embryo recoveries were performed on all donors which exhibited estrus. Healthy donors produced more total ova and cleaving embryos and had a higher ovum recovery rate, fertilization rate and pregnancy rate from embryos transferred than did cows classified as infertile. While ovum number was not affected during 10 repeated superovulations, fertilization rate and embryo number decreased. The number of ova recovered from healthy cows was affected by season, and from infertile cows by the day of the estrous cycle on which FSH was started and by the number of days since calving. More ova were recovered from infertile cows synchronized with prostaglandins prior to superovulation than following a natural estrous cycle. The number of embryos recovered from infertile cows was affected by age and from healthy cows by daily milk production. Fertilization rates in both healthy and infertile cows were affected by age, time since calving, daily milk production, day of cycle FSH was injected and season. There was no effect of the day of recovery on the number of ova or embryos recovered from healthy or infertile cows. The paper contains nine tables with extensive data.

319. On the Negative Feedback Regulation of Gonadotropins in Castrate and Intact Cattle With Comparison of Two FSH Radioimmunoassays.

W.R. Butler, L.S. Katz, J. Arriola, R.A. Milvae and R.H. Foote

Two homologous radioimmunoassays for bovine follicle stimulating hormone (bFSH) were utilized in comparing the differential regulation of FSH and luteinizing hormone (LH) in response to ovariectomy or administration of gonadal steroids in cattle. There appeared to be significant LH cross-reactivity in one of the bFSH systems (bFSH-HS-2-17), but not in the other (bFSH-BP3). Concentrations of FSH in plasma measured by these two systems suggested both qualitative and quantitative differences. Following ovariectomy in heifers, LH concentrations in plasma were increased by 7.5 h, while FSH (measured in the bFSH-BP3 system) was not significantly elevated until 18 h. Administration of 200 μg of estradiol-17β to ovariectomized heifers inhibited levels of FSH in plasma but large doses of testosterone (100 mg), androstenedione (400 mg) and dihydrotestosterone (800 mg) had no effect. Similarly, LH was not affected by the androgens, while estradiol induced LH surges, leading to increased mean LH concentrations. In contrast to the results in heifers, LH concentrations in plasma from steers were inhibited by administration of androgens as well as by estradiol. In steers, FSH (bFSH-BP3) was marginally inhibited by estradiol and not at all by the androgens. Differences in the secretory patterns of FSH and LH also occurred in intact heifers during the estrous cycle. The 72-h period preceding estrus (follicular phase) was characterized by rapidly declining serum progesterone concentrations, followed by concurrent increases in both LH and estradiol. The circulating levels of bFSH (BP3) tended to decline during this interval. Overall, during the estrous cycle, progesterone levels were positively correlated with bFSH-BP3 (r=.37) and negatively correlated with LH (r = -.39). The gonadotropins were not significantly related (r = -.15). These relationships are consistent with the concept that LH controls the final stages of follicular development in cattle and that FSH may exert only a permissive effect.

320. Embryo Transfer and New Biotechnology.

R.H. Foote

The transfer of embryos in cattle has become a major industry in the U.S.A. and is now practised in many countries. In 1983 it is expected that about 60,000 calves will be born in North America as a result of embryo transfer (ET). The nonsurgical approach has made this practical in the field, particularly in lactating cows. The nonsurgical approach also has been used successfully in horses while the surgical approach is required in smaller domestic animals such as sheep, goats, and swine. The potential value of ET is manifold and includes the following: (1) increasing selection on the female side, (2) controlling the number of progeny per female, (3) introducing new genotypes into different areas of the world, (4) controlling disease and, (5) providing new genetic and physiological research opportunities. The value of each embryo can be further enhanced by several modern biotechnologies, such as sexing of the young embryo, "splitting" the embryo and freezing the embryo. The oocyte or "ootid" can be utilized in vitro for fertilization studies, including overcoming certain problems of infertility. In addition, the improvement in conditions for handling and culturing oocytes and eggs provides new opportunities for microsurgery. Bits of genetic material or haploid or diploid genomes can be inserted or removed under appropriate conditions, leading to a variety of genetic conditions of great potential value to the experimental geneticist and practical animal breeder.

321. New Developments in Embryo Transfer and Related Technology.

R.H. Foote

Embryo transfer is the most recently applied biotechnology in the effort to more efficiently provide high-quality protein foods to a malnourished, growing world population. About 60,000 calves in North America will result from embryo transfer (ET) in 1983. It is currently an adjunct to, not a replacement for, artificial insemination. When judiciously used, it can improve the genetic merit of livestock, overcome certain forms of infertility, assist in control of diseases and provide opportunities for designing special breeding programs. Expansion of ET technology has allowed research to improve and control the kind of embryo transferred and the time and place

that this can be done most expeditiously. Current technologies being used are bisection of embryos and freezing of embryos. Sexing of embryos may soon be commercially feasible. These advances are valuable in new animal reproduction and genetic studies, plus assist in meeting the needs and desires of progressive cattle breeders. For example, identical multiplets could be prepared and sexed, then some male embryos frozen while identical males are produced by ET. After sampling, the bulls could be slaughtered. After progeny testing, genetic copies of the best bulls could be taken from the freezer, implanted in a recipient female, born and raised for further semen production and breeding. Each technique applied to an embryo has a possible cost, either in money (labor, equipment and supplies) or as an effect on the embryo. Therefore, planning should precede application. Techniques of the molecular biologists, combined with microsurgery, may permit the synthesis of new genetic types, such as complete homozygotes and those embryos with special gene complexes inserted. An overview of present practices and future possibilities are presented in this report.

322. **Improvement of Reproduction in Large Dairy Herds**. R.H. Foote

Managers of large dairy herds should have a good understanding of physiology of reproduction, feeding and nutrition, genetics, herd health and general management and people management skills. The first step is to set realistic goals. The second step is to have permanent easy to see identification of all animals so that they can be properly managed. The next step is to have appropriate yards, housing and other facilities. Computers are essential for having all the relevant information on each animal available at the fingertips of the manager. Techniques and procedures must be in place and known by the work force so that the goals can be implemented. Feeding, detection of estrus, breeding and prepartum handling of cows in large herds are simplified by grouping cows relative to production, open for breeding, pregnant, and dry. Proper housing includes a clean, "sterilized" maternity area. For a high conception rate more emphasis should be placed upon the semen used and the training of the inseminator. If the insemination is done by the herdsman, thorough training on semen handling and on insemination procedures is essential.

323. **General Evaluation of Male Reproductive Capacity**. R.H. Foote

Breeding soundness examinations have become very important in males as either through natural breeding, and more so through artificial insemination, they affect the reproductive outcome of many females. Factors to consider are the following: 1) reproductive history of the male and close relatives; 2) thorough physical examination, especially of the testis (size, shape, consistency); 3) normal penis; 4) endocrine profile in the blood; 5) serving ability; 6) semen evaluation of several samples; and 7) tested for various diseases. The most important single component is the testes. A combination of linear measurements or scrotal circumference combined with a tonometer test for firmness or ultrasound evaluation is desirable, especially in older bulls. Testis characteristics are positively correlated with sperm production and fertility. Selected semen characteristics also are important, but they are subject to much greater sampling errors. At the particular time each male is collected there may be an effect of age, season (especially in seasonal breeders), previous illness, health that day, teasing, the teaser, the collector and other conditions at the moment of semen collection, plus variation in subsampling the semen collected. We must recognize these and use all the skill at our disposal to reduce these random errors for they will contribute to the validity or lack of validity of our assessment of male reproductive capacity.

324. **Optimization of Procedures for Separation of Motile and Nonmotile Bull and Rabbit Spermatozoa With Bovine Serum Albumin Gradients.**

R.J. Wall, D.A. Jerrard, J.J. Parrish and R.H. Foote

The effect of equipment design, separatory media, and time and temperature of separation were studied. Discontinuous 4%/10% bovine serum albumin (BSA) gradients were used to isolate highly motile spermatozoa in rabbit and bull semen. For all conditions tested, motility of spermatozoa collected from the 4% BSA gradient layer (top) was less than or equal to the motility of the unseparated controls.

Fractions collected from the 10% BSA gradient layer contained highly motile spermatozoa. In experiment 1, washed bull spermatozoa were diluted with phosphate-buffered saline (PBS) containing 2% BSA or 4% BSA before being fractionated on BSA columns contained in test tubes. Inclusion of BSA in PBS tended to reduce loss of motility during washing, but the proportion of sperm recovered was highest in PBS. In experiment 2, motility and recovery of buck spermatozoa collected from the 10% BSA gradient region tended to be higher when fractionation temperature was 30°C as compared to 35°C, and motility was significantly higher when incubation time was 30 min as compared to 1 hr. The proportion of sperm recovered was unaffected by incubation time. In experiments 1 and 2, 41 of 48 separations resulted in at least one fraction containing spermatozoa with motility greater than or equal to 90%. In the third experiment, the surface area on which bull and buck spermatozoa were layered was increased by forming the 4%/10% BSA gradients in conical supports. Separation of sperm on conically shaped columns was not as effective as on cylinders. The use of cylinders to support the BSA gradients and a separation time of 30 min at 30°C is recommended.

325. **Toxicologic Studies With Male Sheep Grazing on Municipal Sludge-Amended Soil.** D.E. Hogue, J.J. Parrish, R.H. Foote, J.R. Stouffer, J.L. Anderson, G.S. Stoewsand, J.N. Telford, C.A. Bache, W.H. Gutenmann and D.J. Lisk

Growing sheep were grazed for 152 d on grass-legume forage growing on soil that had been amended with municipal sewage sludge from Syracuse, N.Y., at 224 metric tons per hectare. Cadmium was higher, but not significantly ($p > 0.05$), in tissues of sheep fed the sludge-grown forage as compared to controls. No significant differences between the sludge or control treatments were found in weight of the complete or cauda epididymis or in percent progressive motility of cauda epididymal sperm. The sludge-treatment group had significantly larger testes ($p<0.025$) when expressed as a percentage of body weight, and higher blood uric acid values ($p<0.05$). There were no observable changes in tissue ultrastructure of liver, kidney, muscle, or testes as examined by electron microscopy in either of the treatment groups. There were no significant differences for rate of animal weight gain, carcass weight, dressing percentage, or quality or yield grade of the carcasses between the treatment groups.

326. **Buffers and Extenders: What Do They Do? Why Are They Important?** R.H. Foote

What do sperm need to survive? They need to be maintained in an appropriate temperature, in fluids with the appropriate osmotic pressure, pH and buffering capacity, energy source, sperm concentration (and macromolecule concentration), microbiological control, toxic materials excluded and an appropriate gas phase. The media or extenders to provide the optimal environment will change from ambient temperature to cooling to 5°C and finally with freezing in liquid nitrogen. Much is known about what sperm require, but this is not fully understood and no completely synthetic medium has been formulated which is equivalent to those containing complex mixtures, such as egg yolk or milk.

327. **Manipulating Cow Uterine Response at the Time of Insemination.** R.H. Foote, M.D. Cooper, E.C. Schermerhorn and S.K. Newman

Clitoral massage consistently causes an immediate uterine contraction and this can be demonstrated repeatedly. This does not cause release of oxytocin. Insemination of 2,090 control animals (74.3% nonreturns) and 2,049 massaged animals (74.0% nonreturns) by 18 technicians failed to demonstrate that treatment had any effect on the nonreturn rate of cows or heifers. The nonreturn rate by technician varied considerably (68 to 83%). The overall skill of the individual technician and dairy herd manager, likely have a greater effect on fertility than individual components of the insemination process measured here.

328. **Uterine Contractions and Fertility Following Clitoral Massage of Dairy Cattle in Estrus.**
M.D. Cooper, S.K. Newman, E.C. Schermerhorn and R.H. Foote

Lactating and nonlactating Holstein cows received 30 s of gentle clitoral massage to determine its effect on uterine contractility and oxytocin concentrations in blood. Clitoral stimulation caused an immediate single uterine contraction, and this could be repeated at 2.5-min intervals. Oxytocin concentrations did not change during intensive blood sampling following clitoral massage. Thus, the uterine response is interpreted as neurally mediated, rather than mediated hormonally. Eighteen full-time inseminators were allotted to two groups, balanced so that each day half were inseminating control and half treated animals (5 s of clitoral massage following insemination). The 56-day nonreturns for 2090 controls were 74.3% versus 74.0% for 2049 animals receiving clitoral massage. Heifers were more fertile than cows (81.9% versus 70.6% nonreturns), but there was no indication that clitoral massage altered the nonreturn rate in either age group. Also, the time of day animals were inseminated did not affect nonreturn rate.

329. **Efficacy of TEPA as a "Sperm Label" for Competitive Fertilization Studies.**
R.J. Wall, C.A. Carberry and R.H. Foote

In this paper, the conditions necessary to use TEPA [tris (1-aziridinyl)] effectively as a label for spermatozoa in competitive fertilization are established. The fertilizing ability of rabbit spermatozoa treated with 0 and 0.8 mg TEPA/ml was compared at insemination doses of 1, 5, 20, and 40 $\times$ 10^6 spermatozoa. Fertility was assessed by collecting ova from 64 does 48 to 52 h after insemination. TEPA blocked all but 4% of the ova from developing when 1 $\times$ 10^6 spermatozoa were inseminated, but fertility was reduced. When 5 $\times$ 10^6 spermatozoa were inseminated following treatment with 0, 0.6 or 1.2 mg of TEPA/ml, the fertility was 83, 74 and 50% ($P<0.05$), and the percentage of ova containing more than four blastomeres was 83, 11 and 5% ($P<0.05$), respectively. The 0.6% TEPA level was selected for a competitive fertilization trial. Equal numbers of sperm from pure Dutch-color and albino sires were combined so that either both types were untreated, only the 'albino' semen was treated, only the 'Dutch' semen was treated, or both were treated. Does were inseminated with 5 $\times$ 10^6 sperm and allowed to kindle. The litter sizes were 5.6, 3.1, 2.7, and 0 young, and the proportion of Dutch-color progeny was 63, 97, 0 and 0%, respectively, confirming the effectiveness of TEPA as a "label." Only one of 60 young born resulted from fertilization by a TEPA-treated spermatozoon, demonstrating that few embryos fully escape the TEPA block. Thus, the TEPA concentration and sperm numbers were established to use TEPA effectively as a label for spermatozoa in competitive fertilization studies.

330. **A Specific Oligoteratozoospermia in a Bull: The Sperm Tail Stump Defect.**
J. Arriola, L.A. Johnson, M. Kaproth and R.H. Foote

The first known case in the United States of a bull with the sterilizing oligoteratozoospermia known as "sperm tail stump defect" is reported. Ejaculates of semen were characterized by a watery appearance, extremely low sperm concentrations, sperm akinesia, and 100% abnormal spermatozoa. Different degrees of partial tail development were observed. The most common abnormalities were a short midpiece remnant or a cytoplasmic droplet-like rounded body replacing the midpiece and tail. Also a high percentage of sperm head abnormalities were found. Testicular histology revealed seminiferous tubules with a low rate of spermatogenesis. Elongation of the spermatids did not proceed normally and no normal tail development was observed. The paper includes 17 figures of the defects and associated testicular tissue.

331. **Prostaglandin $F_2\alpha$-induced Estrus in Open Cows and Presumed Abortion in Pregnant Cows With Unobserved Estrus in a Herd Monitored by Milk Progesterone Assay.**
D. Cavestany and R.H. Foote

Data were obtained in a large Florida herd of about 1800 Holstein cows. All cows were inseminated by the herdsman who did the pregnancy

checks and who also administered drugs. The herdsman injected 103 cows with prostaglandin $F_2\alpha$ during the time this herd was under continuous observation by the authors who were conducting an unrelated research project. These cows consisted of 86 open (never bred) cows which had no estrus observed during the first 70 days postpartum, or no second estrus observed within 30 days after a previous estrus, and 17 cows previously inseminated. Two-thirds (57) of the 86 open cows were in estrus within 4 days. The 17 previously inseminated cows appeared to be pregnant, based upon progesterone profiles, when these were inadvertently given prostaglandin $F_2\alpha$ by the herdsman. Progesterone declined in all cows and they were in estrus in 7 ± 4 days (mean $\pm$ standard error). This result of presumed abortion reflects the luteolytic effectiveness of the drug and the importance of instructing any laymen users to follow necessary precautions to avoid undesirable effects.

332. The Use of Milk Progesterone and Electronic Vaginal Probes as Aids in Large Dairy Herd Reproductive Management.

D. Cavestany and R.H. Foote

In a large herd 427 cows were examined for reproductive condition 26 to 34 days after calving. Progesterone profiles were obtained from a total of over 5000 milk samples which were taken twice weekly for 4 weeks, starting at the time of examination, and again 21, 23, and 25 days later. Researchers lived on the farm during the experiment to sample cows and record all data. Electronic probe measurements of cervical-vaginal mucus also were obtained. All cows were inseminated artificially with frozen semen from one organization. Eleven percent of the cows were not inseminated in the estrual phase as determined by substantial concentrations of progesterone in milk (MP), and 2% were pregnant and aborted following insemination. The MP 23 to 25 days after insemination essentially was 100% accurate in predicting nonpregnancy and was preferable to samples on day 21. When used in conjunction with MP on the day of insemination pregnancy prediction 23 to 25 days after insemination was 84% accurate. Nonreturn rate 60 days after insemination was 7% higher than palpated pregnancy rate. The use of electronic probes under large herd conditions was labor intensive and did not give repeatably distinctive values at estrus.

333. Effect of Season and High Environmental Temperature on Fertility of Holstein Cattle.

D. Cavestany, A.B. El-wishy and R.H. Foote

The breeding records and meteorological data for cows with first services between July 1, 1979, and June 30, 1980, in a large Florida herd were analyzed to determine the relationship between temperature and breeding efficiency. Seasonal high environmental temperatures were associated with low breeding efficiency. Increased maximum temperature from 29.7°C during April to 33.9°C during July was associated with a decrease in conception rate on first service from 25 to 7%. Also, the average number of inseminations per conception, based on pregnancy diagnosis 6 to 8 wk after breeding, was higher from May to August (4.5 to 5.3) than from September to April (2.3 to 3.5). Days open were longer for the cows first inseminated during May, June, and July (173, 171, and 167 days, respectively) than during other months (99 to 149 days). Temperature decreases of any magnitude for 1 to 3 days before or after the day of breeding, when maximum temperatures on the day of breeding were $\geq$27°C, were associated with higher pregnancy rates. Also, similar temperature decreases around the time of breeding, below the previously mentioned high maximum temperatures for 20 days before the day of breeding, were accompanied by higher conception rates. Fertility was consistently lower under all temperature changes when maximum temperatures on the day of breeding were $\geq$33°C.

334. Reproductive Performance of Holstein Cows Administered GnRH Analog HOE 766 (Buserelin) 26 to 34 Days Postpartum.

D. Cavestany and R.H. Foote

During the fourth week postpartum, 443 healthy Holstein cows milked thrice daily were randomly divided among four groups to receive 0, 2, 8 and 32 μg of the GnRH analog HOE 766. Intervals from calving to first estrus and to first breeding, from breeding to conception and conception rates at

first breeding were calculated to measure treatment response, and progesterone was measured in the fat-free portion of milk samples collected twice weekly during the first 4 wk following treatment. Uterine involution at the time of treatment was estimated by palpation per rectum. Twenty percent of the cows examined were classified as having delayed uterine involution (abnormal). By analyzing milk progesterone patterns it was determined that 38% of the animals were in the luteal phase of an estrous cycle when treated. Cows without luteal tissue (<1 ng of progesterone/ml milk) given 8 or 32 μg of HOE 766 increased in progesterone to ≥1 ng/ml within 7 d in 77 and 72% of the cows compared with 40 and 57% for cows receiving 0 and 2 μg ($P<.05$). This increase in progesterone was followed by a normal estrous cycle within 4 wk in a higher proportion of cows treated with the two higher doses of GnRH analog (87 and 86%) compared with 67 and 70% of those receiving 0 or 2 μg of the analog ($P<.005$). There were no treatment differences ($P>.05$) for other traits analyzed, but cows with a normal progesterone cycle were observed in estrus and were bred sooner ($P<.01$) than those with irregular progesterone patterns. It was concluded that the GnRH analog hastened the onset of normal ovarian cycles in cows milked thrice daily.

335. **Motility and Fertility of Frozen Bull Spermatozoa in Tris-Yolk and Milk Extenders Containing Amikacin Sulfate.** K. Ahmad and R.H. Foote

Amikacin sulfate, an aminoglycoside antibiotic, effective against some streptomycin-resistant organisms, was tested in bull semen extended with whole milk and egg yolk-Tris extenders in a 6 × 5 × 2 factorial arrangement. Semen from six bulls was frozen in extenders containing 500, 1000, and 2500 μg/ml of amikacin, a control containing 500 IU/ml of penicillin plus 1000 μg/ml of streptomycin plus 500 IU/ml of polymyxin, and control plus amikacin (1000 μg/ml). Semen was frozen in .5 ml polyvinyl chloride straws in liquid nitrogen vapor and stored in liquid nitrogen for 2 wk. Straws were thawed in a water bath at 35°C for 30 s. Postthaw motility of spermatozoa in each treatment was assessed using two straws by two observers independently. At amikacin concentrations of 500, 1000, or 2500 μg/ml, the percentage of motile spermatozoa averaged 47, 45, and 36% in whole milk and 52, 46, and 46% in egg yolk-Tris. Control and control plus amikacin (1000 μg/ml) were 49 and 47% in whole milk and 48 and 52% in egg yolk-Tris. Only amikacin in milk at 2500 μg/ml, depressed sperm motility. In a field trial with whole milk the nonreturn rate to 1182 first service inseminations with semen treated with amikacin (500 μg/ml) plus control antibiotics was 70.4% compared with 70.5% for 1949 first service inseminations with control semen. Amikacin can safely be added to whole milk and egg yolk-tris extenders to control streptomycin-resistant organisms.

336. **Normal Development of Fetuses Resulting From Holstein Semen Processed for Sex Separation.** R.H. Foote

Semen from two high fertility Holstein bulls with extensive histories in artificial insemination was specially processed for the purpose of sexing and then frozen. The semen was used to inseminate 200 open crossbred beef heifers carefully selected for reproductive soundness from a much larger group. Animals were inseminated alternately with the semen from one of the two bulls and subsequently slaughtered. There were 96 fetuses, ranging in age from 63 to 88 days, which were carefully examined for general appearance, fetal crown-rump length and body weight. There was no effect of sire on fetal size, but the difference between fetal sexes was significant, with the male fetus being appreciably heavier. The regression of fetal length on fetal age was linear and the regression of fetal weight on fetal age was curvilinear. Fetal age accounted for 92% to 96% of the variation in fetal weight and also in crown-rump length.

337. **Counting Mammalian Spermatozoa in Biological Fluids Containing Particulate Matter.** J.E. Parks, E. Ehrenwald and R.H. Foote

Accurate counting of spermatozoa in biological fluids by particle counters requires elimination of competing background. This was accomplished by dissolving cell organelles and other lipid and proteinacious material with sodium dodecyl sulfate

to leave the sperm nucleus. Progress of selective dissolution of interfering background versus sperm nuclei was monitored by phase contrast microscopy. Rabbit semen was diluted in 2.5% sodium dodecyl sulfate (wt/vol) and then 1:1 (vol/vol) with .5 M sodium hydroxide. Suspensions were incubated, diluted 1:200 in .1 M sodium citrate-.1% Triton-X, and counted after 5, 10, and 20 min with a Coulter Counter. All treatment times resulted in similar mean counts, ranging from 367 to 369 $\times 10^6$ sperm/ml. These means were slightly higher than the 350×10^6 sperm/ml for hemacytometer counts, but the correlation with hemacytometer counts was $r \geq .98$. Bull semen was diluted in either 1) citrate-Triton-X, 2) .25 M Tris-20% egg yolk, or 3) heated whole milk and further treated with 10% sodium dodecyl sulfate and .5 M sodium hydroxide. After 15 min, treated samples were diluted 1:200 in citrate-Triton-X and counted. Following treatment, estimated sperm concentration in the three diluters was not different from that of untreated sperm in citrate-Triton-X. The procedure was successfully applied to bull sperm diluted in milk and packaged in .5-ml French straws used routinely for artificial insemination.

338. **Fertility Differences Among Male Rabbits Determined by Heterospermic Insemination of Fluorochrome-labeled Spermatozoa.**

J.J. Parrish and R.H. Foote

Spermatozoa from different bucks were stained with different fluorochromes, mixed, and inseminated heterospermically. By altering the interval between insemination and luteinizing hormone injection, spermatozoa were allowed to reside in the female tract approximately 5, 10, or 15 h prior to ovulation. The number of functional spermatozoa, from each male of a pair used, that was transported to the site of fertilization was estimated by counting total number of differently stained spermatozoa that surrounded or fertilized each oocyte. Spermatozoa from split ejaculates within a male competed against each other equally, indicating that the staining procedure did not affect fertilization or functional spermatozoal transport rates. Three pairs of males with high initial semen quality (>80% motility) differed in fertility primarily due to functional spermatozoal transport. Spermatozoal survival in the female tract and capacitation time played a role in differences in male fertility when heterospermic insemination occurred at variable times relative to ovulation. Differences in fertilization not accounted for by spermatozoal transport ratio raised the possibility that rate of egg penetration due to acrosomal enzyme differences may be important in determining male fertility. Therefore, total acrosin, hyaluronidase, and arylsulfatase activity in spermatozoa from specific bucks used in fertilization experiments were determined. Although there were trends favoring high fertility when enzyme content was higher, the difference was significant only for arylsulfatase in one buck.

339. **Measurement of Semen Quality, Fertility, and Reproductive Hormones to Assess Dibromochloropropane (DBCP) Effects in Live Rabbits.**

R.H. Foote, E.C. Schermerhorn and M.E. Simkin

Thirty-six sexually mature Dutch rabbits were divided into six equal groups to receive in the drinking water 5 days/week for 10 weeks 0, 0.94, 1.88, 3.75, 7.50, and 15.00 mg of DBCP/kg body wt. General health, body weight, semen quality (four ejaculates per male per week), and libido were measured throughout. Fertility, blood follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured the last week and cauda epididymal sperm were examined at sacrifice. There was no effect of DBCP on general health or body weight. There was considerable variation in ejaculate volume, percentage motile sperm, and sperm concentration per milliliter within groups and among weeks. However, between the first 2 weeks and the last 2 weeks of the experiment sperm output had increased by 19% in the three lower DBCP groups and decreased by 16% in the three higher DBCP groups ($p < 0.01$). The proportion of sperm with abnormal tails also increased as DBCP dosage increased. Fertility was unaffected. FSH was elevated ($P < 0.01$) in the group receiving 15 mg/kg of DBCP, which is consistent with the impairment of spermatogenesis. Libido, LH, and testosterone levels were not affected. Sperm morphology was the most sensitive indicator of a DBCP effect in the live animal, being affected at a daily oral intake $\geq$ 1.88 mg DBCP/kg body wt.

340. **Postthaw Survival and Fertility of Frozen Bull Spermatozoa Treated With Antibiotics and Detergent.** K. Ahmad and R.H. Foote

Five factorial experiments were conducted, each with 10 bulls, three extenders, and six antibiotic treatments to determine safe levels of new antibiotics to add to bull spermatozoa. Dicloxacillin, cephapirin, ceforanide, gentamicin, and minocin were added at five concentrations to whole milk, egg yolk-Tris, and egg yolk-Tris detergent (sodium triethanolamine lauryl sulfate) extenders with penicillin and streptomycin as a control. Semen was extended to 20×10^6 sperm/ml, packaged in .5-ml straws, frozen and stored in liquid nitrogen, and thawed at 35°C for 30 s. Two people assessed postthaw percentage of motile spermatozoa from two straws per treatment. Bulls and extenders always affected sperm motility significantly. Percentage of intact acrosomes of spermatozoa was assessed in selected treatments but did not differ in any experiment. Sodium triethanolamine lauryl sulfate tended to improve cryopreservation of spermatozoa. Percentage of motile spermatozoa in milk declined when concentrations of dicloxacillin exceeded 200 μg/ml or when gentamicin, as Gentocin®, exceeded 500 μg/ml. Minocin was toxic at all levels tested in egg yolk and was nontoxic to sperm in milk extender at concentrations ≤ 100 μg/ml. Cephapirin and ceforanide were innocuous at all concentrations tested (200 to 2000 μg/ml). Fertility with cephapirin (500 μg/ml) added to an extender containing control antibiotics resulted in a 56-d nonreturn rate of 75.5 versus 72.1% for the control, based upon a total of 1279 first inseminations.

341. **Use of Quantitative Testicular Histology to Assess the Effect of Dibromocholoropropane (DBCP) on Reproduction in Rabbits.** R.H. Foote, W.E. Berndtson and T.R. Rounsaville

Dibromocholoropropane (DBCP) was administered orally to 36 sexually mature male Dutch Belted rabbits assigned at random to one of six groups to receive 0, 0.94, 1.88, 3.75, 7.5, or 15.0 mg DBCP per kilogram of body weight daily 5 days per week during a 69-day treatment period. Animals were euthanized and necropsied on Day 70. Body weights and weights of the kidneys, liver, epididymides, and accessory sex glands were not influenced by DBCP treatment. The highest dosage reduced mean paired testes weight to 45% of control values ($p<0.01$). Mean seminiferous tubular diameter was reduced by 15 and 29% with the two highest dosages of DBCP, respectively ($p<0.01$), and the percentage of seminiferous tubules containing elongating and round spermatids, as the most advanced cell type present, was decreased ($p<0.01$). The number of leptotene primary spermatocytes per Sertoli cell, and the numbers of spermatogonia, young and old primary spermatocytes, and Step 1 spermatids per Stage I seminiferous tubular cross section likewise indicated a general depression ($p<0.01$) of spermatogenesis. Based upon the number of Step 1 spermatids per cross section at Day 70, production of sperm at this time by rabbits receiving the highest dose of DBCP was estimated to be less than one-fourth of control values. No significant effect ($p>0.05$) of 0.94 mg DCBP/kg on any parameters was found, so this could be considered to be the no effect dose. However, the regressions and mean values are interpreted to indicate that the no effect level of DBCP may be less than 0.94 mg DBCP/kg of body weight. It is suggested that the most sensitive tests described here be included in future screening tests for potential effects of agents on male reproduction.

342. **Endocrine and Other Non-Infectious Factors Associated With Infertility in Cattle.** R.H. Foote and T.J. Reimers

This report is organized into several sections. First, we will discuss briefly many factors which may limit reproduction in a herd. It is important to identify these on a herd basis, thus distinguishing them from specific organic disorders affecting individual cows for which submission of samples to a diagnostic laboratory may be of help in characterizing a particular cow problem. Second, we will describe the normal endocrine changes which occur at parturition and during the postpartum period until pregnancy is established. This is important to provide background for diagnosing various endocrine disorders in cows and in providing a rationale for treatment. Third, the importance of maintaining a proper energy balance for successful reproduction of

high producing cows will be included. Fourth, specific endocrine disturbances will be described. Fifth the discussion will include multiple methods for detecting pregnancy and early embryonic death. With the development of equipment and kits for field use many diagnoses can be made directly on the farm. By sending additional samples to a diagnostic laboratory, and by consultation with the laboratory's experts, additional insight into individual cow as well as herd problems can result. In addition to concentrating on the cow problems, if bulls are used these should be carefully checked. With artificial insemination the quality of semen (if held on the farm) should be checked, detection of estrus, time of insemination and insemination techniques all are important.

343. **Sexual Behaviors, Estrus Detection and Conception of Heifers Synchronized by Progesterone Intravaginal Device (Prid) and Synchromate-B**. K.S. Im, C.K. Kim, H.J. Voss, S. Allen, X. Zheng and R.H. Foote

Seventy four Holstein heifers were randomly assigned over three trials to PRID-7 + PG-6 and Synchromate-B-9 regimens to synchronize estrous cycles for embryo transfer. Sexual behaviors: mounting, standing, orientation, chin-resting, sniffing, licking, rubbing and butting, vaginal swelling and mucus discharge were observed between 06-08, 12-14 and 18-20 h on the 1st day and 00-02, 06-08, 12-14 and 18-20 h on the 2nd day after removal of hormones. Synchromate-B treatment (81.6%) showed a higher synchronized estrus than the PRID treatment (77.8%). Standing estrus was observed within 74 h after PG injection of PRID animals and within 52 h after removal of Synchromate-B implants. About 68% of heifers with PRID and 74% of heifers with Synchromate-B showed standing estrus between 0-14 h on the 2nd day after removal of the hormones. Synchromate-B resulted in a tighter synchrony of standing estrus than with PRIDs. Frequency of mounting and standing per head during the observation period was 22.3 and 16.6 with the PRID and 28.1 and 13.6 with Synchromate-B. The PRID showed a peak in active mounting at 18-20 h on the 1st day; however, the Synchromate-B peaked at 0-2 h on the 2nd day after removal of hormone. Active standing was shown between 18 h on the 1st day to 20 h on the 2nd day with PRIDs; however, this was between 0-14 h on the 2nd day after removal of the hormone with Synchromate-B. There was a slight difference in the pattern of active mounting and standing during estrus between PRIDs and Synchromate-B. Conception rate of synchronized heifers receiving fresh and frozen embryos by non-surgical and surgical methods was higher with Synchromate-B (62.5%) than with PRIDs (38.5%). Chin-resting showed the highest incidence among 6 sexual behavioral components in the both treatments. Synchromate-B showed a higher incidence of chin-resting (16.6%) than PRIDs (10.7%). The Synchromate-B group showed also a higher incidence of orientation, sniffing and butting than the PRID group. Synchromate-B resulted in more active sexual behaviors than PRID. The pattern of incidence of chin-resting, licking and butting was almost symmetrical with PRIDs with their peak values at 6-8 h on the 2nd day; however in Synchromate-B chin-resting and sniffing was symmetrical with their peak values at 12-14 h on the 2nd day after removal of hormone. There was a tendency to increase vaginal swelling with time of synchronized estrus in the both treatments. Incidence of mucus discharge with Synchromate-B was slightly higher than with PRIDs.

344. **Reproductive Practices and Results in Dairies Using Owner or Professional Inseminators**. E.C. Schermerhorn, R.H. Foote, S.K. Newman and R.D. Smith

In study 1, information on breeding practices of owner-inseminators in 234 herds of Holstein cattle was obtained by mail survey. Improved timing of insemination during estrus and convenience were reasons dairy farmers gave for performing insemination themselves. Many also used natural service bulls or professional inseminators to breed some cows. Cows in less than half of the herds were observed specifically for estrus at scheduled intervals. Cows were frequently inseminated more than once per estrus; 65% of the inseminations were within 2 h of milking. Mean services per conception for cows in 135 herds responding to a second questionnaire were 1.7 and mean calving interval was 12.8 mo. Study 2 involved only herds and associated records in Dairy Herd Improvement. Herds using only professional inseminators were randomly selected so as to match similar owner-serviced responding and

nonresponding tested herds in Study 1. Reproductive efficiency varied greatly among herds with a slight decrease as the size of the herd increased. The 1.70 services per conception achieved by professional technicians was only slightly better than 1.74 services per conception for direct service personnel. Dairy farmers in large herds who did not respond to a special questionnaire handled more cows per worker, had more services per conception, and had a slightly longer calving interval.

345. **Effect of Oxytocin, Prostaglandin $F_2\alpha$ and Reproductive Tract Manipulations on Uterine Contractility in Holstein Cows on Days 0 and 7 of the Estrous Cycle.** M.D. Cooper and R.H. Foote

The influence of various treatments on cattle at the time of natural estrus vs estrus induced by prostaglandin $F_2\alpha$ ($PGF_2\alpha$) or at d 7 of the estrous cycle were studied; the latter is when embryo transfer often is performed. Eight lactating and 25 nonlactating, normal cycling cows were tested many times while in estrus and 7 d after estrus. A balloon was positioned in the body of the uterus to record changes in intrauterine pressure following clitoral massage, cervical massage, vaginal distention, electrical stimulation of the cervix and vagina, tailhead rubbing, udder massage and the injection of oxytocin or $PGF_2\alpha$. Blood oxytocin and intramammary pressure were measured. There were no differences between cows in estrus spontaneously or those induced, so these groups were combined. Intravenous oxytocin injections of .5, 1, 2, 4 and 15 IU increased blood levels of oxytocin. Intramam-mary pressure was increased by all oxytocin doses, but $\geq$ 2 IU was required to cause substantial changes in uterine contractions. As expected, the peak contractions during control periods for cows in estrus were high, averaging 31 mm Hg vs 11 mm Hg on d 7. None of the manipulations of the reproductive organs caused detectable oxytocin release or increases in intra-mammary pressure, contrasting to responses to massage of the udder. Clitoral massage increased peak uterine pressure by 32 to 60% in four experiments. It did not induce luteinizing hormone release. The contraction was immediate, was not sustained and could be obtained repeatedly, suggesting a reflex response. Treatment with $PGF_2\alpha$ increased intramammary pressure. It increased uterine pressure on d 7, but had no effect at estrus. This contrasts with injected oxytocin, which resulted in the highest amplitude for cows in estrus, although the response on d 7 was greater in proportion to the low activity in controls at that time. It is concluded that manual manipulation of the reproductive tract (other than clitoral massage) has little effect on uterine contractility.

346. **Fertility of Cooled and Frozen Rabbit Sperm Measured by Competitive Fertilization.** J.J. Parrish and R.H. Foote

The fertility of rabbit sperm that had been cooled to 5°C or frozen and thawed was determined by competitive fertilization. Treatments were identified by labeling sperm either with fluorescein isothiocyanate (FITC) or tetramethylrhodamine B isothiocyanate (TRITC). Sperm from different treatments were mixed and used in a competitive insemination experiment. Does were inseminated 5, 10 or 15 h prior to ovulation. Time of ovulation was controlled by injections of luteinizing hormone. The functional sperm transport, as determined by the number of sperm transported to the site of fertilization and capable of fertilizing oocytes, was estimated by counting the total number of differently stained sperm that surrounded or fertilized each oocyte. The fertility of sperm cooled to 5°C was not affected ($p>0.05$) as compared to fertility of uncooled sperm. Functional sperm transport at all times of insemination and fertilization ratio at insemination 10 or 15 h before ovulation were reduced ($p<0.05$) for frozen-thawed vs. cooled sperm. No difference in fertilization ratio ($p>0.05$) occurred, however, when does were inseminated 5 h before ovulation. While sperm survival and capacitation time appeared to play roles in fertility of frozen-thawed sperm, the most important factor was reduced functional sperm transport. However, fertility of frozen-thawed sperm was improved when the time from insemination to ovulation was reduced.

347. **Effect of Dilauroylphosphatidylcholine on the Acrosome Reaction and Subsequent Penetration of Bull Spermatozoa into Zona-Free Hamster Eggs.**
J.K. Graham, R.H. Foote and J.J. Parrish

Incubation of bull sperm with liposomes made with phosphatidylcholine (PC) containing fatty acyl chains of either 10 (PC10) or 12 (PC12) carbons resulted in greater than 90% of the sperm exhibiting an acrosome reaction (AR) within 15 min. Liposomes of PC10 rapidly destroyed sperm motility while PC12 acrosome-reacted sperm remained motile for several h. Liposomes of PC with ≥ 14-carbon fatty acyl chains had no effect on the AR or motility of sperm. The AR was not induced by lysophospholipids, because lysophospholipids were not detected in the PC liposomes, and the AR did not occur when lysophospholipids were tested at the same concentration as PC12. The concentration of PC12 necessary to induce maximal numbers of acrosome-reacted sperm varied with the concentration of sperm. The effect of PC12 on sperm also varied with the ratio of live to dead sperm in a sample. When 3×10^6 bull sperm/ml were treated with 0, 10, 20, and 30 μM PC12 for 7 min prior to addition to zona-free hamster eggs, 6, 6, 98, and 77% of the eggs were penetrated, respectively. Lipid concentrations of 0 μM and 10 μM did not affect the AR, whereas higher levels induced the AR in sperm. This procedure can quickly provide acrosome-reacted bull sperm for use with various in vitro fertilization procedures and for assessment of male fertility.

348. **Dose-Response Shift in the Ability of Gilts to Remain Pregnant Following Exogenous Estradiol-17β Exposure.**
W.F. Pope, M.S. Lawyer, W.R. Butler, R.H. Foote and N.L. First

Sixty mated gilts were assigned to a 2 × 6 factorial arrangement (n = 5) of day of injection (d 9 and 10 vs 12 and 13; d 0 = first day of estrus) and dose of estradiol-17β (0, .125, .5, 2, 8 and 32 $mg \cdot gilt^{-1} \cdot d^{-1}$). Gilts were slaughtered on Day 30, and percent embryonic survival was calculated. A 64-fold shift in the dose-response curve for percent embryonic survival illustrated that the adverse effects of exogenous estradiol-17β were less when administered on d 12 and 13 as compared with d 9 and 10 (day × dose, $P<.01$). This experiment demonstrated that the uterine-embryonic environment of d 12 and 13 pregnant gilts was more tolerant of exogenous estrogen alterations than that of d 9 and 10 pregnant gilts.

349. **Superovulation Practices and Related Current Research.** R.H. Foote

There are many factors that can cause variable superovulatory response. To minimize sources over which the operator has some control there are at least 9 items to pay strict attention to. These are as follows: 1) use ideal donor cows; 2) donor cows properly fed and managed for good reproduction; 3) reproductive status of the donor properly diagnosed to initiate treatment; 4) proper administration of treatment to induce superovulation in each cow; 5) use of highly fertile semen; 6) insemination properly timed and performed; 7) excellent techniques for recovering and handling embryos; 8) effective techniques to identify fully functional embryos; and 9) excellent transfer techniques to fully competent recipients. It is not possible to completely control all the steps listed. Particularly animals vary greatly, even those that appear to be of similar age, breed and in good health. Different batches of PMSG, FSH, and FSH:LH vary. Once an organization has a batch that works well they should obtain several years of supply, if possible. Current experimental techniques of using cloned hormones, inhibin, follicle growth inhibitors, embryo sexing and cloning are discussed.

350. **Effect of Several Lipids, Fatty Acyl Chain Length, and Degree of Unsaturation on the Motility of Bull Spermatozoa after Cold Shock and Freezing.** J.K. Graham and R.H. Foote

Diluents containing sonicated liposomes of purified phosphatidylserine (PS), phosphatidylcholine (PC) with varying fatty acyl chain lengths and double bonds and cholesterol (CH) alone or in combination, or egg yolk lecithin were evaluated for protection of bull sperm during cold shock produced

by rapid cooling from 25 to 0°C during freezing and thawing. Bull semen was washed twice and diluted to 50×10^6 sperm/ml in diluents containing no lipid, 0.5 or 5 mM sonicated lipid or 20% egg yolk and plunged into ice water to cold shock the sperm. Sperm so treated were frozen using conventional methods. The percentage of progressively motile sperm (MS) was estimated prior to cooling, after cold shock, and after freezing and thawing. Lipids with fatty acyl chains of less than 12 carbons were toxic to sperm cells. Phosphatidylserine alone or in combination with PC or CH, but not PC or CH alone, protected sperm from cold shock as well as did egg yolk lecithin liposomes or egg yolk. Liposomes of PS/PC or PS/CH were not better than PS in protecting sperm from cold shock. Lipid concentrations of 0.5 mM were more effective than liposomes at 5 mM in protecting sperm during freezing and thawing. During freezing, PS alone or in combination with PC partially protected sperm, but only PS/CH was as effective as egg yolk in protecting sperm from freeze-thaw damage. It is concluded that defined diluents, particularly those containing PS, may be useful in studies of cryobiology of spermatozoa.

351. **Dilauroylphosphatidylcholine Liposome Effects on the Acrosome Reaction and In Vitro Penetration of Zona-Free Hamster Eggs by Bull Sperm: I. A Fertility Assay for Fresh Semen.** J.K. Graham and R.H. Foote

Fresh sperm from five bulls having nonreturn rates ranging from 48% to 77% were treated with 15.7, 21.0, 26.2, 31.5, 36.7, and 42.0 μM dilauroylphosphatidylcholine (PC12) to induce the sperm acrosome reaction (AR). Treated sperm were incubated 3 hr with zona-free hamster eggs at 39°C prior to fixation. The eggs were then stained and examined for sperm penetration. Differences in the percentages of motile sperm and of sperm exhibiting an AR among bulls were small when compared on a within-liposome-concentration basis. Increasing the PC12 concentration from 15.7 μM to 42.0 μM increased the percentage of sperm exhibiting an AR for all bulls. At the lowest lipid concentration (15.7 μM), the percentage of eggs penetrated by sperm from the five bulls was 6% to 36%, with 0% in controls. When sperm were incubated with increasing lipid concentrations, the egg penetration rate increased to over 80%, and the total number of sperm increased to over 100 per 36 eggs in each treatment for every bull. These penetration rates decreased at the highest lipid concentration. A correlation between the PC12 concentration maximizing egg penetration and the nonreturn rate of -.63 was found. The correlation between the PC12 concentration maximizing the total number of penetrated sperm per treatment and the bull nonreturn rate was -.96. It was concluded that PC 12 liposomes induce the AR in bull spermatozoa, which enables them to penetrate zona-free hamster eggs. High fertility bulls required less lipid to induce the AR than did lower fertility bulls. Consequently, this assay of fresh semen could provide a laboratory method to estimate the fertility of a bull.

352. **Dilauroylphosphatidylcholine Liposome Effects on the Acrosome Reaction and In Vitro Penetration of Zona-Free Hamster Eggs by Bull Sperm: II. A Fertility Assay for Frozen-Thawed Semen.** J.K. Graham and R.H. Foote

Frozen-thawed sperm from five bulls with fertility rates ranging from 48% to 77% were treated with seven concentrations of dilauroylphosphatidylcholine (PC12) liposomes to induce an acrosome reaction (AR) that enabled sperm to penetrate eggs. Treated sperm were incubated with liposomes for 7 min prior to insemination of zona-free hamster eggs in vitro. Sperm and eggs were incubated 3 hr at 39°C prior to fixation, staining, and examination for sperm penetration and nuclear decondensation. The percentage of motile sperm immediately after thawing as well as after treatment with liposomes had a low correlation with sire fertility ($r=.39$ and $\leq .63$, respectively). The percentage of sperm exhibiting an AR was more highly correlated with fertility ($r \leq -.85$). Similar correlations were found between fertility and the penetration rates of zona-free hamster eggs or the total number of penetrating sperm. When data for two high and for two lower fertility bulls were each grouped to increase information per data point the correlation between the PC12 concentration giving the maximum proportion of eggs

penetrated and fertility was r=.92 (P<.05). The correlation between the PC12 concentration producing the most total sperm penetrating the eggs and fertility was r=.97 (P<.05). It was concluded that PC 12 liposomes induced an AR in bull sperm frozen-thawed in egg yolk extender. Frozen-thawed sperm from low fertility bulls require less PC12 to induce the AR and to penetrate zona-free hamster eggs than do sperm from higher fertility bulls. These differences in lipid requirements may help to provide a quick, direct laboratory assay method to estimate the fertility of frozen bull semen.

353. **Effect of Donor-Embryo-Recipient Interactions on Pregnancy Rate in a Large-Scale Bovine Embryo Transfer Program.** J.F. Hasler, A.D. McCauley, W.F. Lathrop and R.H. Foote

The effects on pregnancy rate of 23 factors relating to time, embryos, donors, and recipients in a commercial bovine embryo transfer program were analyzed retrospectively. Over 6½ years, embryos were recovered on 1625 occasions from 825 different cows and heifers. Unfrozen embryos were transferred surgically to virgin Holstein heifers of a relatively uniform size and age, maintained under a single management system in the state of Pennsylvania in the USA. Transfer of 7652 embryos resulted in an average pregnancy rate of 71.3% with small, but significant differences among years and months. There were no differences among years, however, in the mean quality, stage, or age of the embryos transferred, or in the mean age of donors, number of embryos recovered, or the mean number of times that a recipient was used. The pregnancy rate was not affected by breed, fertility or lactational status of the donor, or day of the estrous cycle on which superovulatory treatment began; however, embryos from cows over 15 years of age resulted in lower pregnancy rates. While there was no influence of total number of ova recovered or percent fertilization, the number of fertilized ova had a small effect. Moreover, there were differences due to the morphological quality and state of development of the embryos, and due to the interval from the donor's estrus to recovery. Pregnancy rates were lower with embryos recovered 9 or more days after estrus. Although duration of culture had no effect, Ham's F-10 was superior to modified PBS. Although the interval from treatment to estrus as well as the number of previous estrus synchronization treatments had no influence, recipients induced with prostaglandin to be in estrous-cycle synchrony with the donor had a distinctly higher pregnancy rate than those in natural synchrony. Estrous synchrony between the donor and recipient and the interval from the recipient's estrus to transfer affected the pregnancy rate, but the side and quality of the corpus luteum of the recipient and the number of times that a heifer had been used as a recipient did not. Interactions between the following factors influenced the pregnancy rate: 1) day of flush and embryo quality, 2) day of flush and stage of development of the embryo, 3) embryo quality and estrous synchrony, 4) stage of embryonic development and interval from the recipient's estrus to transfer, and 5) stage of embryonic development and estrous synchrony. The paper contains 12 tables and six figures.

354. **Relationship of Sire Fertility to Acrosome-Reacted Motile Spermatozoa After Treatment with Liposomes.** A.P. Davis and R.H. Foote

The relationship between bull nonreturn rate and percent acrosome-reacted and percent motile sperm treated with 24.8, 34.8, and 44.8 uM dilauroylphosphatidylcholine liposomes was examined. Frozen-thawed spermatozoa from six bulls, with fertility ranging from 64 to 78%, were incubated at 39°C with the liposomes for 0, 7 and 15 min. Bulls differed in percentages of motile sperm, and this decreased over time, especially at the highest concentration of lipids (44.8 uM). Bulls differed initially in their percentage of sperm with intact acrosomes, and in their linear rate of increase in percent acrosome-reacted sperm. At 24.8 uM of lipid the rate of increase was nearly linear, whereas at higher concentrations the maximum acrosome reaction was approached during short incubations. The variables a) decline in percent motile sperm and b) increase in percent acrosome-reacted sperm, used singly, predicted fertility most accurately when measured on sperm treated with 44.8 uM and 24.8 uM of lipids, respectively. However, the highest relationship ($R^2 \geq .97$) was established by combining the acrosome and motility measurements of sperm exposed to 34.8 uM PC12. Thus, potential fertility of bulls may be predicted by applying this assay.

355. **In Vitro Fertilization and Embryo Transfer in Domestic Animals: Applications in Animals and Implications for Humans**. R.H. Foote

Extensive research has been conducted in domestic animals, particularly in cattle, in the reproductive technologies of sperm handling, capacitation and acrosome reaction, superovulation, embryo handling, sexing, bisection, cryopreservation and transfer. Because of the economic importance of cattle these technologies have been tested and improved under clinical conditions. The results of employing these procedures are available on tens of thousands of pregnancies and offspring. This information has implications in applying some of the same technologies in human reproduction. The large number of normal progeny produced in cattle after a long prenatal development period similar to humans provides some assurance that these technologies, carefully applied, are safe. The basis for these conclusions are documented in the references.

356. **Penetration of Zona-Free Hamster Eggs by Liposome-Treated Sperm from the Bull, Ram, Stallion, and Boar.**
J. K. Graham, R.H. Foote and S.R. Hough

Spermatozoa from each of four rams, four stallions, and three boars (six semen samples) were treated with dilauroylphosphatidylcholine (PC12) liposomes and compared with control bull sperm to induce the acrosome reaction (AR) and study possible penetration of the sperm into zona-free hamster eggs. Diluted sperm were incubated with several concentrations of PC12 for 7 min at 39°C prior to insemination of the hamster eggs in vitro. The sperm from the bull were diluted to 10^6 cells/ml, as previously studied. Sperm from the ram, stallion, and boar were diluted to 6×10^6 and 20×10^6 cells/ml. After addition to the eggs, the sperm concentration was reduced by 75 percent. Inseminated eggs were incubated with sperm for 3 h at 39°C prior to being fixed, stained, and observed for sperm penetration. At an initial concentration of 6×10^6 cells/ml, bull sperm treated with 36.7 μM PC12 achieved an egg penetration rate of 92%, whereas under nearly identical conditions stallion spermatozoa achieved only 54% egg penetration. Under similar conditions, ram spermatozoa failed to penetrate eggs, but when the initial sperm concentration was increased to 20×10^6 cells/ml, sperm incubated with 51.1 μM PC12 achieved 52% egg penetration. Boar spermatozoa treated with PC12 at either sperm concentration failed to exhibit an AR or penetrate hamster eggs. In general, as PC12 concentration increased the percentage of sperm with an AR increased and sperm motility decreased. It is concluded that 1) PC12 liposomes are effective in inducing the AR in sperm from the bull, ram, and stallion, but under conditions tested are ineffective with boar sperm; 2) these acrosome-reacted spermatozoa will penetrate zona-free hamster eggs and so may provide insight into the fertilizing ability of sperm from individual males of several species.

357. **Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-treated Egg Yolk and Whole Egg Extenders.**
J. Arriola and R.H. Foote

Bull sperm in Experiment I were added to a standard egg yolk-tris extender containing 0, .25, .5, 1.0, 1.5 and 2.0% (vol/vol) of the detergent mixture, sodium and triethanolamine lauryl sulfate. Glycerol was added in one step to the initial extender or in three steps after cooling semen to 4°C. The extended semen was packaged in .5 ml French straws and frozen over static nitrogen vapor. Thawing was done at 4, 30 and 60°C. There was little difference due to the method of adding glycerol provided detergent was present. The percentages of motile sperm after freezing in the six concentrations of detergent, in ascending order, were 31, 45, 52, 51, 48, and 36. The percentages of motile sperm following thawing at 4, 30 and 60°C were 36.5, 46.0 and 48.6. Acrosome retention also was better preserved with the higher thaw temperatures. A second experiment, similar in design to the first one, was conducted with whole egg-tris. The best results were in whole egg-tris containing .125% detergent where post-thaw motile sperm of 70.4% greatly exceeded 30.7% without sodium and triethanolamine lauryl sulfate. Fertility of sperm frozen in yolk-tris-detergent was equivalent to whole milk used as a control.

358. **Quantification of Bovine Sperm Separation by a Swim-up Method. Relationship to Sperm Motility, Integrity of Acrosomes, Sperm Migration in Polyacrylamide Gel and Fertility**. J.J. Parrish and R.H. Foote

The number of bovine spermatozoa separated in a swim-up procedure was quantified using an electronic cell counter. In an initial test of the swim-up procedure, non-frozen sperm samples with different ratios of live to dead cells were prepared and tested for the number of spermatozoa counted by the swim-up procedure. In ejaculates from six bulls, the number of spermatozoa swimming up was related to the number of live cells present (R^2=0.97). Next, sperm quality of frozen-thawed semen immediately after thawing was measured at 37 C by swim-up sperm count, sperm motility, spermatozoa with an intact acrosome and migration in polyacrylamide gel and then compared with the fertility of the semen used for artificial insemination. Twenty-nine ejaculates of frozen-thawed semen from 11 bulls were evaluated. Correlations with fertility were highest on an ejaculate basis for motility (r=0.41, P=0.05) and for swim-up sperm count (r=0.35, P=0.06). On a bull basis, swim-up sperm count had the highest correlation with fertility (r=0.59, P=0.06). In a multiple regression model to predict male fertility that included all described measures of semen quality, a R^2 value of 0.69 was obtained. This is the first report showing that the ability of spermatozoa to swim out of a more dense medium (whole milk-glycerol extender) into culture media is quantitatively related to in vivo fertility.

359. **Reproductive Patterns in Cyclic and Pregnant Thyroidectomized Mares.** J.E. Lowe, R.H. Foote, B.H. Baldwin, R.B. Hillman and F.A. Kallfelz

Three Quarter-horse mares were thyroidectomized at about 1.5 years of age. Three similar intact mares served as controls. The study continued through two breeding seasons. The thyroidectomized mares were lethargic, rear limbs were oedematous and hair coats were coarse. They displayed a tranquil oestrous behaviour when exposed to a stallion and were only mildly antagonistic when not in oestrus. Length of oestrous cycles varied but most often they were 19-24 days long. Duration of oestrus (mean ± s.e.m.) for the control and the thyroidectomized mares was 12.9 ± 2.9 and 11.7 ± 2.2 days respectively (P>0.05). The peak of LH during oestrus was as high as 60 ng/ml blood serum with no difference between the two groups. Peak progestagen on Day 7 after ovulation for controls was 9.0 ± 1.6 ng/ml and was not different (P>0.05) from the peak of 6.3 ± 1.7 ng/ml for thyroidectomized mares on Day 8. Pregnancy was achieved in both groups of mares, including the use of semen from a thyroidectomized stallion. Thyroxine was detectable in one pregnant thyroidectomized mare during the last two-thirds of pregnancy only.

360. **Plasma and Uterine Progesterone and Embryo Survival in Rabbits Following Asynchronous Transfer to Unilaterally Ovariectomized Recipients.** M.G. Battista, W.F. Pope and R.H. Foote

Two experiments were conducted with a total of 176 rabbits to test the null hypotheses that 1) proximity of the corpora lutea to each uterine horn would not affect embryo survival and that 2) circulating and uterine progesterone content would not be affected by hemiovariectomy. Donor rabbits were inseminated and injected with luteinizing hormone, and 3.5, 4.0 and 4.5 d later embryos were transferred to Day 4 hemiovariectomized recipients. The synchronous Day 4 transfers were controls. There was no difference in survival of embryos transferred to the ipsilateral (intact) or contralateral (ovariectomized) side (P > 0.05). Also, there was a nonsignificant (P > 0.05) advantage in survival to Day 29 of embryos 4.5 d old when transferred (0.9 fetuses per side) in competition with 3.5-d-old embryos (0.6 fetuses per side). Progesterone content of the ipsilateral uterus was 59.7 ± 5.9 ng/g of wet tissue versus 62.2 ± 8.0 ng/g for the opposite side (P > 0.05). Likewise, the circulating concentrations of progesterone were similar in both pregnant and nonpregnant intact and hemiovariectomized rabbits, peaking around Day 12 (Day 0 = day of insemination). We concluded that, in contrast to cattle, there is no systemic gradient in progesterone and no local effect of the corpus luteum on embryo survival in rabbits.

361. Postthaw Motility, Acrosomal Integrity, and Fertility of Antibiotic-treated Frozen Bull Spermatozoa.

K. Ahmad, R.H. Foote and M. Kaproth

New antibiotics, including tiamulin, which has a high antimycoplasma activity, were tested to determine safe levels to add to bull semen frozen in straws. In Experiment 1, only raw semen (unextended) was treated with cephapirin. In Experiments 2 and 3, whole milk (WM), egg yolk-tris (EYT), and egg yolk-tris-sodium and triethanolamine lauryl sulfate (EYT-STLS) extenders were treated with an amikacin-cephapirin mixture and tiamulin. Penicillin and streptomycin were added to control extenders. After storage for 2 wk in liquid nitrogen, frozen semen was thawed at 37°C. Postthaw percentage of motile spermatozoa was affected by bulls, extenders, and antibiotic treatments. Addition to raw semen of 500 to 1000 ug/ml of cephapirin improved postthaw percentage of motile spermatozoa and percent of intact acrosomes. Fertility of 5796 cows with semen frozen in WM with added cephapirin resulted in a 72.1% 59-d nonreturn rate, compared with 72.5% for the control. Sperm motility and the proportion of sperm with normal acrosomes was unaffected by 500 ug/ml each of amikacin and cephapirin. However, the maximum innocuous concentration of tiamulin was 200 ug/ml. The addition of STLS detergent to EYT improved cryopreservation of spermatozoa.

362. Homospermic Versus Heterospermic Insemination of Zona-free Hamster Eggs to Assess Fertility of Fluorochrome-Labeled Acrosome-reacted Bull Spermatozoa.

A.P. Davis, J.K. Graham and R.H. Foote

Fresh spermatozoa from six bulls, with fertility ranging from 64 to 78%, (based upon 59-day nonreturn rates for 159,448 cows inseminated) were mixed with zona-free hamster eggs in 15 heterospermic pair inseminations. Five of the bulls were used in homospermic insemination studies. Prior to incubation, spermatozoa from each bull were labeled with contrasting fluorescent stains pretested for effects on spermatozoa. Equal numbers of spermatozoa were mixed and treated with liposomes of dilauroylphosphatidylcholine to induce the acrosome reaction. Spermatozoa from split ejaculates within a male competed against each other equally in the hamster egg test, indicating that the staining procedure did not affect egg penetration rates. Bulls differed in their egg penetration rates when their sperm were inseminated either homospermically or heterospermically, but the differences in the homospermic inseminations were not significantly correlated with sire fertility. The number and percentage of sperm which penetrated eggs, and the number of eggs penetrated in the heterospermic competitive tests were highly correlated with fertility ($r \geq 0.86$). Therefore, egg penetration rates from heterospermic inseminations appear to be valuable indicators of fertility and much more sensitive predictors than results from homospermic inseminations.

363. Antibiotics for Bull Semen Frozen in Milk and Egg Yolk Extenders.

K. Ahmad and R.H. Foote

In six experiments, gentamicin, clindamycin, amikacin, minocin, tylosin, and Linco-Spectin^R were tested for their effect on motility and fertility of frozen bull spermatozoa. Antibiotics were added to raw (unextended) semen and non-glycerol portions of the three commonly used semen extenders, whole milk, egg yolk-tris and egg yolk-citrate. Semen was frozen in .5 ml straws, stored in liquid nitrogen, and thawed at 37°C for 30 s. Postthaw percentage of motile spermatozoa in antibiotic-treated semen was different for individual bulls in each experiment. There was a small but significant depression in the percentage of motile sperm at the higher concentrations of clindamycin and Linco-Spectin^R tested in whole milk and with minocin in the two egg yolk extenders. In general, nonspermicidal concentrations of each antibiotic were established. Of five antibiotics tested for fertility (12,577 inseminations), only gentamicin reduced fertility on the basis of 59-d nonreturn rates.

364. Production of Identical Twin Rabbits by Micromanipulation of Embryos.

X. Yang and R.H. Foote

The research was conducted to improve micromanipulation procedures with rabbit embryos, including the production of genetically identical progeny. In the first experiment embryos in different

stages of development were used for micromanipulation by removing half of the blastomeres with a beveled aspirating pipet. Embryos 74 to 78 h postovulatory, in the late compacted morula or early blastocyst stage, were demonstrated to be best for micromanipulation. When embryos at this stage were halved 77% (64/83) developed into blastocysts compared to 78% (65/83) for the intact control. In the second experiment, the survival of demi-embryos in original versus foreign zonae were tested. Young born from the demi-embryos transferred within original zonae (33%) were not significantly different from those transferred in foreign zonae (24%). Significantly more offspring, however, were obtained from intact control embryos (58%, $P < 0.01$). In the third experiment, identical monozygotic twins were produced from Day 3 embryos following modification of the aspirating pipet by further sharpening it to a fine point with a microforge. Eleven young (34%) were obtained following microsurgery compared to 36% for intact control embryos transferred. Among the demi-embryos, a pair of albino and a pair of Dutch-belted young were identical twins.

365. Motility and Fertility of Bull Sperm Frozen-Thawed Differently in Egg Yolk and Milk Extenders Containing Detergent. R.H. Foote and J. Arriola

The purpose of this study was to examine survival and fertility of frozen bull sperm processed in several extenders containing a detergent mixture, sodium and triethanolamine lauryl sulfate. Addition of .5% vol/vol) detergent to whole milk was completely spermicidal. When 10% egg yolk (vol/vol) was included in the glycerol or nonglycerol fractions or both fractions of milk, sperm were equally well preserved in the presence of .5% detergent, and sperm survival following thawing at 4°C was dramatically improved by the detergent. This improved result with the 4°C thaw temperature also was obtained by adding .5% detergent to an egg yolk-tris extender. Thawing temperatures of 30 and 50°C (latter for 15 s) were generally superior to 4°C. A fertility trial with whole milk, whole milk-10% yolk, whole milk-10% yolk-.5% detergent, egg yolk-tris and egg yolk-tris-.5% detergent was conducted. The 59-day percent nonreturns, respectively, were 73.4, 65.8, 65.6, 69.5 and 73.4. Whole milk or egg yolk-tris plus detergent were equally superior to the other extenders for bull semen.

366. Lipids of Plasma Membrane and Outer Acrosomal Membrane from Bovine Spermatozoa. J.E. Parks, J. Arion and R.H. Foote

Plasma membrane (PM), primarily from the anterior sperm head, and outer acrosomal membrane (OAM) were isolated from ejaculated bovine spermatozoa and the major lipid classes were characterized. Whole sperm (WS) lipids were analyzed for comparison. PM was removed by nitrogen cavitation and purified by sucrose density gradient centrifugation. The OAM was removed by centrifugation through hyperosmotic sucrose and recovered by sucrose density gradient centrifugation. The PM contained primarily spherical vesicles from the region overlying the OAM and was enriched 9- and 13-fold in 5'-nucleotidase and alkaline phosphatase activity, respectively, compared to the original cavitate. The OAM was recovered as cap-like structures with associated ground substance. Protein, phospholipid and cholesterol (PR, PL and CH as $\mu g/5 \times 10^9$ sperm) were 300, 467 and 93 for PM and 276, 111 and 25 for OAM, respectively. Corresponding values for WS (mg/5 $\times 10^9$ sperm) were 31.4, 6.63 and 0.72. The PR/PL (w/w) and CH/PL (mol/mol) ratios were 0.66 and 0.38 for PM; 2.48 and 0.26 for OAM; and 4.39 and 0.22 for WS. Cholesterol was the only free sterol detected by gas liquid chromatography in WS, PM and OAM with traces of CH sulfate present in all three preparations. Glycolipid tentatively identified as sulfogalactolipid was detected by thin layer chromatography (TLC) in PM but not OAM. Phospholipid composition of WS and membranes was determined by TLC. Cardiolipin (3% of PL) was present in WS only. Choline, ethanolamine and inositol phosphoglycerides (CP, EP, PI, PIP, PIPP); sphingomyelin (SP); phosphatidylserine (PS); and lysophosphatidylcholine (LPC) were present in WS, PM and OAM. Approximately 50% of total PL was CP in all preparations; SP was 13% of PL in PM and 17% in OAM ($P<.05$); EP was 7% of PL in PM and 10% in OAM ($P<.05$). The differences in composition between PM and OAM is discussed with respect to capacitation and ability of sperm to undergo the acrosome reaction.

367. Is A Superovulated Oocyte Normal?

R.H. Foote and J.E. Ellington

Superovulatory success rates in cattle have improved only slightly in the last ten years. Basic to our goal of increasing numbers of transferable embryos, is an understanding of the effects of superovulation on oocyte development. This paper endeavors to review what is known of the quality or 'normalcy' of superovulated oocytes and hypothesizes protocols that may, with further understanding, yield higher numbers of viable embryos per superovulatory cycle. As a result of reviewing 52 papers several conclusions can be drawn. Superovulating cattle can cause abnormal follicular steroidogenesis, premature maturation and ovulation of oocytes, deviant systemic hormone profiles and other ovario-uterine environmental changes. In spite of these deviations from the physiologic norm, thousands of superovulated bovine oocytes go on to produce normal offspring each year. In order to advance beyond the "stalemate" which now exists in superovulating cattle, we need to develop a much deeper more precise knowledge of ovarian function and oocyte development. Superovulated oocytes mature in a different environment than unstimulated oocytes. As we begin to understand the interactions of this environment more fully it is hopeful that we can provide a more healthy "normal" development pattern for our future embryos.

368. An Enzyme-linked Immunoassay of Milk Progesterone as a Diagnostic Aid in Embryo Transfer Programs.

S.E. Allen and R.H. Foote

The objective of this study was to use an enzyme-linked immunosorbent assay to measure progesterone (P_4) in donor cows as an indicator of reproductive normality and response to superovulatory treatment. Forty-eight Holstein cows were superovulated and blood and milk samples were collected four times: 1) at initial follicle stimulating hormone treatment, 2) just prior to administration of prostaglandin F_2 alpha, 3) at estrus and 4) at the time of embryo recovery, 6 to 7 d after estrus. Cows were inseminated twice during estrus. The P_4 profiles (ng/ml) obtained at the four collections, were respectively, as follows: blood plasma, 2.4, 2.7, 0.2 and 11.2; defatted milk, analyzed by radioimmunoassay, 3.7, 3.8, 0.5 and 14.6; preserved whole milk analyzed by ELISA, 6.3, 7.8, 0.7 and 19.1; frozen whole milk analyzed by ELISA, 9.8, 10.0, 0.8 and 21.1. All four assays resulted in the expected pattern of P_4 values for most cows at the four stages sampled. Approximately 10% of the cows had P_4 concentrations outside of the expected range of values. A majority of these cows were aberrant, based upon behavior and palpation of the reproductive organs. The rapidly available P_4 results from the ELISA were useful in making appropriate decisions regarding management of donor cows.

369. Preservation and Fertility Prediction of Spermatozoa. R.H. Foote

This paper is a review, with 76 references, of sperm preservation at body temperature, ambient temperature, 0 to 5°C and at -196°C. The role of phospholipids and cholesterol and phase changes in their liquid-crystalline structure to gels during cooling as this relates to cold shock is discussed. Lipoproteins and phospholipids appear to bind, at least loosely, to sperm membranes and help to maintain integrity of the sperm membrane during phase transitions. Various theories and experimental results to explain freezing damage is discussed. many tests of semen quality to predict fertility are described. The importance of large numbers to reduce the binomial variance of pregnancy (0 or 1) must be stressed, as the standard deviation in fertility due to binomial variation when only 25 females per treatment are inseminated is ± 10%.

370. The Interaction Between Bluetongue Virus and Bovine Embryo Transfers and Also Susceptible Superovulated Donor Cows and Synchronized Recipient Heifers.

J. Gillespie, R. Foote, D. Schlafer,
S. Quick, E. Dougherty, E. Schiff,
P. Powers, C. Hall, S. Allen and H. Voss

Serotype 11 BTV does not penetrate the intact zona pellucida of 6-7 day old bovine embryos. The virus has a definite affinity for the surface of the zona as evidenced by marked virus clustering. This property makes the use of EM slightly more sensitive than cell culture for viral detection. The

presence of a high concentration of virus in the semen causes widespread dissemination of virus in various reproductive tissues in the donor cows including the ovary. Despite the detection of virus in the ovary at day 6, one cow had 7 normally-appearing embryos which were free of detectable virus. Acutely infected cows or exposed heifers, may have virus in some reproductive tissues and the blood but these limited animal trials did not establish whether fertilization was affected by the virus. The data generated in these experiments with BTV certainly implies the risk of transmitting BTV through the use of bovine embryo transfers with an intact zona pellucida is extremely low. To eliminate the risk entirely, it would be ideal to use bovine embryo transfers from donor cows that are in areas free of the disease.

371. **Related AI Ventures**. R.H. Foote

The use of human and artificial intelligence, computer networks and joining forces with DHIA, along with improving current techniques of harvesting and dispensing semen are all avenues for increasing the success of A.I. The AI organizations could choose to engage in other areas of breeding such as sexing and embryo transfer, embryo splitting and development of other animal biotechnologies. These can be combined into global programs of selecting, testing and selling germ plasm.

372. **Characterization of Short Luteal Cycles in the Early Post-Partum Period and Their Relation to Reproductive Performance of Dairy Cows**.
S. Eger, M. Shemesh, H. Schindler, S. Amir and R.H. Foote

The ovarian activity of primiparous and multiparous Israeli-Friesian dairy cows was monitored by progesterone (P_4) determination in fat-free milk twice weekly, beginning at calving and continuing until pregnancy was confirmed by palpation, but not later than 150 days post partum. The cows were observed for standing oestrus, thrice daily. Based on the distribution of progesterone concentrations concurrent with inseminations at behavioral oestrus resulting in conceptions or not, 0.5 ng/ml was taken as the dividing value between luteal and non-luteal origins of progesterone. Resumption of luteal activity occurred by 35 days post partum in 99% and 93% of the primiparous and multiparous cows, respectively. The rate of short luteal cycles ($\leq$ 11 days) was about 54% in the first, 24% in the second and 20% in the third-fifth luteal cycles post partum. The duration of first luteal cycles was associated with post partum interval. Neither length nor maximum progesterone concentration of the cycle preceding insemination was associated with conception rate. The results indicate that short luteal cycles, whenever they occur, can be attributed to a functional corpus luteum and not to ovary malfunction.

373. **Cholesterol Efflux From Bovine Sperm. I. Induction of the Acrosome Reaction With Lysophosphatidylcholine After Reducing Sperm Cholesterol**.
E. Ehrenwald, J.E. Parks and R.H. Foote

Methods were developed to quantitatively reduce the cholesterol (Chol)/phospholipid (PL) ratio of bovine sperm and to determine the effectiveness of this treatment in capacitating sperm. Washed sperm (2×10^8) were incubated in 1.0 ml of modified Tyrode's solution (TS) containing unilamellar liposomes of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and [^{14}C]-Chol (35:35:30 molar ratio, 300 nmol total PL). [^{3}H-]-triolein was included as a nonexchangeable marker. After 90 min at 39°C, a 13% net exchange of [^{14}C]-Chol from liposomes to sperm was observed (n = 4), and sperm motility was 80%. Sperm were then washed and 50 $\times 10^6$ sperm were incubated as before with PC/PE liposomes containing no Chol. After 90 min, sperm were separated from liposomes by centrifugation. Measurement of [^{14}C]-Chol in the liposomes (supernatant) and parallel gas chromatographic analysis of extracted, saponified liposomes (n = 4) indicated that 30% of sperm Chol was removed by this procedure. Chol efflux reduced sperm velocity by more than 50%. Sperm incubated with no liposomes (control), with liposomes containing Chol (+Chol), and with Chol-free liposomes (-Chol) were washed and resuspended in TS with 0.2% BSA and 30 µg lysophosphatidylcholine (PC)/mg bovine serum albumin (BSA). Percent sperm undergoing the acrosome reaction (AR) upon incubation with LPC-BSA was used as a measure of sperm capacitation. After 60 min of exposure to LPC-BSA at 39°C, the

mean (± SE) percent motile sperm for control, +Chol, and -Chol treatments was 57.0 ± 4.9, 60.0 ± 4.7, and 57.0 ± 6.8, respectively. Corresponding values for percent AR were 14.0 ± 3.4, 20.3 ± 4.4, and 39.7 ± 1.2. These results suggest that loss of Chol from bovine sperm may be an early step in sperm capacitation in this species.

374. **Cholesterol Efflux From Bovine Sperm: II. Effect of Reducing Sperm Cholesterol on Penetration of Zona-Free Hamster and In Vitro Matured Bovine Ova.**
E. Ehrenwald, J.E. Parks and R.H. Foote

Several reports have indicated that sperm capacitation includes loss of membrane cholesterol (Chol) with a concomitant decrease in the Chol-to-phospholipid (PL) ratio. Methods were developed for quantifiable removal of bovine sperm Chol, which predisposed sperm to induction of the acrosome reaction upon addition of lysophosphatidylcholine (LPC). The objective of this study was to evaluate the effect of Chol removal from bovine sperm on penetration of zona-free hamster and intact bovine ova in vitro. Washed ejaculated bovine sperm were incubated (2 h, 39°C) in a modified Tyrode's solution (TALP) containing 1) Chol-free liposomes (-Chol, 50 × 10^6 sperm and 600 nmol phospholipid/ml); 2) liposomes containing 30 mol% Chol (+Chol, 2 × 10^8 sperm and 300 nmol total lipid/ml); or 3) no liposomes (Control). We have previously shown that net Chol efflux from sperm is 31% of the total sperm Chol with -Chol liposomes and less than 1% with control media. Sperm were then washed twice and challenged with LPC bound to bovine serum albumin (BSA) using celite as a carrier. Treated sperm (25 × 10^6) were incubated immediately with either zona-free hamster ova (HO) or in vitro matured bovine ova (BO) in 50-µl droplets of TALP under medial fluid in an atmosphere of 5% CO_2 in air (3 h, 39°C). Ova were fixed in ethanol:acetic acid, stained with 1% orcein, and examined. Percent penetration (%P) of HO (X ± SEM) for 30 and 40 µg of LPC/mg BSA was 59.4 ± 5.3 and 82.9 ± 5.4; 38.5 ± 5.6 and 52.3 ± 4.7; and 16.0 ± 4.6 and 23.2 ± 5.6 for -Chol, Control, and +Chol treatments, respectively (n = 3). %P of BO (X ± SEM) for 30, 35, and 40 µg of LPC/mg BSA was 43.3 ± 5.4, 70.7 ± 7.5, and 81.5 ± 5.1 for -Chol and 16.4 ± 6.9, 36.2 ± 6.9, 36.2 ± 6.9, and 44.2 ± 8.6 for Control treatments, respectively (n = 3). In a second set of experiments %P of BO (X ± SEM) was 63.6 ± 6.8, 31.8 ± 4.9, and 10.5 ± 3.4 for -Chol, Control, and +Chol treatments, respectively, when 40 µg LPC/mg BSA was added (n = 2). %P and the number of sperm per fertilized ovum were consistently higher for the -Chol treatment for both HO and BO ($P < .01$). These results demonstrate that Chol removal from bovine sperm plays a role in bovine sperm capacitation.

375. **Factors Limiting Reproductive Efficiency in Selected Laboratory Animals.** R.H. Foote and E.W. Carney

The reproductive characteristics of the male and female mouse, rat, hamster and rabbit are reviewed. Obviously these species have developed remarkable reproductive capabilities as they have competed successfully in the wild. The total reproductive capacity of a particular species, breed, or strain of laboratory animals is a function of the number of young that can be produced per unit of time per female, the reproductive life span, and the generation interval. The number of young produced per unit of time is a function of litter size, gestation length, and the interval from parturition until the establishment of successive pregnancies. Litter size alone can be affected by the fertility of males, ovulation rate and fertility of the females, and embryo and fetal mortality. In turn, even under the controlled conditions of laboratory animals, the diet, lighting, housing, sanitation, handling and general reproductive management affect the ability of the animals to achieve their reproductive potential. Thus, the tables of reproductive characteristics and other data presented must be considered as approximations or representative of selected conditions under which the data were obtained. Under laboratory conditions various experimental treatments such as superovulation and embryo transfer in the females, semen collection and artificial insemination of controlled numbers of sperm, timed relative to ovulation, and many other useful studies can be performed.

376. **Production of Chimeric Rabbits from Morulae by a Simple Procedure.**
X. Yang and R.H. Foote

Experiments were conducted to develop a simple and reliable technique to produce chimeric rabbits from morula stage embryos. In Experiments 1 and 2, an in vitro test of viability was initially performed by culturing embryos to the blastocyst stage. Ninety-three percent of the "chimeric" embryos developed to the blastocyst stage compared to 94% for controls when embryos were manipulated soon after collection (Expt. 1). Eighty-one percent chimeric embryos and 78% control embryos developed to blastocyst stage when embryos were held at room temperature for 4 hr (Expt. 2). In Experiment 3, enough morula-stage embryos were available from true breeding Dutch-belted and albino rabbits to form potentially 67 diverse "color" pairs. These micromanipulated pairs of morulae were successfully combined to produce 64 chimeric embryos (96%, 64/67). They were transferred to the uteri of seven recipient does and three became pregnant producing 13 young. Four of the young exhibited substantial overt chimerism (31%) and one more was a possible chimera.

377. **Objective Analysis of Stallion Sperm Motility.** D.J. Jasko, T.V. Little, K. Smith, D.H. Lein and R.H. Foote

An image-analysis system utilizing a microcomputer and CellSoft® computer-assisted semen analysis software package was evaluated to assess stallion sperm motility characteristics. Analyses were performed at 37°C on a 6 µl drop of diluted semen placed on a glass slide and covered with an 18 mm^2 coverslip. Four groups of 25 cells each per slide, four slides per ejaculate and four ejaculates from each of three stallions were analyzed in a nested model. The percentage of motile sperm cells, mean velocity (µg/sec), mean linearity, and mean angular head displacement (µm) were measured. Statistical analysis of variance components showed that within ejaculates, more variation was accounted for in the differences among groups of 25 cells than among slides. Predicted standard deviations calculated for combinations of slides and groups of cells showed that a combination of two slides from which a total of 400 cells were analyzed resulted in a mean intra-assay coefficient of variation (CV) of 5.7% for the four measured variables. The following are individual coefficients of variation: percentage of motile cells (7.8%), mean velocity (6.4%), mean linearity (1.9%) and mean angular head displacement (6.6%). When ejaculate differences were included in the model and predicted standard deviations were calculated for a single ejaculate, the mean inter-assay CV was 9.2%. Mean velocity (6.4%) and mean linearity (4.7%) were more repeatable among ejaculates than either the percentage of motile sperm (14.4%) or angular head displacement (11.2%). It was concluded that this system is precise enough to determine differences in motility characteristics of stallion semen samples.

378. **Optimal Replication for Histometric Analyses of Testicular Function in Rats or Rabbits.** W.E. Berndtson, C. Neefus, R.H. Foote and R.P. Amann

Quantitative evaluations of testicular histology can provide sensitive endpoints for determining toxicity of chemicals to the male reproductive system. But, the numbers of observations per testis or number of animals per treatment group often are selected by tradition or availability, rather than on a statistical basis. Therefore, we studied the number of observations per male (sampling intensity) and number of animals per treatment (replication) needed to detect treatment effects of given magnitude, with predictable error probabilities, using data from Sprague-Dawley rats and Dutch-belted rabbits that received 0.0, 0.94, 1.88, 3.75, 7.5, or 15.0 mg/kg body wt of 1,2-dibromo-3-chloropropane (DBCP). Data for one testis from 102 rats and 34 rabbits were available. For each testis, observations included measurement of the minor diameter of 50 seminiferous tubules, counts of the number of leptotene primary spermatocytes per 250 Sertoli cells, and counts of spherical spermatids within 20 seminiferous tubular cross sections. Tabular data are presented showing optimal numbers of observations per testis and animals per treatment group as a function of the difference to be detected and selected probabilities for Type I and II errors. In general, precise assessments required far fewer observations per testis than are used routinely. However, due to

the inherent variability among animals, the number of animals required per treatment tended to be greater for experiments with the rabbit, and increased substantially for both species when detection of small differences had to be ensured. The data presented should enable investigators to design experiments of chose sensitivity and precision while making cost-effective use of animals and labor.

379. **In Vitro Capacitation and Fertilizing Ability of Ejaculated Rabbit Sperm Treated with Lysophosphatidylcholine**. C.K. Kim, K.S. Im, X. Zheng and R.H. Foote

Four experiments were replicated 1) to establish dose-response relationships between lysophosphatidylcholine (LPC), sperm motility and the acrosome reaction (AR), 2) to evaluate the influence of rabbit serum (RS) on these endpoints, 3) to compare buck differences in induction of the AR and 4) to examine fertilizing ability in vitro of sperm tested under the first three objectives. Semen was collected from Dutch-belted rabbits, washed once by centrifugation, resuspended and preincubated for 2 or 4 hr in a chemically defined medium (DM), DM plus 20% RS or BSA-free DM plus 20% RS at 37°C. At the end of preincubation LPC was added to the preincubated sperm at concentrations from 0 to 100 ng/ml. Sperm were examined .5 to 4 hr later for AR and sperm motility. For in vitro fertilization, sperm and ova were coincubated in DM up to 24 hr after insemination and in a more complex medium for another 24 hr. Addition of LPC to 4-hr preincubated sperm was more effective for inducing the AR than addition to 2-hr preincubated sperm. A significant increase ($P<.05$) in the AR occurred in 15 and 30 min following exposure to 100 and 80 ng of LPC per ml, respectively, but the higher concentration of LPC decreased sperm motility. Addition of 20% RS to DM with or without BSA surprisingly inhibited the AR but maintained sperm motility, as expected. Bucks differed ($P<.05$) in the initial percentage and the induced percentage of AR sperm. For the AR the optimal concentration of LPC per ml was 80 ng, but for in vitro fertilization 60 ng tended to be superior.

380. **Timed Breeding of Rabbits with Fresh and Frozen-Thawed Semen and Evidence of Acrosome Alteration Following Freezing and Thawing.** Y. Chen, X. Yang and R.H. Foote

The objectives of this research were to compare extenders for freezing rabbit spermatozoa, to study the effect of freezing on the acrosome and to test the possibility that an effect on the acrosome could alter the optimal time for insemination relative to expected ovulation time. In Experiment 1 the percentage of motile spermatozoa frozen in 0.5 ml straws was higher ($P<0.05$) following freezing and thawing in an egg yolk-acetamide extender than in an egg yolk extender with glycerol and dimethylsulfoxide (43% versus 32%, respectively). Only the yolk-acetamide extender was used subsequently. Freezing and thawing in this extender (Experiment 2) caused a small but significant ($P<0.05$) increase in spermatozoa with damaged or missing acrosomes. In experiment 3, 78 does were inseminated with fresh or frozen-thawed semen 0, 10, 15 or 20 h after being given an ovulating dose of a gonadotropin releasing hormone (Buserelin). Overall, there was no difference ($P>0.05$) in the pregnancy rate between does inseminated with fresh or frozen semen. The percentage of does pregnant (12 does per group) with fresh semen inseminated at 0 and 10 h after Buserelin was 75 and 75, and correspondingly for frozen-thawed semen was 100 and 42. Litter size and the number of young born was greater ($P<0.05$) for both semen treatment groups at 0 h (the normal time for insemination) than at 10 h, which was the expected time of ovulation. Thus, if freezing did reduce the time required for capacitation, it was not detectable by these experiments.

381. **Value of Testicular and Sperm Profiles in Optimizing Reproductive Success: Lessons Learned from Selective Breeding Programs of Domestic and Laboratory Animals.** R.H. Foote

The economic potential of the biotechnology of artificial insemination for genetic improvement

and disease control was recognized in the United States 50 years ago. To fully exploit this technology in dairy cattle, researchers worked closely with industry to study testicular function, best methods for harvesting and evaluating semen, techniques for preserving and inseminating sperm and finally methods for mass recording of fertility information. Methods of testicular and semen evaluation have been discovered which are highly correlated with other reproductive characteristics, including fertility. This has resulted in an unprecedented opportunity to study experimentally imposed conditions and naturally occurring environmental conditions upon reproductive efficiency in the male. The selection and testing of sires has resulted practically in the elimination of genes which produce lethal or severely depressed performance. The use of one male for the controlled insemination of many females also has facilitated studies on reproductive problems in the female. While less selective breeding programs have been conducted with the rabbit relative to reproductive function, the rabbit has been used extensively as a model for large domestic animals and humans. The rabbit is the smallest laboratory animal which can be trained easily to ejaculate into an artificial vagina and use the semen for insemination. This permits longitudinal and correlative studies to be performed relating testis function and epididymal transport to sperm output, semen quality and fertility. Spermatogenesis of both bulls and rabbits is sensitive to toxicants. The effects can be monitored by testicular biopsy and cytological evaluation of the testis, by semen collection and semen evaluation and by in vitro and in vivo fertility testing. Gross effects on the testis are detectable in the live animal by measuring testis size and by obtaining ultrasound profiles.

382. Buserelin in a Superovulatory Regimen for Holstein Cows. I. Pituitary and Ovarian Hormone Response in an Experimental Herd.

H.J. Voss, S.E. Allen, R.H. Foote, P. Im, C-K. Kim and P. Aquadro

Two experiments were conducted with frequent blood sampling in standard superovulatory regimens using follicle stimulating hormone (FSH) and prostaglandin F_2 alpha (PGF) to study the effects of the gonadotropin releasing hormone analog, Buserelin, on changes in FSH, luteinizing hormone (LH), progesterone (P_4) and estradiol (E_2). In Experiment I, Buserelin (20 ng) was administered to a total of 28 dry Holsteins. One group was treated with Buserelin 36 and 60 h after PGF administration, a second group was treated 60 h after PGF, and a third group served as the controls. In Experiment II, 30 dry Holsteins received Buserelin (10 ng). One group was treated 48 h after PGF, a second group at 54 h after PGF, a third group 24 h after estrus was first observed and a fourth group was a control. The general pattern of a decrease in P_4 following PGF, an increase in E_2, the onset of estrus, an LH peak, and finally, an increase in P_4 in superovulated cows was observed. Buserelin consistently produced a sharp LH peak at 36 h when given 36 h after PGF. At later intervals, it produced either a major or minor peak depending upon whether a spontaneous LH peak had already occurred. There was too much individual cow variation in the interval from PGF to a spontaneous LH peak to consistently induce a uniform LH peak, except when Buserelin was given 36 h after PGF, which may be early for normal oocyte maturation. There was no treatment effect on FSH, and embryo recovery rate was unaffected by treatment ($P>0.05$).

383. Buserelin in a Superovulatory Regimen for Holstein Cows: II. Yield and Quality of Embryos in Commercial Herds.

R.H. Foote, S.E. Allen and B. Henderson

The gonadotropin releasing hormone analog, Buserelin, was tested in a superovulatory regimen in cows by administering 8 ng of it at the following times: Group I (12 cows), 48 h after the first prostaglandin F_2 alpha (PGF) injection; Group II (11 cows), 54 h after PGF; Group III (10 cows), 24 h after standing estrus was first observed; and Group IV (12 cows), served as superovulated controls. The cows were lactating Holsteins between 45 and 143 d post partum, with at least one estrus prior to superovulation. The number of embryos collected from Groups I, II, III and IV 7 d after estrus averaged 4.5, 8.1, 6.4 and 5.6, respectively ($P>0.05$). The fertilization rate in the three groups receiving Buserelin was 83 versus 76% for controls ($P<0.10$). Blood and milk samples taken just before starting follicle stimulating hormone treatment, at the

expected estrus and at the time of embryo recovery were tested for progesterone concentration, and results from a rapid ELISA test were useful in identifying cows that a) were unsuitable for superovulation, b) should have been in estrus but were not observed standing and c) produced few, if any, embryos.

384. **A Spectrophotometric Procedure for the Determination of Objective Measurements of Equine Spermatozoan Motility**. D.J. Jasko, K. Smith, T.V. Little, D. Lein and R.H. Foote

A spectrophotometric procedure was developed and evaluated for the objective measurement of equine spermatozoan motility. A 100 µl sample of a sperm suspension, prepared by the removal of seminal plasma, was layered under a column of optically clear medium in a specially designed spectrophotometric cuvette maintained at 37°C. Changes in light transmittance above the interface of the sperm suspension and medium were recorded on chart paper. As sperm cells swam into the medium, a decrease in light transmittance was recorded as a deflection on the chart paper. Chart recordings were analyzed for the height (cm) and time (min) to the peak deflection. To standardize the procedure, a fixed number of cells ($1x10^9$) were used to prepare suspensions of $300x10^6$ cells/ml. Coefficients of variation for mean values obtained under these conditions after the evaluation of five ejaculates from a given stallion were estimated at between 10 and 12%. Correlations between swim-up measurements and computer-assisted semen analysis (0.57 to 0.66) demonstrated that the percentage of motile cells and mean velocity (µm/sec) of motile cells influenced swim-up measurements. Described here is a simple and inexpensive procedure to determine objective measurements of spermatozoan motility that may have application in semen evaluation and fertility testing in the stallion.

385. **The Technology and Costs of Deposits**. R.H. Foote

The human population is increasing. Food requirements will increase with an increasing emphasis on technology to provide for human needs. Some of the enhanced animal sources of food will come from genetic selection, and improved agronomic and grazing practices, for example. Genetic engineering to produce transgenic animals, as models for studying diseases, to produce pharmaceutical products and to produce selected lines of food producing animals will result in patented techniques to produce such animals. The animal products will be patented. The pros and cons of patenting and patent laws are discussed. Patent laws require not only a description, but also in the case of biological material that such material be available, i.e. a deposit. One of the special uses of the development of successful cryopreservation of embryos is that a transgenic mouse, for example, can be maintained by preserving a bank of embryos and depositing them at the American Type Culture collection in Rockville, MD 20852. The ability to reconstitute hundreds of strains of mice from frozen embryos has been demonstrated by the staff at the Roscoe B. Jackson Laboratories in Bar Harbor, ME.

386. **Fertility of Fresh and Frozen Rabbit Semen Inseminated at Different Times is Indicative of Male Differences in Capacitation Time**. Y. Chen, J. Li, M.E. Simkin, X. Yang and R.H. Foote

Some reports indicate that sperm from different males differ in capacitation time, and other reports suggest that freezing sperm may affect their capacitation time. These two variables were specifically studied in a fertility trial with 96 does inseminated with approximately 1.6 million motile fresh or frozen sperm from three different bucks, 15, 10, 5 and 0 h before expected ovulation. Fresh semen averaged 84% unstained (live) sperm and 88% had normal acrosomes; corresponding values for frozen sperm were 44 and 54%. Based upon pregnant does average litter size with fresh semen was 5.5 and with frozen semen was 4.8 ($p>0.05$), but overall does frozen semen produced fewer young, ($p<0.05$). Based upon all does and semen used, average litter size resulting from insemination at 15, 10, 5 and 0 h was 2.8, 4.2, 3.8 and 1.7, and for bucks was 4.0, 1.8 and 3.6. There was no interaction of type of semen with the other variables in the model ($p>0.05$). Bucks and time of insemination affected both the proportion of does pregnant and litter size ($p<0.01$).

A major interaction between bucks and time of insemination ($p<0.01$) was due apparently to both differential sperm survival and probable capacitation time among bucks. This major interaction should be considered in designing in vitro and in vivo fertility studies, and for selecting males for use in artificial insemination.

387. Comparison of Persistence of Seven Bovine Viruses on Bovine Embryos Following In Vitro Exposure.

J.H. Gillespie, D.H. Schlafer, R.H. Foote, S. Quick, E. Dougherty, E. Schiff and S. Allen

The ability of seven cytopathic strains of bovine viruses to adhere to the zona pellucida of six-to-eight day-old bovine embryos were compared. Embryos were exposed to virus by placing them either in virus suspensions or by culturing them on infected bovine turbinate cultures for 18-24 hour. After exposure to bovine virus diarrhea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), bluetongue virus (BTV), pseudorabies virus (PRV), vesicular stomatitis virus (VSV), parainfluenza 3 virus (PI_3), or bovine enterovirus virus (BEV), the embryos were tested for virus by culture in bovine turbinate cells and by morphological examination using electron microscopy (EM). A special technique to minimize loss of embryos processed for EM was developed. More embryos had viral particles on the surface of the zona pellucida after exposure to 18-24 hour infected cell cultures than did embryos exposed to viral culture suspensions. The most dramatic finding was that BTV adhered in large numbers to the surface of the zona pellucida of exposed embryos. IBRV, PRV, and VSV comprised an intermediate group, with virions occasionally detected on the surface of exposed embryos after 5 washes. Therefore, extensive washing is required. The PI_3 and BEV were easily removed from embryo-exposed virus by washing. BVD was difficult to identify morphologically, making assessment by EM unreliable. There was no evidence that any one of the seven viruses penetrated the intact zona pellucida. Using a micromanipulator, 42 embryos were also directly inoculated through the zona pellucida with ± 50 picoliters of virus inoculum or medium. Virus was detected by cell culture assay and by EM in nearly all embryos inoculated with 0.5 $TCID_{50}$ of IBRV or 5 $TCID_{50}$ of PRV.

388. Experimental Transmission of Bovine Viral Diseases by Insemination With Contaminated Semen or During Embryo Transfer.

D.H. Schlafer, J.H. Gillespie, R.H. Foote, S. Quick, N.N. Pennow, E.P. Dougherty, E.I. Schiff, S.E. Allen, P.A. Powers, C.E. Hall and H. Voss

Three experimental approaches were used to study transmission of blue tongue (BT), infectious bovine rhinotracheitis (IBR) and bovine virus diarrhoea (BVD) viruses. These were insemination with contaminated semen, experimental infection of embryo donor cows, or transfer of embryos experimentally exposed to virus in vitro to normal recipients. Parameters assessed included number and quality of embryos produced, virus detection (isolation and electron microscopy), serology and histopathology. All superovulated susceptible cows inseminated with semen containing blue tongue virus (BTV) (n=2) or infectious bovine rhinotracheitis virus (IBRV) (n=2) became infected. One cow inseminated with semen containing BTV produced seven virus-free seven-day-old embryos; the second cow failed to produce any embryos. One of two cows inseminated with semen containing IBRV produced two underdeveloped, virus-free embryos while no embryos were produced by the second cow. One of two cows inseminated with semen containing bovine viral diarrhoea virus (BVDV) became infected. Two poorly developed, virus-free seven-day-old embryos were recovered from one of these cows. Superovulated susceptible cows inoculated either intramuscularly with BTV (n=3) or intranasally with IBR virus (n=2) became infected. Virus was isolated from some tissues of two BTV-infected cows, neither of which produced embryos. A third BTV-infected cow produced two virus-free embryos collected at necropsy five days after inoculation. One of two cows experimentally infected with IBR virus, produced three embryos but virus was not detected either by electron microscopy (1 embryo) or in cell culture by cytopathic alterations (1 embryo). Virus was, however, detected in the embryo by using an IBR nucleic acid probe in material taken from a cell culture exposed to the embryo, emphasizing the

importance of using highly specific and sensitive probes. Six seven-day-old embryos that were exposed in vitro to BTV (n=3) or IBRV (n=3 cows) for 18-24 h and subsequently washed a maximum of three times were transferred to synchronized recipients. Blue tongue virus was isolated from the blood and vaginal swab samples taken at day seven from one heifer but not from two other. None of the three heifers became pregnant, but all three developed specific serum-neutralizing antibody titers. IBRV was isolated from the reproductive tissues and blood or spleen of two cows and nether cow produced embryos. No virus was recovered by cell culture from selected tissues of the third cow or from a liver of two viable 59-d-old fetuses recovered at necropsy. These latter findings underscore the critical IETS recommendations for 10 washes as currently recommended to prevent possible transmission of diseases to susceptible recipients.

389. **Bovine Oviductal Fluid Components and Their Potential Role in Sperm Cholesterol Efflux**. E. Ehrenwald, R.H. Foote and J.E. Parks

Bovine oviductal fluid (OF) was collected and analyzed throughout the estrous cycle, and the capacity of the protein and lipoprotein components to support cholesterol efflex from bovine sperm was evaluated. Blood was collected and assayed for progesterone (P_4) to monitor the estrous cycle. Protein and lipoprotein separation was achieved by density gradient centrifugation. Two major bands were identified. The first ($1.056 < \alpha_{20} < 1.140$ g/ml) corresponded to bovine and rabbit plasma high-density lipoprotein (HDL) based on distribution in the density gradient and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The second band ($1.235 < \alpha_{20} < 1.243$ g/ml) consisted predominantly of oviductal fluid albumin (OFA). Oviductal fluid protein concentration increased as serum P_4 decreased around the time of estrus. Mean OF protein concentration was 21.3 mg/ml when serum P_4 was lower than 0.5 ng/ml and 6.9 mg/ml when serum P_4 was greater than 0.5 ng/ml. An inverse log relationship was found between HDL protein concentration and serum P_4. Unesterified cholesterol (UC), cholesteryl ester, and phospholipid (PL) content of HDL for HDL protein concentrations of 3-56.1 μg/ml were 1.35-46.2 μg/ml, 1.91-44.48 μg/ml, and 1.69-59.8 μg/ml, respectively. Phosphatidylcholine and phosophatidylethanolamine were the major PLs present in the HDL fraction and their molar ratio (4:1 mol/mol) was relatively constant through the estrous cycle. The OFA fraction of the same samples accounted for more than 90% of total protein and for most of the variation in OF protein. To determine the ability of OF components to serve as sperm cholesterol acceptors, OF samples were incubated 1:1 (v/v) with and without 4×10^8 bovine sperm in 1.0 ml of modified Tyrode's solution and OF for 2 hr at 39°C. After incubation, HDL and OFA fractions were isolated and analyzed for changes in protein and lipid content. After OF, samples were incubated with sperm, an increase in UC was found in the HDL fractions. UC in HDL increased by 12.1 ± 1.0 μg/ml ($X \pm SE$) when serum P_4 was ≤ 0.5 ng/ml. For samples corresponding to higher serum P_4, the increase in UC was 3.60 ± 0.89 μg/ml. Values for UC in HDL were corrected for the contribution of UC from OFA of OF samples. Cholesterol efflux from sperm has been implicated in the process of sperm capacitation. These results indicate that HDL from OF is elevated during the follicular phase of the estrous cycle and can serve as an acceptor for bovine sperm cholesterol.

390. **Maturation, Fertilization and Development of Bovine Oocytes in Vitro Using TCM199 and a Simple Defined Medium with Co-Culture**. C.I. Kim, J.E. Ellington and R.H. Foote

Bovine oocytes obtained from ovarian follicles (2 to 5 mm in diameter) from slaughtered cattle were cultured in TCM199 with 10% heat-inactivated estrous cow serum (ECS) for 24 to 25 h at 39°C under 5% CO_2 in air. The 10% ECS was selected on the basis of preliminary studies in which in vitro fertilization rates of oocytes with 10, 15 and 20% ECS in the medium were 46, 30 and 31%, respectively ($P<0.05$). Of 120 oocytes cultured for 24 to 25 h, 63% were classified as being in Metaphase II. The rate of oocytes matured in vitro was 55% (69/125), the proportion of penetrated oocytes which contained male and female pronuclei was 94% (65/69), and the incidence of polyspermy was very low (0 to 9%). Of 122 oocytes fertilized in vitro and cultured

in TCM199 medium with 10% fetal bovine serum for 7 d, 53% were cleaved, but only 2% developed beyond the 16-cell block. However, in simple semi-defined Chatot-Ziomek-Bavister medium co-cultured with bovine oviduct epithelial cells (BOEC), 75% of 138 oocytes cleaved, and 38% of those which cleaved developed into morulae or blastocysts. The results of this study indicate that co-culture with BOEC exerted a pronounced beneficial effect on development of in vitro fertilized bovine oocytes through the 16-cell block. The medium required in the co-culture system was simple and semi-defined.

391. **Bovine 1-2-Cell Embryo Development Using a Simple Medium in Three Oviduct Epithelial Cell Co-culture Systems**. J.E. Ellington, E.W. Carney, P.B. Farrell, M.E. Simkin and R.H. Foote

These studies were designed to develop a co-culture system using a simple medium to promote 1-cell bovine embryo development through the 8-16-cell stage to morula and blastocyst stages. Monolayers for co-culture were prepared from bovine oviduct epithelial cells (BOEC). In-vivo fertilized 1-2-cell embryos and ova (384) were surgically collected from superovulated cows. In Experiment 1, embryos co-cultured in a simple glucose- and serum-free medium (CZB) developed with better embryo quality scores than embryos co-cultured in Ham's F10 with serum, and with a greater percentage developing past 8-16-cells than embryos co-cultured in CMRL-1066 with serum ($p<0.05$). In Experiment 2, embryos co-cultured with fresh BOEC monolayers averaged more ($p<0.05$) cells than did embryos in co-culture with frozen-thawed BOEC monolayers or in BOEC conditioned medium. Without glucose in the simple medium for the first 48 h of culture, more embryos blastulated ($p<0.01$) by Day 5.5 of culture (Day 6.5 of donor's estrous cycle), than embryos in the same medium with glucose present throughout. More embryos in Experiment 3 tended to hatch in BOEC co-culture ($p<0.10$) than in conditioned medium. These results show that a chemically simple medium with fresh BOEC monolayers can provide a significant benefit for co-culture of early bovine embryos.

392. **Calving Interval and Milk Production**. H. Schindler, S. Eger, S. Amir, E.C. Schermerhorn and R.H. Foote

This study was based upon 893 cows in the ARO research herd and a cooperating commercial herd with random assignment at the end of the preceding lactation to be first inseminated either 60 to 90 days or 120 to 150 days after calving. This resulted in different length of calving intervals and the objective was to determine this effect on milk production. All cows were production tested and milk yields in the groups were comparable. The differences in the cumulative yields between calving cycles of different length were not the same at each production interval. Their fluctuations were associated with the number of days dry and of days in milk at each period. During the greater part of the first year the two breeding groups had similar cumulative yields, but during the greater part of the 2nd year the early group had the higher yields. It was only at the end of the first and the 2nd year that the late group had an advantage. Thus the yield of the early group precedes the late group during the grater part of the 2nd year and accordingly advances income. In the non-responding fractions of the cooperating herd a selection process occurred eliminating cows with a marginal production which did not justify their further maintenance after a prolonged open period. Thus, among the cows with a prolonged open period only those with higher yields remained in the herd. Therefore, the late conception of these cows is not associated with high production, but rather is the result of a selection which allowed these cows to survive the culling process.

393. **Use of Reproductive Biotechnologies to Solve Problems of Reproduction of Farm Animals**. R.H. Foote

This paper integrates the hereditary (selection) and management practices that are key components of programs to obtain efficient reproduction of farm animals under different global conditions. The species discussed are dairy cattle, beef cattle, sheep and swine. The male component is discussed relative to heritability of testis size ($h^2 = .67$), and its high correlation with sperm output (0.85 to 0.95). In

addition to proper collection and handling of semen various experimental techniques, such as the induction of the acrosome reaction, are discussed. Likewise the key components in feeding and managing the female, careful detection of estrus, use of detection aids and synchronization of estrus are discussed. Selected use of embryo transfer on a global basis to move desirable germ plasm effectively, considering the climatic and nutritional conditions worldwide, are included. Control of disease is a major concern. From this synopsis the following general conclusions are these: 1) The most important biological component of any system of animal reproduction is the manager with knowledge, applied skills and ability to act. 2) Biological and bioengineering technologies are advancing the art of animal breeding and reproduction. 3) The need for knowledge and understanding is central to progress. New technological tools should be used in carefully designed experiments to explore the unknown and communication should be fostered so shared information is put into the hands of potential users.

394. **Improved Developmental Potential of Rabbit Oocytes Fertilized by Sperm Microinjection Into the Perivitelline Space Enlarged by Hypertonic Media**. X. Yang, J. Chen, Y-Q. Chen and R.H. Foote

The objectives of the present study were: a) to develop a simple and more efficient technique for sperm microinjection than is currently available, using the rabbit as a model and b) to evaluate the development of rabbit oocytes fertilized by single or multiple sperm microinjection. Hyperosmotic sucrose in phosphate-buffered saline (SPBS) was employed to dehydrate oocytes to increase the perivitelline space for sperm microinjection and prevent possible injury to the vitellus. In the first experiment, 58% (n=29) oocytes treated with 0.5 M SPBS developed to morulae following multiple sperm microinjection compared, respectively, to 47% (n=34) and 60% (n=15) for control IVF with or without sucrose exposure ($P>0.05$). Blastocyst development from microinjected oocytes, however, was much lower ($P<0.05$) than those of controls (14% vs 42% and 40%, respectively). Sham operation by puncturing the zona pellucida of the sucrose treated oocytes with the microinjection pipet did not increase parthenogenesis ($P>0.05$). In Experiment 2 a smaller size injection pipet and shorter sucrose exposure time after sperm microinjection resulted in 41% (n=42) of the oocytes developing into blastocysts for the microinjection group, whereas only 21% (n=24) developed to blastocysts in the control IVF group ($P<0.05$). When relatively older oocytes (17 hr post ovulation injection) were used to test if microinjection could reduce the time to fertilization and cleavage (Expt. 3), an average of 27% (n=63) blastocysts resulted from microinjection versus 0% (n=28) for the control IVF group.

395. **Development and Survival After Transfer of Cow Embryos Cultured from 1-2-cells to Morulae or Blastocysts in Rabbit Oviducts or in a Simple Medium with Bovine Oviduct Epithelial Cells**. J.E. Ellington, P.B. Farrell, M.E. Simkin, R.H. Foote, E.E. Goldman and A.B. McGrath

This study compares development of bovine 1-2-cell embryos in either bovine oviduct epithelial cell co-culture (BOEC) with a semi-defined medium, or in ligated oviducts of rabbits (RO). Embryos were surgically collected from superovulated donor cows 40-48 h after the beginning of estrus and randomly distributed between two treatments: 1) BOEC using a glucose- and serum-free simple medium (CZB) or 2) surgical transfer to RO. Embryos were cultured or incubated for 5 days. In Experiment 1, embryo quality scores and total numbers of cells in the two groups were compared. In Experiment 2, pairs of similarly treated morulae were transferred to each of 10 or 12 recipients in the RO and BOEC groups, respectively. Total cell counts per embryo in both groups averaged 52 ($P>0.05$), and the in-vitro BOEC culture system was equivalent to the RO in promoting embryo development for all characteristics measured. Embryo survival, as determined by ultrasound between days 39-43 post-oestrus, in 13 ideal recipients was 57% for embryos cultured in BOEC and 58% for embryos incubated in RO. None of the nine less desirable recipients were pregnant for either group. These data establish that a high rate of development from the zygote to morulae in BOEC with only a simple medium can produce normal pregnancy rates in cattle.

396. Survival of Bisected Rabbit Morulae Transferred to Synchronous and Asynchronous Recipients.

X. Yang and R.H. Foote

The rabbit was used as a model to test the concept that temporal asynchrony is required to establish physiological synchrony when embryos are bisected to produce demi-embryos. In preliminary studies with intact embryos it was confirmed that embryos harvested on Days 2, 3, 4 or 5 (Day 0 = day of breeding) can be transferred with ± 1 day of asynchrony to the uteri of recipient rabbits. Three experiments were conducted with bisected embryos. In Experiment 1, 192 bisected and 194 control Day 3 embryos were transferred to uteri of Day 2, 2.5 and 3 recipients (ovulated 0, 12, and 24 h after the donors) with 14% of the bisected and 39% of the intact embryos ($P<.05$), resulting in young. Only 4% (2/48) of the Day 3 bisected embryos versus 39% ($P<.05$) of the intact Day 3 embryos survived in the uterus of Day 2 recipients. In Experiment 2, Day 3 bisected and intact embryos were transferred to the oviducts of Days 3, 3.5 or 4 recipients, speculating that the oviduct might provide a more neutral environment than the uterus. However, embryo survival was very low, excepting for the intact embryos transferred to synchronized recipients (42% young born). In Experiment 3, 150 intact and 162 (81 pairs) bisected Day 3 embryos collected from uteri were transferred to uteri of Day 2.5, 3.0 and 3.5 recipient does. Significantly more pregnancies (100% vs 47%, $P<.01$) and young born (56% vs 19%, $P<.01$) resulted from intact embryos than from bisected embryos, irrespective of the uterine age. Asynchrony (± 0.5 days) did not affect pregnancy rate or young born from Day 3 intact embryos, but bisected embryos are more sensitive to uterine or oviductal environment than whole embryos as survival was higher ($P<.05$) when they were transferred to Day 2.5 recipients.

397. Co-culture of Rabbit One-cell Embryos With Rabbit Oviduct Epithelial Cells.

E.W. Carney, C. Tobback and R.H. Foote

Rabbit 1-cell embryos were co-cultured with rabbit oviduct epithelial cells (ROEC) to determine if ROEC can enhance embryo development in vitro. Primary ROEC were cultured in serum-free media at 39°C in a 5% CO_2/95% air environment. In Experiment 1, 1-cell embryos were co-cultured in Ham's F10 with freshly collected or 4-day-old cultures of ROEC seeded in plastic culture wells or on collagen membranes (Cellagen^R, ICN). One-cell embryos cultured without ROEC served as controls. After 65 h in culture, embryos were stained with Hoechst 33342 to determine the number of cells per embryo. Cell numbers were higher ($P<0.035$) in all co-culture treatments when compared to controls. Optimal development was obtained by co-culture with 4-day-old ROEC grown on plastic ($P<0.003$). In Experiment 2, Ham's F10, Medium 199, and CZB with glucose medium were compared for their ability to support embryo development in the presence or absence of 4-day-old ROEC grown on plastic. Cell number and the percentage of embryos becoming blastocysts were significantly ($P<0.001$) higher for embryos cultured in Medium 199 compared to the other media tested. In Medium 199, co-culture with ROEC resulted in only a slight non-significant increase in cell number over culture in Medium 199 alone (110 vs. 96 cells). However, the percentage of embryos reaching the blastocyst stage when co-cultured in Medium 199 with ROEC (49%) was nearly twice ($P<0.02$) that of embryos in Medium 199 without ROEC (26%). In Experiment 3, transfer of embryos cultured in Medium 199 with or without ROEC for 24 or 48 h resulted in no significant differences in post-transfer development. These data indicate a beneficial effect of ROEC on blastogenesis, and a salvage effect of ROEC on cell proliferation in embryos grown in a less supportive medium such as Ham's F10.

398. Determination of the Relationship Between Sperm Morphologic Classifications and Fertility in Stallions: 66 Cases (1987-1988).

D.J. Jasko, D.H. Lein and R.H. Foote

The analysis of breeding records and sperm morphologic classifications from ejaculated semen during 99 stallion seasons, over a 2-year period, revealed a significant correlation ($r = 0.34$, $P < 0.01$) between the percentage of morphologically normal sperm in ejaculates and the per cycle fertility estimate of the stallions studied. In addition, the

percentage of sperm classified as having major defects (abnormal heads, proximal droplets, and abnormal midpieces) was significantly inversely correlated ($r = -0.36$, $P < 0.01$) with the same fertility estimates. Multiple variable regression demonstrated that the variation in 2 morphologic features classified as major defects, abnormal heads, and proximal droplets, accounted for the largest amount of variation in fertility. It appears that in stallions, a large percentage of ejaculated sperm with major defects or other defects in combination with major defects is associated with a larger reduction in fertility than is associated with other defects.

399. **Effects of Superovulation, Embryo Recovery, Culture System and Embryo Transfer on Development of Rabbit Embryos In Vivo and In Vitro.** E.W. Carney and R.H. Foote

Uninterrupted development of rabbit embryos *in vivo* was studied in 7 superovulated and 7 normally ovulating (GnRH-treated) does, while another 7 does were superovulated and 1-cell embryos were collected from them at 19 h after LH to compare development in *vivo* and *in vitro*. Embryos from the last group were either cultured in the presence or absence of rabbit oviduct epithelial cells for 65 h in Medium 199, or were immediately transferred to recipients. At 84 h after LH or GnRH, blastomere number, embryo volume and stage of development were assessed for all embryos. Intrazonal embryo volumes were significantly reduced in embryos recovered from superovulated donors. Superovulation also had a negative effect on embryo cell numbers. However, this reduction was more severe in embryos remaining *in vivo* in superovulated donors until 84 h after LH than it was in embryos transferred to non-superovulated recipients at the 1-cell stage (19 h after LH). The embryo recovery procedure apparently caused little harm to the embryos, except that the mucin layer on flushed and immediately transferred embryos was significantly thinner than that of embryos residing continuously *in vivo*. Co-culture with rabbit oviduct epithelial cells resulted in improved development *in vitro*, but this development was still significantly retarded compared with embryos developing *in vivo*.

400. **A Comparison of Two Computer-Automated Semen Analysis Instruments for the Evaluation of Sperm Motion Characteristics in the Stallion.**
D.J. Jasko, D.H. Lein and R.H. Foote

Two commercially available computer-automated semen analysis instruments (CellSoft Automated Semen Analyzer and HTM-2000 Motion Analyzer) were compared for their ability to report similar results based on the analysis of pre-recorded video tapes of extended, motile stallion semen. The determinations of the percentage of motile cells by these instruments were more similar than the comparisons between subjective estimates and either instrument. However, mean values obtained from the same sample may still differ by as much as 30 percentage units between instruments. Instruments varied with regard to the determinations of mean sperm curvilinear velocity and sperm concentration, but mean sperm linearity determinations were similar between the instruments. We concluded that the determinations of sperm motion characteristics by subjective estimation, Cellsoft Automated Semen Analyzer, and HTM-2000 Motility Analyzer are often dissimilar, making direct comparisons of results difficult.

401. **Potential of Hypertonic Medium Treatment for Embryo Micromanipulation. I. Survival of Rabbit Embryos in Vitro and in Vivo Following Sucrose Treatment.**
X. Yang, Y. Chen, J. Chen and R.H. Foote

Rabbit zygotes and embryos were exposed to hypertonic sucrose in phosphate-buffered-saline (SPBS). In experiment 1, 144 zygotes shrank to 32 to 36% of their initial volume in 1.0 M SPBS within 30 min. Neither hypertonic treatment with 0.5 M or 1.0 M SPBS nor micropuncture of the zona pellucida after shrinkage affected embryo development into blastocysts in vitro (88%, 83% and 82%, respectively), compared to that of the controls (93%, $P > .05$). In experiment 2, 252 2- to 4-cell and 177 morula-stage embryos were exposed to isotonic PBS

control or 0.5 M, 1.0 M or 1.5 M SPBS for 30, 60, 90, 120 and 150 min before transfer to PBS (290 mOsm). Embryo development was significantly reduced ($P < .05$) when embryos were exposed in 0.5 M and 1.0 M SPBS for more than 60 min or in 1.5 M SPBS for more than 30 min. In experiment 3, morulae exposed for 60 min to 0.5 M or 1.0 M SPBS shrank to 37 to 39% or 32 to 35% of their initial volume and then expanded to 87 to 94% or 81 to 90% of their initial volume, respectively, after being returned to isotonic PBS for 60 min, but embryos in 1.5 M SPBS had erratic osmotic behavior. In experiment 4, 192 2- to 4-cell embryos exposed to 0.5 M SPBS for 0, 30 and 60 min before transfer to oviducts of recipients resulted in the production of 39%, 42% and 31% young, respectively ($P > .05$). Exposure of embryos to 0.5 M sucrose for 60 min does not compromise developmental potential and can simplify procedures.

402. **Potential of Hypertonic Medium Treatment for Embryo Micromanipulation. II. Assessment of Nuclear Transplantation Methodology, Isolation, Subzona Insertion and Electrofusion of Blastomeres to Intact or Functionally Enucleated Oocytes in Rabbits.**
X. Yang, L. Zhang, A. Kovács, C. Tobback and R.H. Foote

The objective of this research was to study efficiency of embryo development following transfer of blastomeres into the perivitelline space of oocytes. Single blastomeres from 8-, 16- and 32-cell embryos were obtained following mucin coat and zona pellucida removal by combined treatments with pronase and acidic phosphate-buffered saline (PBS, pH = 2.5). Blastomeres were separated by pipetting with a fire-polished micropipette following incubation in Ca^{++}-free PBS for 15 min at 39°C. This procedure resulted in over 97% blastomere separation. For ease of blastomere insertion, oocytes were placed in droplets of 0.5 M sucrose in PBS (SPBS) during micromanipulation. To functionally enucleate oocytes some were stained with Hoechst 33342 DNA stain and irradiated. A single 8- or 16-cell blastomere was aspirated into an injection pipette (35 nm or 25 nm at the tip, respectively) and inserted into the perivitelline space of an irradiated or non-irradiated oocyte, but not fused with the oocyte. This micromanipulation procedure did not affect development of individual blastomeres into blastocysts or trophectoderm vesicles when compared with cultured control single blastomeres ($P > .05$). When the inserted blastomere was induced to fuse with an intact non-irradiated oocyte under an electric field, 56 to 57% were fused and 39 to 45% of the fused and activated oocytes developed to morulae or blastocysts. When an inserted blastomere (from 8- to 32-cell embryos) was induced to fuse with a functionally enucleated oocyte treated by Hoechst 33342 staining, followed by washing and UV-light irradiation, 63 to 66% of them were fused, but only 15 to 22% developed to the morula or blastocyst stage. This research demonstrated that the use of hypertonic medium treated oocytes greatly improved the ease and success rate of blastomere subzona insertion, but the value of functionally enucleated oocytes as recipient cells for nuclear transfer requires further investigation.

403. **Co-culture of Rabbit 2-cell Embryos with Rabbit Oviduct Epithelial Cells and Other Somatic Cells.**
E.W. Carney, C. Tobback, J.E. Ellington and R.H. Foote

Rabbit 2-cell embryos were co-cultured in Basal Synthetic Medium II + 10% fetal bovine serum with one of the following: primary cultures of rabbit oviduct epithelial cells (ROEC), a rabbit kidney epithelioid cell line (RK13), a rabbit epidermal epithelioid cell line (Sf1), or a rabbit skin fibroblast-like cell line (RAB9). Embryos cultured in medium alone served as controls. After 4 d of culture at 39°C in 5% CO_2 in air, 77-93% of the rabbit embryos which were co-cultured with somatic cells had reached the blastocyst stage, and 60-76% were hatching through their zonae pellucidae. These percentages, however, were not significantly different ($P>.05$) from those of embryos in medium alone, of which 90% had reached the blastocyst stage and 83% had hatched. Mean intrazonal embryo diameters also did not differ significantly among treatments (239-302 nm). Bovine 1-8-cell embryos were

also co-cultured with ROEC. This stimulated 60% of these embryos to develop beyond the so-called "16-cell block" in vitro, whereas 0% of the embryos cultured in medium alone developed past this block. Evaluation of the ROEC cultures by light microscopy, immunocytochemistry, and gel electrophoretic analysis of conditioned medium, together with the positive results with bovine embryos, indicate that the ROEC culture partially simulates oviductal conditions in vivo. Therefore, our results suggest that oviduct epithelial cells may play a less pivotal role in regulating early development in the rabbit than in the cow. However, other factors besides those provided by the ROEC cultures must also be important for optimal development, as both rabbit and bovine embryos develop more slowly in co-culture than in vivo.

404. **1;16 Tandem Translocation with Trisomy 16 in a Brown Swiss Bull.**
A. Kovács, R.H. Foote and D.H. Lein

A new tandem translocation was found in a Brown Swiss bull. The abnormally long acrocentric autosome has no interstitial C-band and is a result of a 1;16 tandem fusion. The chromosome number is 60, including a pair of normal chromosomes 16 and thus a trisomy 16 is present. The karyotype of this bull is included in the paper.

405. **Comparison of Six-day Bovine Embryo Development in Uterine Tube (Oviduct) Epithelial Cell Co-culture Versus in Vivo Development in the Cow.**
J.E. Ellington, P.B. Farrell and R.H. Foote

A study was designed to evaluate and compare the appearance of embryos recovered from donor cows on Day 6 to embryos from in vivo fertilized cow zygotes developed to Day 6 on uterine tube (oviduct) epithelial cell co-culture using serum-free CZB medium. Embryo stage of development and quality score were assessed. Hoechst 33342 DNA stain was then used to determine the total number of blastomeres, the number of poor nuclei and the number of nuclei in mitosis. Mean cell counts did not differ for the 70 embryos evaluated in each group (65 cells in vivo, 61 cells in vitro). The percentage of transferable embryos (excellent, good or fair quality), in each group also did not differ (57% in vivo, 56% in vitro). There were no significant differences in any of the measured parameters. Our findings suggest that co-culture of in vivo produced cow zygotes can result in embryos comparable in developmental stage and quality to embryos developed in vivo in the cow for 6 d.

406. **A Procedure for Cryopreservation of Hamster Oocytes Yielding Highly Conserved Oocytes Suitable for Sperm Penetration Tests.**
C. Tobback, S. Hough and R.H. Foote

Hamsters were superovulated and their oocytes frozen in Dulbecco's phosphate-buffered saline (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Hyclone, Logan, UT or Gibco Laboratories) and 1.5 molar 1,2-propanediol. In several experiments 94% of the hamster oocytes survived freezing and were equivalent to fresh oocytes in sperm penetration. In routine use of the procedure described, also 94% of 1340 frozen-thawed oocytes were satisfactory for sperm penetration of several species. Frozen oocytes eliminate problems and costs of maintaining hamster colonies with variable responses on individual days.

407. **Method for Obtaining Bovine Zygotes Produced In Vivo.** J.E. Ellington, P.B. Farrell, M.E. Simkin, R.H. Foote

A superovulatory and surgical protocol was developed for recovery of bovine zygotes. Holstein cows and heifers were given follicle stimulating hormone and cloprostenol to induce superovulation. Surgical cannulation and lavage of the uterine tube was performed 40-48 hours after the start of standing estrus. In general, cows had more corpora hemorrhagica than heifers, but a higher percentage ($P<0.05$) of the recovered ova were infertile. Several heifers were subjected to the procedure twice and embryo recovery rates were equivalent both times.

408. **Improved Development of Rabbit One-cell Embryos to the Hatching Blastocyst Stage by Culture in a Defined, Protein-free Culture Medium**. E.W. Carney and R.H. Foote

In Exp. 1, Medium 199 and Medium RD (RPMI-1640 and Dulbecco's MEM, 1:1 v/v) were compared in a 2x2 factorial design by supplementing each with 15 mg bovine serum albumin (BSA)/ml or 1 mg polyvinyl alcohol (PVA)/ml. All media contained 5 ng insulin/ml, 5 ng transferrin/ml, 5 ng selenium/ml (ITS), and 10 ng epidermal growth factor (EGF)/ml. One-cell embryos were cultured at 39°C with 5% CO_2 in air for 65 h and then stained with Hoechst 33342 to determine blastomere number. Embryos in Medium 199 developed poorly ($P<0.001$) when PVA was used instead of BSA (30 *vs* 76 cells/embryo), but developed rapidly in Medium RD with PVA or BSA (118 and 121 cells). Similar results were obtained in Exp. 2 in BSA- and PVA-free medium. In Exp. 3, the development of 1-cell embryos after 65 h in unsupplemented (protein-free) Medium RD (68% blastocysts, 117 cells) did not differ ($P>0.37$) from that obtained using Medium RD with insulin, ITS or EGF alone. Culture in protein-free Medium RD resulted in 82% of the 1-cell embryos forming blastocysts and 40% hatching through the zona pellucida. A test of viability of 1-cell embryos cultured in this medium for 48 or 65 h and transferred to recipients resulted in 5/18 (28%) and 3/24 (12%) Day-15 viable fetuses. Blastocyst cell counts after culturing 1-cell embryos for 65 h in Medium RD indicated that cell division was more rapid than that obtained previously in this laboratory. This is the first report of rabbit embryo development from the 1-cell to the hatching blastocyst stage in a defined protein-free culture medium.

409. **The Repeatability and Effect of Season on Seminal Characteristics and Computer-aided Sperm Analysis in the Stallion.**
D.J. Jasko, D.H. Lein and R.H. Foote

The within-stallion repeatability and effect of season on sperm movement characteristics, determined by computer-aided sperm analysis (CASA), were compared with those of other seminal characteristics. The computer-aided determinations of sperm movement were more repeatable than the seminal characteristics of gel-free volume and sperm cell concentration based on coefficients of variation obtained from the analysis of multiple ejaculates from the same stallions. A significant ($P < 0.05$) seasonal effect on the computer-aided movement characteristic of mean sperm linearity was observed, with a reduction in sperm linearity in the winter months. The percentage of motile and progressively motile cells and mean sperm velocity determined by CASA also tended to be lower in the winter months. These changes in sperm movement characteristics paralleled changes in other seminal characteristics. The use of CASA may have value in the potential fertility evaluation of a stallion in that it can provide relatively precise quantification of sperm movement characteristics from the evaluation of only a few ejaculates. Some CASA derived sperm movement characteristics may be lower during the physiological nonbreeding season than during the breeding season.

410. **Behavior of Bull Spermatozoa in Bovine Uterine Tube Epithelial Cell Co-Culture: An In Vitro Model for Studying the Cell Interactions of Reproduction**. J.E. Ellington, A.W. Padilla, W.L. Vredenburgh, E.P. Dougherty and R.H. Foote

Freshly ejaculated bull semen was centrifuged and spermatozoa were resuspended in modified sperm TALP. Bovine uterine tube epithelial cell monolayers (BUTC) were obtained from cows in the periovulatory phase of estrus. In Experiment 1, sperm aliquots were assigned to culture wells containing either BUTC, BUTC-conditioned TALP, or control TALP. Sperm heads attached to the monolayers within 1 h of co-culture. Attached spermatozoa showed vigorous tail motion. At 5, 8 and 11 h of incubation at 39°C, the percentage of unattached sperm cells with intact acrosome membranes and percentage of motility of these cells was measured. Sperm-BUTC co-cultures were also fixed in situ for electron microscopy. Unattached spermatozoa in co-culture had more ($P<0.05$) acrosomal membrane

loss, showed hyperactive motion and had an overall decrease in motility as compared to sperm cells in control or conditioned medium. Evaluation by electron microscopy showed BUTC attached spermatozoa to behave in the co-culture system similar to reports for spermatozoa found in uterine tubes in vivo. Microvilli of the BUTC appeared to actively entrap the spermatozoa. Mucus-type granules could be seen on acrosomal regions and vesiculation of acrosomal membranes was seen in some cells. In Experiment 2, 43% of the 12×10^6 sperm cells added to 2-cm^2 BUTC bound within 4 h of co-culture. By 7 h of co-culture 19% of the previously bound sperm cells had been released from the BUTC. Released cells had limited motility and were mostly dead (73%). Sperm cells remaining on the monolayer at 7 h showed vigorous tail motion. They were gradually released from the BUTC over 48 h. Spermatozoa in co-culture interacted with the BUTC in a manner much like that seen in vivo, and sperm capacitation changes were stimulated by this interaction.

411. **The Usefulness of New Biotechnologies for Livestock Improvement**. R.H. Foote

This paper provides an overview, with 96 references, of the potential application of a variety of emerging animal biotechnologies to improve livestock production. The benefits of animal biotechnology will only be fully realized when various management practices provide a suitable environment, including meeting nutritional requirements. The various current animal biotechnologies (artificial insemination, estrous cycle regulation, embryo splitting, embryo freezing and embryo transfer) and new biotechnologies (sexing sperm and embryos, capacitating sperm, in vitro fertilization, chimeras, embryonic stem cells, transgenic animals and cloning), along with new information on chromosome mapping, genetic markers and the production of animals as pharmaceutical factories are included. Some conclusions follow. Biotechnology applied to animals through artificial insemination and embryo transfer has provided a basis for capitalizing on the many potentials of sexing, cryopreservation of embryos and genetic engineering of animals. Cost effective ways of using new products to improve animal health and efficiency are a challenge. There are numerous problems to be overcome before many of the techniques, particularly production of transgenic livestock, become practical. One of the most important is to have a better understanding of the way genes control body function. Production of animal models and products to carry out experiments not possible prior to the surge in genetic engineering technology will assist in revealing currently unknown mechanisms of importance in sustaining life and production. These studies are costly. Information needs to be shared among all sectors of research groups, industry, government and producers. We are on a collision course with population increases that are increasingly difficult to sustain. We need the political and agricultural leaders of all nations to determine what is important for their country and how each can cooperate with the others to protect the planet earth in the process, so that her ability to provide for future generations will not be diminished. Then our children and children's children can "bless" us for providing them with an opportunity to have a decent and respectful life.

412. **Extender and Centrifugation Effects on the Motility Patterns of Slow-cooled Stallion Spermatozoa.** A.W. Padilla and R.H. Foote

Slow-cooled stallion spermatozoa, with and without seminal plasma removed by centrifugation, were diluted in Kenney's extender (KE) containing non-fat dry skim milk with glucose and antibiotics, or in KE supplemented by adding a modified high potassium Tyrode's medium (KMT). Four ejaculates from each of four stallions were collected and divided factorially across these four treatments. Percentage of motile sperm (MOT), path velocity (VEL) and linearity (LIN) immediately after treatment (0 h) and after storage at 4°C for 24, 48 and 72 h were evaluated objectively by use of a HTM-2030 sperm motility analyzer. Stallions were a significant source of variation ($P < .01$) throughout. After cooling, effects of stallion, extender, centrifugation and their interactions were all significant ($P < .01$). The MOT at 0, 24, 48 and 72 h for centrifuged KE were 74, 47, 39 and 24%; for uncentrifuged KE

were 76, 56, 50 and 37%; for centrifuged KMT were 76, 75, 72 and 64% and for uncentrifuged KMT were 80, 50, 26 and 13%, respectively. The extender by centrifugation interaction, after 24, 48 and 72 h of storage, accounted for half or more of the variation. Whereas centrifugation of semen extended in KE appeared to be harmful to sperm, MOT in sperm extended in KMT after centrifugation was remarkably conserved for 72 h and was superior to all other treatments ($P < .05$). This extender is promising for preserving liquid stallion semen where it must be transported over time and space before use in artificial insemination.

413. **The Effects of Gonadotropin-Releasing Hormone (GnRH) on Pregnancy Rate in Dairy Cattle. A Critical Review**. W. Heuwieser, C.L. Guard, J.D. Ferguson, R.H. Foote and R. Mansfeld

The objective of this article was to review the current literature of the influence of gonadotropin releasing hormone (GnRH) and GnRH agonists on conception rate in dairy cattle. The application of GnRH or agonists at artificial insemination (first and subsequent inseminations) and between days 7 and 34 after parturition were considered. The variations between studies were discussed as well as different mechanisms concerning the influence of GnRH for establishing pregnancy. From a critical point of view the routine use of GnRH or agonists at the time of first or subsequent breeding or during the postpartum period cannot be supported.

414. **Factors Affecting Response of Groups of Dairy Cows Managed for Different Calving-conception Intervals**. H. Schindler, S. Eger, M. Davidson, D. Ochowski, E.C. Schermerhorn and R.H. Foote

Factors which positively or adversely affect the response of cow groups to different planned conception periods were analyzed and quantified. Cows were randomly assigned to groups for breeding at postpartum intervals of 35 to 59, 60 to 90 and 120 to 150 days in a research herd, or 60 to 90 and 120 to 150 days in a cooperating commercial herd, with a planned preceding dry period of 60 days in both herds. Breeding at 35 to 59 and 60 to 90 days post partum resulted in a lower conception rate among multiparous cows but had no effect on primiparous cows. Genital disorders such as metritis, retained placenta, vulva inflammation and vaginitis, did not affect the start of luteal activity or length of the subsequent cycles, but caused a significant delay of the first behavioral estrus and, consequently, a delay of the first insemination in the earlier breeding groups. About 30% of the cows in the different categories were inseminated three or more times and were considered to be repeat breeders. Response rates (number of cows conceiving on time/number assigned) in the later breeding group improved due to higher submission rates (number of cows submitted on time/number assigned), and the conception rates were higher (number of cows conceiving on time/number submitted). The rates in all groups were adversely affected by repeat breeding and genital disorders. The latter delayed the first insemination and the conception rate.

415. **Botanical Composition and Nutrient Content of Diets Selected by Goats Grazing on Desert Grassland in Northern Mexico**. M. Mellado, R.H. Foote, A. Rodriquez and P. Zarate

The diets selected by esophageal-fistulated indigenous goats grazing on desert grassland in northern Mexico were characterized throughout the year, in terms of botanical composition, nutrient content and preference indices. Browse (primarily *Parthenium incanun*, *Agave lechuguilla*, *Buddleja scordioides*, and *Atriplex canescens*), constituted over 80% of the goats' diet throughout the year except 57% during the rainy season in April. Grasses made up less than 10% of the diet for most of the year except during the dry period in October. *Bouteloua karwinskii* was the grass most utilized. *Sphaeralcea angustifolia* was the most important forb in goats diet (23% of the diet in April). Goats showed the highest preference for *Atriplex canescens*, *Buddleja scordioides*, and *Sphaeralcea angustifolia*. Nutritional adequacy of forage for the

greater part of the year was poor. Goats were not able to attain the N.R.C. recommended total consumption of protein for pregnancy and lactation (levels of protein ranged from 7.1 to 12.1%). Phosphorus and energy intake were estimated to be inadequate, even for maintenance. Despite these constraints, goats productivity in the area is acceptable. Perhaps indigenous goats have lower nutrient requirements than given by N.R.C. (1981).

416. Reproductive Efficiency of Nubian Goats Throughout the Year in Northern Mexico.

M. Mellado, R.H. Foote, and A. Gomez

Reproductive performance of 40 Nubian goats under intensive management and bred throughout the year during 5 years was studied with respect to seasonality of reproduction in this breed of goats in northern Mexico. Conceptions occurred year round but distribution of monthly fertility rates differed significantly ($P<0.05$) among the quarters of the year (54% for the first and 85% for the last quarter). Mean parturition interval was 301 days (S.E.=3.7). The estimated interval between time of initial exposure of does to bucks and time of conception was significantly different ($P<0.05$) among months in which does were bred, being longest (24 days) in April and shortest (3 days) in November. Mean prolificacy was 1.77 kids per kidding (S.E.=0.05), with no significant variation throughout the year. Precipitation and temperatures had significant correlations with fertility ($P<0.05$). Under uniform feeding conditions throughout the year, reproduction in Nubian goats at 25°N in Mexico is less affected by season than in goats in more northern latitudes. Rainfall and temperature seemed to be factors influencing sexual activity in the goats.

417. Effects of Age and Season on Mortality of Goats Due to Infections and Malnutrition in Northeast Mexico.

M. Mellado, R.H. Foote, J.N. De Tellitu

Influences of age and season on occurrence of deaths diagnosed as due to pneumonia, enteritis, pneumoenteritis and malnutrition in goats were studied. Data were from 251 necropsies of dairy and native goats in semiconfinement and on a good health management program in northeast Mexico. Annual mortality was 21.5%. Pneumonia accounted for the majority of the losses (55% of all deaths). Using the log-odds method, trends were noted for the youngest animals to be at increased risk of dying due to enteritis (10 times higher than adult animals, $P<0.05$). Adult animals had the highest risk of dying due to pneumonia and malnutrition (two times higher than young animals, $P<0.05$). Deaths diagnosed as due to enteritis were significantly higher in summer ($P<0.01$) and the occurrence of deaths due to this disease were significantly correlated ($P<0.05$) with temperature and precipitation. Significant seasonal patterns in deaths due to pneumonia were not detected. These results suggested that summer months favor the occurrence of deaths due to enteritis and that kids are the most susceptible animals. The data obtained will facilitate planning future studies designed to identify specific causative agents and reduce their presence.

418. Lactational Performance, Prolificacy and Relationship to Parity and Body Weight in Crossbred Native Goats in Northern Mexico.

M. Mellado, R.H. Foote and E. Borrego

Seventy-six native crossbred dairy goats were used to study milk production of these goats in a dual-purpose system under range conditions without concentrate supplementation in northern Mexico. Relationships between lactation performance, litter traits and parity, body weight and body measurements also were determined. Mean milk yield was 140 kg in six-month lactations. The fourth lactation yield was the highest. Body weight before kidding and parity combined accounted for 44% of the total variation in lactation yield. Mean litter size for first, second, third and fourth parities was 1.5, 1.5, 1.8 and 1.8 kids per kidding. Litter size and litter weight were positively correlated with parity, although only 7% of the variation in litter size and 14% of the variation in litter weight was associated with parity. It was concluded that larger goats in good body condition would be advantageous in goat operations

that emphasize milk production under Mexican range conditions.

419. **Penetration of Frozen-Thawed, Zona-Free Hamster Oocytes by Fresh and Slow-Cooled Stallion Spermatozoa.** A.W. Padilla, C. Tobback and R.H. Foote

A method for preparing stored unfrozen stallion sperm for the zona-free hamster oocyte penetration test (HOPT) and a subsequent comparison of fresh and stored sperm by the HOPT were evaluated. In Experiment 1, sperm from four stallion ejaculates, cooled to 4°C and stored for 24 h, were treated with 60, 90 and 120 nM of dilauroylphosphatidylcholine (PC12) liposomes to initiate the acrosome reaction. The percentage of motile and acrosome-reacted (AR) sperm were recorded after 8, 15 and 30 min of incubation at 39°C, using automated image analysis equipment. Liposome concentration did not affect motility during 8 or 15 min incubations. Sperm samples treated with 120 nM PC12 had fewer ($P<0.05$) motile sperm after 30 min and had a higher ($P<0.05$) percentage of AR sperm at all times than did samples treated with 60 nM PC12. In Experiment 2, sperm cooled and stored for 0, 24 and 72 h from five stallion ejaculates were treated with 120 nM PC12 for 8 min and incubated with frozen-thawed, zona-free hamster oocytes. There was no difference ($P>0.05$) in the percentage of eggs penetrated by sperm stored for 0, 24 or 72 h (77, 80 and 75%), but the average number of penetrating sperm per penetrated egg was lower ($P<0.01$) after 72 h of storage (5.9 and 6.1 vs 2.9). Results of this study indicate that stallion sperm can be stored for at least 24 h at 4°C without change in sperm characteristics measured here, and the HOPT test may be useful in indicating a decline in fertilizing potential with prolonged storage.

420. **Ethical Concerns of New Animal Biotechnologies.** R. H. Foote

What is ethics? My old Webster dictionary defines ethics as "1. A treatise on morals. 2. The science of moral duty; broadly the science of ideal human character. 3. Moral principles, quality or practice." And morals is defined as "establishing principles of right or wrong in behavior." No reasonable person will argue with the importance of distinguishing between right and wrong, but there is a wide range of opinions on almost every area of endeavor as to what is right or wrong action. Certainly it is of great importance that scientists be highly concerned with ethics, both to secure their own well-being and to serve as role models for others. The scientific method demands of us that we plan to use the best methods available to test a particular hypothesis or respond to a particular need. The major ethical concerns for any technology applied to animals are 1) that the objectives of these biotechnologies be used to produce superior or especially useful animals, 2) that each animal produced will not itself be at risk, 3) that if some animals produced are at risk, they will be properly cared for, 4) that the impact of these rapid changes on social and economic well-being of the human population will be addressed and 5) that the genetic base (gene pool) of any species of animal being selectively propagated be preserved (OTA, 1987) to provide future opportunities for maintaining species and producing individuals most compatible with changing environments. Rollin (1986) states that in his view "the genetic engineering of animals in and of itself is morally neutral, very much like the breeding of animals, or indeed, like any tool. If it is used judiciously to benefit humans and animals, with foreseeable risks controlled, and the welfare of the animals kept clearly in mind as a goal and a governor, it is certainly morally non-problematic and can provide great benefits. On the other hand, if it is used simply because it is there, in a manner guided at most only by considerations of economic expediency and 'efficiency,' or by quest for 'knowledge for its own sake,' with no moral thinking tempering its development, it could well substantiate the worst rational fears encapsulated in 'the Frankenstein thing,'"

421. **Biochemical Changes in Bull Spermatozoa During Capacitation In Vitro.** I.G. White, L. Belanger, S. Hough, J. Ellington and R.H. Foote

To evaluate the metabolic changes of bull spermatozoa (SPZ) during capacitation in vitro, SPZ

were incubated for 0, 5 or 10 hours in the presence (co-culture) and absence (control) of monolayers of bovine oviduct epithelial cells, which promote capacitation-like changes in vitro. There was little change in the oxygen uptake of the SPZ after 5 hours, but after 10 hours there was a decrease, particularly in the co-cultured sample. After 5 hours there was little change in the cyclic adenosine monophosphate (cAMP) concentration of the co-culture or control SPZ, but by 10 hours the levels of cAMP decreased in both the co-cultured and control SPZ (P=0.06). The concentration of adenosine triphosphate (ATP) was somewhat decreased after 5 hours in both the co-cultured and control SPZ and the percentage of decline was much higher after 10 hours. Overall, there was no significant change in oxygen uptake or cAMP and ATP levels specifically associated with capacitation of bull SPZ.

422. Comparison of Spermatozoal Movement and Semen Characteristics With Fertility in Stallions: 64 Cases (1987-1988).

D.J. Jasko, T.V. Little, D.H. Lein and R.H. Foote

Information pertaining to evaluation of single ejaculates of semen and records for 2 consecutive breeding seasons were obtained. In all, data for 99 individual breeding seasons (n = 43 Standardbreds and 56 Thoroughbreds) were evaluated. Included in each semen evaluation was examination of semen characteristics and computer-aided analysis of spermatozoal movement characteristics. On the basis of the analysis of breeding records for 4,175 mates (7,017 estrous cycles), a per-estrous cycle fertility rate was calculated from data for 96 of the breeding seasons. Stallions with lower fertility than the mean overall season fertility had significantly ($P < 0.01$) lower mean values for subjective appraisal of the percentage of motile and progressively motile spermatozoa and for percentage of morphologically normal spermatozoa. Lower mean values were obtained for computer-aided movement analysis of the percentage of motile and progressively motile spermatozoa, and for mean velocity of motile spermatozoa. Semen characteristics, including spermatozoal movement characteristics, and fertility were significantly ($P < 0.05$) correlated for Thoroughbred and Standardbred stallions when analyzed individually and when data for both breeds were combined. Characteristics most highly correlated ($P < 0.01$) with fertility data for both breeds combined were: subjective appraisal of the percentage of motile ($r = 0.40$) and progressively motile ($r = 0.46$) spermatozoa; percentage of morphologically normal spermatozoa ($r = 0.36$); and computer-aided analysis of percentage of motile spermatozoa ($r = .34$). However, on the basis of evaluation of a single ejaculate for each stallion, the variation in the characteristics only accounted for approximately 20% of the observed variation in fertility rate.

423. Viability and Acrosome Staining of Bull, Boar and Rabbit Spermatozoa.

A. Kovács and R.H. Foote

A practical and reliable staining procedure was developed to distinguish live-dead and the acrosomal status of bull, boar and rabbit spermatozoa. The first stain with trypan blue or Congo red is rapid and avoids artifacts. This stain is precipitated by neutral red during 2 min required for fixation. The precipitate gives a highly distinguishable black color resistant to the subsequent rinsings and long time required for staining of the acrosome with Giemsa. Ten classes of spermatozoa are distinguished (live and dead with intact acrosomes, with loose acrosomes, with damaged acrosomes, with no acrosome and with no acrosome and no postacrosomal ring). The intact acrosomes are purple, the loose acrosomes are dark lavender and the damaged acrosomes are pale lavender. The anterior part of the head of live spermatozoa with no acrosome is white or light pink and this area of dead spermatozoa is white or pale gray. The postacrosomal ring is red. The postacrosomal area of the head of live spermatozoa is white or light pink and this part of dead spermatozoa is black, dark violet or gray. The procedure was not satisfactory for stallion spermatozoa.

424. Cloning Bovine Embryos.

R.H. Foote and X. Yang

Cloning has many potential advantages. Details of the many steps necessary to produce animal clones are described, and it is not surprising

that the overall efficiency is low. As long as the successful starting point is early embryonic cells the application is limited. If recycled clones can be used efficiently the genetic potential is considerable. This would be greatly increased and costs reduced if embryonic stem cell lines with totipotency can be developed. At the present time success rates of produced cloned cattle are very low (about 1-4%). If cloned cattle become commercially feasible on a large scale, many lines should be maintained and germ plasm banks of embryos established to preserve the available gene pool. The production of large calves from cloned embryos is a serious commercial problem. Simultaneously, this phenomenon presents an exciting challenge to molecular and developmental biologist to determine the mechanism(s) of gene action involved, and to alter procedures to permit the cloning principle to be applied without producing calving problems.

425. **The Germinal Cells.**
R.H. Foote and W.E. Berndtson

This chapter provides a detailed description of testis organization and kinetics of spermatogenesis. Factors affecting germinal cell function and the relative sensitivity to noxious agents precedes a section on difference among species and in routes of exposure. Examples of the effect of dibromochloropropane, ethylene dibromide and triethylenemelamine on male laboratory animals and on man are discussed. Finally, the relative sensitivity of different endpoints and experimental design are discussed. The chapter includes 217 references.

426. **Fertilization of Bovine Oocytes After Microsurgical Injection of Spermatozoa.** W. Heuwieser, X. Yang, S. Jiang and R.H. Foote

The objective of this study was to compare the fertilization rate of bovine oocytes matured in vitro (22, 25 or 28 hours) and in vivo (30 to 35 hours after standing estrus) following the microinjection of a single spermatozoon. A single motile spermatozoon was injected into the perivitelline space (Experiments 1 to 9), and a single immotile spermatozoon was injected into the ooplasm (Experiments 10 to 15). A single ejaculate of frozen-thawed semen was used throughout. The spermatozoa were injected either without treatment or after treatment with heparin (100 μg/ml), or Ca ionophore A23187 (0.1 μM), or co-cultured for 5 hours with bovine oviduct epithelial cells (BOEC), or they were co-cultured for 5 hours with BOEC and immobilized by freezing and thawing twice without cryoprotectant, or they remained untreated. Oocytes were placed in a droplet of hyperosmotic solution of 0.1 M sucrose in PBS to enlarge the perivitelline space (Experiments 1 to 9) or in PBS (Experiments 10 to 15). Small amounts of polyvinyl pyrrolidone (PVP) without spermatozoa were injected as a control for parthenogenetic activation. After injection, oocytes were incubated in Medium 199 for 22 hours at 39°C, and they were stained with 1% aceto-orcein and examined for evidence of fertilization or parthenogenetic activation. Low rates (9 to 11%) of fertilization resulted from injection into the perivitelline space of oocytes matured for 22 hours in vitro irrespective of spermatozoa treatment. Fertilization rates were higher in oocytes matured in vivo after injection into either perivitelline space (66%) or ooplasm (74%) than in oocytes matured in vitro (9 to 44% fertilization). Surprisingly, in oocytes matured in vivo, there was no difference in the proportions fertilized by spermatozoa injection into ooplasm and parthenogenetically activated by injection of medium alone (74 and 66%, respectively).

427. **Electron Microscopy and Pedigree Study in an Ayrshire Bull with Tail-stump Sperm Defects.** R.H. Foote, S.R. Hough, L.A. Johnson and M. Kaproth

A tail-stump sperm defect found in an Ayrshire bull was essentially identical to this defect reported previously in a closely related Ayrshire bull. No sperm had normal tails and no sperm were motile. Total sperm per ejaculate averaged 1.1×10^9. The relationship between the two Ayrshire bulls and the similarity of this rare condition is strong presumptive evidence that the condition is inherited. A figure shows the sperm morphology, as viewed by scanning electron microscopy.

428. **Pregnancy Rates After the Use of a Gonadotropin Releasing Hormone Agonist in Bovine Embryo Transfer Recipients**. J.E. Ellington, R.H. Foote, P.B. Farrell, J.F. Hasler, J. Webb, W.B. Henderson and A.B. McGrath

The use of the gonadotropin releasing hormone analog, Buserelin, was evaluated in a commercial embryo transfer program. Virgin Holstein heifer recipients (n=764) at two embryo transfer facilities were randomly allocated to three treatment groups: 1) control animals, 2) heifers injected with 8 μg Buserelin at the time of transfer and 3) heifers receiving 8 μg of Buserelin 4 to 7 days after transfer. Fresh or frozen/thawed embryos were evaluated, equalized across treatments and transferred to recipients on Day 7 or 8 after estrus. Recipient progesterone levels were evaluated on the day of transfer. Pregnancy evaluations were done by palpation per rectum between Days 35 to 60 of gestation. There was no significant difference in pregnancy rates for the three treatments groups, with 68% of the animals pregnant in Group 1, 72% in Group 2 and 66% in Group 3. Progesterone levels at the time of transfer were similar for animals which became pregnant (2.60 ± 0.05 ng/ml) and animals which did not become pregnant (2.74 ± 0.09 ng/ml). There was no significant interaction observed between treatment and embryo quality or progesterone level, suggesting that the luteotrophic action of Buserelin at this early stage did not help support additional pregnancies over those seen in the control group.

429. **Nuclear Totipotency of Cultured Rabbit Morulae to Support Full Term Development Following Nuclear Transfer**. X. Yang, S. Jiang, A. Kovács and R.H. Foote

The rabbit was used as a model for nuclear transfer. A critical step in nuclear transfer is oocyte activation, which was evaluated in this research. Optimal field strength of an electric stimulus for activation was examined. Significantly higher activation rate in all criteria tested was achieved when oocytes were activated electrically with a field strength of 2.4 kV/cm versus 1.2 or 1.8 kV/cm. Also, electrical stimulation with combined alternating current (AC) and direct current (DC) was superior to DC stimulation alone for activation. In another study involving 586 oocytes, exposing oocytes to cytochalasin B for 1 h followed by activation with electrical stimulation significantly improved development to blastocyst stage compared to oocytes without cytochalasin B pre-exposure (38% vs 26%, $P<0.05$). Cytochalasin B exposure alone (control), however, had no effect on activation. Exposing oocytes to activation medium without electrical stimulation also activated some oocytes. In the nuclear transfer experiment, blastomeres from 8-cell embryos cultured for 20 to 24 h to the 32- to 64-cell stage were used as nuclear donor cells. Out of 491 oocytes used, 459 (93%) survived the enucleation and fusion procedure, 370 (81%) fused and 284 (77%) developed into 2- to 4-cell embryos. A total of 243 of these 2- to 4-cell embryos were transferred to 15 pseudopregnant recipients and produced 8 young (3%). Although the efficiency is low, this study demonstrated that rabbit morulae cultured for 20 to 24 h to the 32- to 64-cell stage as nuclear donors for transfer remain totipotent.

430. **Collecting and Processing Semen with Vision, Decision and Revision: Producing and Monitoring a Quality Product**. R.H. Foote

The total semen processing from the bull to the cow as sperm is harvested, processed, distributed and inseminated is critically reviewed. In addition, quality control is essential. Constant checks to know that your organization is doing what you think you are doing is clearly of major importance. Secondly, with todays electronic and computer technology, all records should be available for retrospective analysis, to point out weak links where experiments can be designed to measure dose response, and to establish optimas and limits for future quality control. Fertility can be improved by 1) following the best procedures and 2) by culling ejaculates within bulls and sometimes replacing one bull with another bull of equal merit. While fertility prediction is less than perfect, with judicious selection of semen extended to an appropriate sperm number, fertility can be

improved without increasing the number of bulls. Conversely, more total breeding units could be produced per bull stud without altering fertility, resulting in fewer bulls. Studies with frozen semen, using a combination of criteria in a multiple regression or selection index, also indicate that a substantial portion of nonreturn rates can be accounted for by differences in these criteria. Furthermore, genetic information also can be taken into account so that the overall merit of the gene pool available for use is not compromised. The price of progress is eternal vigilance.

431. **Increasing Carbon Dioxide from Five Percent to Ten Percent Improves Rabbit Blastocyst Development from Cultured Zygotes**. K. Hallden, J. Li, E.W. Carney and R.H. Foote

One-cell rabbit zygotes were cultured at 39°C in Basal Synthetic Medium II (BSM-II) with 5, 10, or 15% CO_2 and humidified air to determine the effect of CO_2 concentration on development in vitro. After 4 d in culture, 37% of the embryos grown in 10 or 15% CO_2 had reached the hatching blastocyst stage, but only 10% of the embryos were hatching when cultured under 5% CO_2 ($p=0.01$). Overall blastocysts, cell numbers were 207, 246 and 205 for the 5, 10 and 15% CO_2 treatments, respectively. In a second experiment to determine if there was a beneficial effect, particularly at the blastocyst stage, of a higher concentration of CO_2, embryos were cultured 4 d in either 5 or 10% CO_2 or for 2 d in 5% CO_2 followed by 2 d in 10% CO_2. The number of blastomeres per embryo and embryo diameter were greater ($p<0.05$) in embryos cultured continuously in 10% CO_2 or in 10% CO_2 only during Days 3 and 4 of culture than in embryos cultured continuously in 5% CO_2. In a third experiment one-cell rabbit zygotes were cultured with 5 or 10% CO_2 in a defined, protein-free medium consisting of 1:1 RPMI-1640 and Dulbecco's MEM. The proportion of embryos hatching and cell counts were significantly greater ($p<0.01$) when cultured in the presence of 10% CO_2. These data indicate that a 10% CO_2 atmosphere exerts a beneficial effect on the development of zygotes into expanding and hatching rabbit blastocysts in vitro.

432. **A Comparison Between In Vitro Fertilization and Microinjection of Immobilized Spermatozoa From Bulls Producing Spermatozoa With Defects**. W. Heuwieser, X. Yang, S. Jiang and R.H. Foote

The objectives of this study were to compare the fertilization rate of bovine in vitro matured oocytes by in vitro fertilization (IVF) and by microinjection of a single spermatozoon (MI) and to relate these rates with fertility reported for these bulls in artificial breeding. Bull A (Holstein) had a nonreturn rate of 75%. Semen from this bull is routinely used in our standard IVF procedure. Bull B (Ayrshire), used regularly in artificial breeding and related to bull D, had a nonreturn rate of 69.2%. Bull C (Brown Swiss), with a chromosomal translocation and trisomy, achieved a nonreturn rate of 42%. Bull D (Ayrshire) produced nonmotile spermatozoa (SPZ) and had an abnormality described as "tail stump defect." No pregnancies sired by bull D have been reported. Oocytes were either fertilized in vitro by capacitated SPZ or by microinjection of a single immobilized SPZ into the ooplasma. SPZ were treated with 0.1 µM A23187 and used for IVF. For microinjection SPZ were cocultured for 5 h with bovine oviduct epithelial cells (BOEC) and then immobilized by freezing and thawing twice without cryoprotectant. A single batch of killed SPZ (stored at -25°C) was used for all microinjections. All oocytes were cultured in Medium 199 for 22 h at 39°C and subsequently fixed, stained, and examined for evidence of fertilization (i.e., female and male pronucleus formation, SPZ decondensation). Fertilization rates following IVF with semen from bulls A, B, C, and D were 80%, 54%, 1%, and 2%, and following microinjection were 39%, 22%, 21%, and 34% respectively.

433. **Synaptonemal Complex Analysis of a Three-breakpoint Translocation in a Subfertile Bull**. A. Kovács, D.A.F. Villagómez, I. Gustavsson, K. Lindblad, R.H. Foote and T.H. Howard

Somatic chromosome analysis of a subfertile Brown Swiss bull demonstrated a three-breakpoint

translocation involving chromosomes 1, 8, and 9 in G- and R-banded karyotypes. Based on standard bovine chromosome nomenclature, the translocation was defined as t(1;8;9)(q43;q13;q26). Synaptonemal complex analysis of the chromosome aberration by electron microscopy revealed a hexavalent configuration in 52 or 53 pachytene cells. Twenty-seven cells (51%) had a completely paired hexavalent configuration showing distinctly non-homologous pairings between normal and/or translocated chromosomes involved in the exchanges. Thirteen cells showed a hexavalent configuration with centrally unpaired chromosome segments but with completely paired terminal arms. In 13 cells (including one at zygotene) the translocation chromosomes formed an open hexavalent, and in one cell there were two completely paired trivalents. Thirty-two cells at diakinesis-MI demonstrated 28 configurations, including one large hexavalent. Testicular histology, testis size, and seminal characteristics were normal. However fertility was reduced (36% below the stud average for this breed).

434. Bovine Oocyte Development Following Different Oocyte Maturation and Sperm Capacitation Procedures.

X. Yang, S. Jiang and R.H. Foote

Various procedures have been reported for successful in vitro maturation and in vitro fertilization (IVM/IVF) of bovine follicular oocytes. Direct comparisons of these different recommended procedures have been rare. In this research, involving a total of 5,128 oocytes, a series of experiments were conducted to compare oocyte maturation, fertilization and development in vitro with two maturation systems (with or without added hormones) and three types of sperm treatment procedures. Oocytes were collected from ovarian antral follicles (2-7 mm in diameter) within 3 hr after slaughter of cows or heifers. Those with intact or at least 4 layers of cumulus cells were selected for IVM/IVF. Oocytes were incubated for 22 hr in either Medium 199 with 7.5% fetal calf serum (M199 + FCS) alone or M199 + FCS with added hormones (M199 + FCS + H; oFSH 0.5 μg/ml, oLH 5 μg/ml and E_2 1 μg/ml) at 39°C in 5% CO_2 and 95% air. For IVF, frozen-thawed sperm were treated with either 0.1 μM calcium ionophore A23187 (A23187) for 1 min or 10 μg/ml or 100 μg/ml heparin (H10 or H100) for 15 min. Our results demonstrated the following: 1) both M199 + FCS and M199 + FCS + H supported maturation development to the metaphase II stage (90-95%, $P>0.05$); 2) when oocytes were matured in M199 + FCS without added hormones, A23187 sperm treatment was superior to H10 or H100 treatment for fertilization and blastocyst development of the inseminated oocytes ($P<0.05$); 3) when oocytes were matured in M199 + FCS + H, A23187 treated sperm again produced a higher fertilization rate than H10 group ($P<0.05$), but the development to the blastocyst stage was similar among all three sperm treatment groups ($P>0.05$); 4) direct comparison of the two maturation systems with A23187 treated sperm resulted in no difference in all criteria measured; however, 5) when compared retrospectively, beneficial effects of added hormones are evident for blastocyst development (but not for fertilization) when sperm were treated with heparin procedures.

435. A Simulation Study of the Effects of the Calving Interval on Milk Yields of Dairy Cows in Fixed Time Periods.

A. Genizi, H. Schindler, S. Amir, S. Eger, M. Zarchi and R.H. Foote

Multiparous cows were assigned before calving to three calving to first insemination intervals of 35 to 59, 60 to 90 and 120 to 150 days. Records of cows conceiving at first or second insemination, were used to construct a model of the lactation curve which incorporated peak production and the effect of progressing pregnancy. The model was used to simulate milk yield during a 4-year period for three production levels and five calving intervals. The model separated the descending part of the lactation curve into a linearly and an exponentially declining component, with the latter becoming distinct at about 20 weeks after conception. Peak yield was negatively correlated with the slope of the linear decline. Within a simulated 4-year period, cumulative milk yields at fixed time periods after calving depended upon the period chosen and the calving-to-conception interval of the cow. Late conceptions resulted in higher cumulative yields at the end of the 1st year, and in lower yields at the 2nd year end, with respect to early conceptions. Smaller differences were found between the intermediate

calving intervals. During the 3rd and 4th years the early conceptions had a distinct advantage. Different rates of the linear decline, obtained for the different production levels, changed the magnitude of the yield differences between the calving intervals but not their relative ranking. The model presented offers a means for the suitable choice of the calving cycle according to the length of the period for which a cow is expected to remain in the herd.

436. **Survival of Bull Spermatozoa Seeded and Frozen at Different Rates in Egg Yolk-Tris and Whole Milk Extenders**. Y. Chen, R.H. Foote, C. Tobback, L. Zhang and S. Hough

Six factorially arranged experiments were designed to study effects of seeding, freezing and thawing rates in whole milk and egg yolk-Tris extenders commonly used for commercial cryopreservation of bull sperm. In these extenders semen normally is supercooled to -13 or -14°C, unless seeded. When sperm are supercooled or seeded mechanically or with immobilized silver iodide, and frozen to -196°C the post-thaw percentages of motile sperm were 59, 57, and 64%, respectively. Freezing rates of -15, -25, and -35°C/min gave similar sperm survival rates and were superior to -5°C/min. For milk, the critical freezing temperature extended to -75°C before transfer to liquid nitrogen gave good results. For egg yolk-Tris, transfer to liquid N_2 was less critical once -50°C had been attained. Thawing of sperm in water baths at +25 and +45°C gave similar results, and both temperatures were superior to +5°C. The post-thaw percentage of motile sperm in egg yolk-Tris was equal or superior to sperm frozen in milk. A freezing rate of -15°C/min to -100°C and thawing at +25°C consistently gave good results.

437. **Nuclear Transfer in Cattle: Effect of Nuclear Donor Cells, Cytoplast Age, Co-culture and Embryo Transfer**. X. Yang, S. Jiang, P. Farrell, R.H. Foote and A.B. McGrath

There are many factors affecting the efficiency of nuclear transfer technology. Some are evaluated here using our novel approach by enucleating oocytes at 20 to 22 hr after in vitro maturation (IVM), culturing the enucleated oocytes (cytoplasts) for 8 to 10 hr or 18 to 20 hr to gain activation competence and then conducting nuclear transfer. In the first experiment, we demonstrated that cumulus cell (CC) monolayer can support some cloned embryos to develop into morulae or blastocysts. Co-culture with CC and bovine oviduct epithelial cell (BOEC) monolayers resulted in no differences ($P>0.05$) in supporting the development of cloned embryos (Experiment 2). When in vitro matured oocytes were enucleated at 22 hr after IVM followed by nuclear transfer 18 to 20 hr later, cleavage and morula or blastocyst development of the cloned embryos were similar to those resulting from the enucleated oocytes which had been matured in vivo (Experiment 3). Frozen embryos as nuclear donor cells worked equally well as fresh embryos for cloning in embryo development which was superior to IVF embryos (Experiment 4). However, fresh embryos resulted in a higher proportion ($P<0.05$) of blastomere recovery than frozen or IVF embryos. Finally, embryo transfer of cloned embryos from our procedure produced a viable calf, demonstrating the commercial value of this novel approach using this technology.

438. **Culture of Rabbit Zygotes into Blastocysts in Protein-free Medium with One to Twenty Per Cent Oxygen**. J. Li and R.H. Foote

Embryos were collected from superovulated Dutch rabbits 19 h after injection of luteinizing hormone and insemination. The embryos were in the one-cell stage at that time and those judged to be normal by the absence of granular cytoplasm and regular shape were distributed randomly within donors into culture dishes containing 500 µl of a macromolecule-free medium consisting of RPMI-1640 and low glucose Dulbecco's modified Eagle's medium (DMEM), 1:1, without a cover of oil. In Experiment 1, O_2 concentrations of 5, 10 and 15%, with 5% CO_2 plus 90, 85 and 80% N_2, respectively, were tested. In Experiment 2, O_2 levels of 1, 5 and 20% were combined with 5% CO_2 and the remaining gas was N_2. Culture was at 39°C. After 84 h in culture, embryos were examined for stage of development and stained with Hoechst 33342 stain so that the number of cells could be counted. In Experiment

1, the proportion of embryos reaching the hatching blastocyst stage after 84 h in culture in 5, 10 and 15% O_2 was 48, 38 and 21%, ($P<0.01$) and corresponding cell numbers per embryo were 258, 226 and 188 ($P<0.01$). In Experiment 2, the proportion of hatching embryos after 84 hr in culture in 1, 5 and 20% O_2 was 67, 72 and 29% ($P<0.01$), respectively. Cell numbers in the 1 and 5% O_2 concentrations were higher than in the 20% O_2 level ($P<0.01$). These results indicate that reduction of O_2 concentration to 5%, well below the frequently used concentration of about 20% O_2 in 95% air, is beneficial to rabbit embryo development from the zygote to the blastocyst stage. The O_2 concentration may be more critical with simple defined macromolecule-free media than in media containing serum.

439. Development of Rabbit Zygotes Cultured in Protein-free Medium with Catalase, Taurine or Superoxide Dismutase.

J. Li, R.H. Foote and M. Simkin

The present study was designed to examine the influence of several potential antioxidants in a synthetic medium completely devoid of macromolecules. Antioxidants may be beneficial as additives to a synthetic medium because they could serve as scavengers of toxic free radicals in media lacking serum or serum albumin or other macromolecules that may serve as scavengers in more complex media. Rabbit zygotes were cultured for 72 h at 39°C in media containing varying concentrations of the antioxidants in a gas phase consisting of 5% CO_2:95% humidified air. At 72 h embryo development was recorded and embryos were fixed and stained with Hoechst 33342 DNA stain to facilitate counting the number of cells. In Experiment 1, concentrations of catalase ranging from 250 to 1000 IU did not affect the proportion of zygotes developing into blastocysts or the cell number ($p>0.05$). In Experiment 2, 2.5 to 10 mM taurine increased the proportion of zygotes developing into expanding blastocysts (70-78% versus 40% for controls) and cell number was increased by taurine from 100 to 154-159 ($p<0.05$). In Experiments 3 and 4 superoxide dismutase was tested over a range of 100 to 2400 IU/ml. The response plateaued at 600 IU/ml, with positive responses in rate of embryo development and growth similar to that found with taurine in Experiment 2. These studies indicate that culture of zygotes into blastocysts in a macromolecule-free medium with 5% CO_2:95% air is substantially improved when agents that could serve as antioxidants are included. Other mechanisms of action are not excluded by these studies.

440. A New Approach to Overcoming a Yearling Holstein Bull's Complete Lack of Motivation to Mounting.

R.H. Foote, G.A. Presicce and C.C. Brockett

A young Holstein bull selected for sampling in artificial breeding was presented with a variety of sexual stimuli which have been used successfully over several years to induce hundreds of yearling Holstein bulls at Eastern A.I. Coop., Inc. to mount "teaser" animals and serve the artificial vagina. This bull was extensively tested with a variety of mounts, and other potential stimuli on 25 occasions and was scheduled for slaughter at 17 months of age because of a total failure to mount. At this time putative pheromones were applied to several teasers, but they did not stimulate mounting. However, the bull was observed to lick the flanks of teasers. Ordinary cane molasses was painted on the mount animal. The bull licked the molasses aggressively. The molasses was placed successively higher on the mount animal until it was on top of the mount near the tailhead. In this position the bull was licking with the chin resting on the teaser and the bull mounted. Thereafter he mounted rapidly and repeatedly without further exposure to the molasses. Excellent semen was obtained. The sperm were frozen and the bull soon produced enough sperm for progeny testing. The novel approach used here was successful in enabling this bull to be quickly entered into the test program, whereas all earlier approaches to stimulate mounting were completely unsuccessful.

441. Fertility of Bull Spermatozoa Frozen in Whole Milk Extender with Trehalose, Taurine, or Blood Serum.

R.H. Foote, Y. Chen and C.C. Brockett and M.T. Kaproth

Fertility of bull semen processed in heated whole milk-glycerol control semen extender with various additives was compared in six field trials.

The additive in field trials 1 and 2 was 25.6 of trehalose/L of the glycerol fraction of whole milk. Whole milk was heated to 95°C for 10 min, cooled and filtered the day before use (trial 1) versus 3 d before use (trial 2). In field trial 3, 3.0 g/L of taurine was added to the glycerol fraction of whole milk. In field trial 4, specially prepared blood serum (15% vol/vol) was included in the glycerol fraction of whole milk. Field trials 5 and 6 were larger fertility studies with trehalose in extenders prepared the day before use (trial 5) and both 1 and 3 d before use (trial 6). Control and treated semen were coded and distributed randomly over a large group of professional inseminators. The 59-d nonreturn rates for control and treated semen, respectively, were as follows: trial 1, 74.1 and 73.7%,; trial 2, 71.3 and 73.1%; trial 3, 74.9 and 70.9% and trial 4, 75.1 and 71.6%. No significant differences resulted in trials 1 to 3, but blood serum decreased the nonreturn rate in trial 4. Trials 5 and 6 resulted in nonsignificant improvement in fertility with added trehalose. Thus, these additives, useful as cryopreservatives or membrane protectors in other systems, did not enhance the fertility of sperm frozen in whole milk.

442. **Effect of Sucrose, Trehalose, Hypotaurine, Taurine and Blood Serum on Survival of Frozen Bull Sperm**. Y. Chen, R.H. Foote and C.C. Brockett

Factorially arranged experiments were conducted to study the effects of adding sucrose and trehalose, known to have cryoprotective properties, and blood serum and antioxidants taurine and hypotaurine on sperm motility after freezing and thawing at different rates. At sugar concentrations of 0.1 M, osmolality of the whole milk (WM) or egg yolk-tris (EYT) freezing medium (without glycerol) was about 370 mosmols. Survival following freezing and thawing was reduced unless osmolality was corrected by adding pure water to reduce osmolality to about 280 mosmols. Then the post-thaw percentages of motile sperm for control WM, and WM with 0.05 M or 0.10 M sucrose, or 0.05 M or 0.10 M trehalose, respectively, were 62, 55, 61, 57 and 62. Thawing semen at 5°C, versus 25°C resulted in 64 versus 70% motile sperm ($P<0.05$). Post-thaw survival of sperm stored at 25°C for 24 h in trehalose-treated WM was superior to WM ($P<0.05$). Hypotaurine and taurine had little effect on sperm survival. Up to 10% (v/v) of heated blood serum was generally beneficial, but gave more variable responses with different bulls. Sperm survival after cooling at -25°C/min was slightly superior to cooling at -15°C/min to -100°C. The effects of the compounds studied on motility of frozen-thawed sperm were small, but if they protect sperm cell membranes, as reported for other types of membranes, they may assist sperm in surviving in the reproductive tract of the cow prior to fertilization.

443. **Current State of the Art and Future of Embryo Manipulation and Engineering to Enhance Breeding Programs in Cattle**. R.H. Foote

The development of artificial insemination of cattle was remarkable because of its early acceptance by the commercial cattle industry as a laboratory procedure (semen evaluation and processing) that was really useful on the farms, and this was accompanied by major economic benefits through control of venereal diseases and eventually major genetic improvement. This set the stage for the application of embryo transfer for the elite (expensive surgical transfer) to the practical nonsurgical procedures. Then came micromanipulated embryos (splitting), cryopreservation and sexing of embryos. Worldwide distribution of germ plasm became possible for the first time. With the development of in vitro techniques of maturing oocytes fertilizing them in vitro and culturing them to the blastocyst stage came the practical procedure of harvesting oocytes from live adult cattle. Calves also could be utilized to test them at a young age and reduce the generation interval. The creation of embryos through nuclear transfer to produce clones has had limited success. The ability to manipulate gametes, zygotes and embryos in vitro has resulted in an opportunity to study early embryo development and the genes regulating early development. It is possible to add genes and to interfere with function of an existing gene. Animals may be produced with characteristics to model diseases associated with genetic deficiencies. These studies have led to somatic gene therapy. Other applications include incorporating genes which produce proteins important for human health, and constructing them so they are activated only in the mammary gland. Most success to date has been

limited to mice, but sheep and goats have been used successfully.

444. **Use of Gonadotropic Releasing Hormone for Ovulating the Rabbit Model.** R.H. Foote and M.E. Simkin

The synthetic decapeptide, GnRH, or analogs do not provoke antibody response and can be used repeatedly to ovulate rabbits. Injection intramuscularly of 2.5 μg of GnRH or 1.2 μg of Buserelin will induce an LH surge almost immediately. This is followed by ovulation 10-12 hours later. In our colony of Dutch rabbits (2.0 to 2.5 kg bodyweight) 100 does injected with Buserelin and inseminated at the same time resulted in 88 litters averaging 5.7 ± 0.3. This result is as good as using LH intravenously and the i.m. route is much simpler.

445. **Effect of Growth Factors on Morula and Blastocyst Development of In Vitro Matured and In Vitro Fertilized Bovine Oocytes.** B.K. Yang, X. Yang and R.H. Foote

Growth factors were studied as a means of increasing the development of in vitro matured (IVM) and in vitro fertilized (IVF) oocytes into morulae or blastocysts. Cell numbers of blastocysts were also counted. In Experiment 1, 2- to 8-cell embryos derived from bovine IVM/IVF oocytes were randomly allotted to one of 3 culture groups: a) synthetic oviduct fluid (SOF); b) SOF + 10 ng/ml epidermal growth factor (EGF); or c) SOF + 100 ng/ml EGF; all 3 culture media contained 10% fetal bovine serum. Culture resulted in 12%, 23% and 14% ($P>0.05$), respectively, developing into morulae and blastocysts. In Experiment 2, 5 ng/ml of transforming growth factor $ß_1$ ($TGFß_1$) added to CR_{1aa} medium containing BSA increased the percentage of blastocysts to 56% vs 40% for the control ($P<0.05$). In Experiment 3, EGF and $TGFß_1$, added singly and in combination to CR_{1aa} did not produce a synergistic effect. More embryos developed into morulae and blastocysts (45%) in a bovine oviduct epithelial co-culture than in any other treatment except in CR_{1aa} + EGF (34%; $P>0.05$). In Experiment 4, 0, 1 and 5 ng/ml of platelet derived growth factor (PDGF) added to CR_{1aa} yielded 39%, 70% and 52% morulae and blastocysts, respectively ($P<0.05$). Cell number was not increased, indicating that growth factors can increase the proportion of embryos that develop without an increase in the cell number.

446. **Behavioral Responses of Bulls Kept Under Artificial Breeding Conditions to Compounds Presented for Olfaction, Taste or With Topical Nasal Application.** G.A. Presicce, C.C. Brockett, T. Cheng, R.H. Foote, G.F. Rivard and W.R. Klemm

The objective of this study was to test practical ways of influencing sexual behaviour of bulls used for artificial insemination. Dairy bulls that were sexually active with regular semen collections were compared with sexually inactive bulls housed elsewhere after being sampled in artificial breeding as yearlings. The behaviour of bulls in response to various volatile compounds and mixtures was recorded. These behaviours included sniffing, licking of sample, tongue manipulation, vocalization and flehmen (Rivard and Klemm, 1990b). Single compounds found as volatile compounds in estrous blood (Rivard, 1991), did not evoke appreciable responses. Mixtures of these compounds evoked some response, but the most powerful stimulus was urine collected from teaser bulls. Most sexually active and inactive bulls responded quickly to urine soaked cloths brought near them. They would attempt to reach the urine sample, sniff it and lick it followed usually by a strong flehmen. Sexually active bulls in their familiar semen collection area with mount bulls paid little attention to urine as compared to licking the teaser bulls. Thus, with this visual stimulus the effect of olfaction stimulus on sexual arousal of dairy bulls was minimal.

447. **Pluripotency of Cultured Rabbit Inner Cell Mass Cells Detected by Isozyme Analysis and Eye Pigmentation of Fetuses Following Injection Into Blastocysts or Morulae.** J.R. Giles, X. Yang, W. Mark and R.H. Foote

Pluripotency of isolated rabbit inner cell masses (ICMs) and cultured (3 days) inner cell mass

(ICM) cells was tested by injecting these donor cells into day 3.5 blastocysts (experiment 1) or day 3 morulae (experiment 2) to produce chimeric embryos. Injected (n=107) and noninjected (n=103) embryos were transferred to opposite uterine horns of the same recipient females. Chimerism was determined by adenosine deaminase (ADA) isozyme analysis on fetal tissue and by eye pigmentation at midgestation. In experiment 1, 53% and 64%, respectively, of blastocysts injected with either ICMs or cultured ICM cells, developed to midgestation, compared with 52% and 48% for controls. Of these fetuses, four (31%) and 1 (6%), respectively, had ADA chimerism. In experiment 2, 38% and 62%, respectively, of the morulae injected with either ICMs or cultured ICM cells, developed to midgestation compared with 46% and 56% for control morulae. Six (43%) chimeric fetuses from morulae injected with ICMs were detected by ADA analysis, but 12 (86%) chimeric fetuses were detected by eye pigmentation, indicating that eye pigmentation was more sensitive a marker for chimerism than our ADA assay. None of the 14 fetuses recovered after injecting morulae with cultured ICM cells were chimeric with either marker. No chimeras developed from control embryos. These studies demonstrate a) that pregnancy rates are not compromised by injection of blastocysts or morulae with ICMs or cultured ICM cells, b) that chimeric rabbit fetuses can be produced by injecting ICMs into either blastocysts or morulae and c) that cultured ICM cells can contribute to embryonic development when injected into blastocysts.

448. **Relationship of Semen Quality, Number of Sperm Inseminated and Fertility in Rabbits.** P.B. Farrell, R.H. Foote, M.E. Simkin, E.D. Clegg and R.J. Wall

The relationship between the total number of sperm inseminated, semen quality and fertility in rabbits was investigated, using fractionated or unfractionated semen and different diluting fluids. Semen was from Dutch-belted males collected twice weekly with an artificial vagina. All does were superovulated except in Experiment 3. In Experiment 1, sperm were fractionated on discontinuous 4% and 10% BSA columns. Sperm from each portion of the gradient, along with unfractionated controls, were diluted to give 0.25×10^6, 0.5×10^6, 1.0×10^6 and 2.0×10^6 total sperm per insemination. In Experiment 2, sperm were diluted with Dulbecco's phosphate-buffered saline (DPBS) to provide 0.10×10^6, 0.50×10^6 and 1.0×10^6 total sperm per insemination, with minimal processing time. In Experiment 3, does were allowed to kindle after inseminating 0.1×10^6 or 1.0×10^6 sperm. In Experiment 4, sperm were diluted with TALP: seminal plasma 1:1 to 0.025×10^6, 0.05×10^6 and 0.10×10^6 total sperm per insemination. Over 2,800 embryos or unfertilized oocytes were obtained either 24 h or 48 h after insemination to measure fertility. Sperm numbers required for normal fertility were 0.50×10^6 in Experiment 1 and only 0.05×10^6 in Experiment 4. This reduction presumably was due primarily to reduced processing time and diluent change. Litter size was normal with 0.1×10^6 sperm (Experiment 3). In Experiment 4, CASA analysis (HTM 2030 system, Beverly, MA) was adapted to successfully screen out some of the "interfering" granules in rabbit semen. The correlation between multiple characteristics of semen quality and fertility was 0.53 ($P<0.01$). This correlation likely will be increased with refinements in instrumentation and reduced sperm numbers per insemination

449. **Harvesting and Utilizing Bull Sperm Power for Maximal Genetic Progress Through Artificial Insemination.** R.H. Foote

By using the best procedures for preparing bulls for frequent semen collection and minimizing losses in semen processing a Holstein sire can ejaculate about 2×10^{12} (2 trillion) sperm per year. With 10×10^6 sperm per insemination, 80% of the semen used and a pregnancy rate of 50%, 80,000 progeny per year per sire would be produced. If the demand were great enough sperm numbers per insemination could be reduced to 5×10^6 with a 1-2% decrease in pregnancy rate. Therefore, by selecting the best bulls genetically, and with average or above fertility, more genetic progress could be made with no economic loss due to depressed fertility. Artificial insemination in rabbits also is a very practical procedure. Where rabbit meat consumption is high use of selected bucks can have a great influence on the industry. Female rabbits conceive at a high rate when inseminated with 1×10^6 sperm. A

male producing 500 × 10^6 sperm per week (some produce 1000 × 10^6 sperm per week) could have its semen used to inseminate 500 does per week. In 50 weeks, with 75% producing an average of 6 weaned young, the number of progeny per buck per year would be 105,000.

450. **Analysis of Polarity of Bovine and Rabbit Embryos by Scanning Electron Microscopy**. H. Koyama, H. Suzuki, X. Yang, S. Jiang and R.H. Foote

Cellular polarization during preimplantation development of the embryo is believed to be a crucial event in the transition of a zygote to a blastocyst stage embryo with morphologically and functionally differentiated cell types. While extensive studies have been done on polarity development in mouse embryos, little information is available in other species, particularly in cattle. The objective of this study was to examine the initiation of polarity by microvilli distribution on blastomeres of cattle and rabbit embryos using scanning electron microscopy (SEM). Bovine embryos were obtained by in vitro fertilization of in vitro matured follicular oocytes. Rabbit embryos of various stages were collected from superovulated rabbits. Intact embryos and isolated blastomeres were examined in both species. Blastomeres from one- to eight-cell embryos in both cattle and rabbits showed no polarity. The onset of transitional polarization of microvillous distribution occurred in some blastomeres of cattle embryos at the 9- to 15-cell stage, but typical, distinct polarity was not manifested until after the 16-cell stage with approximately 40% polar cells per embryo. In the rabbit, blastomere polarity occurred one cell cycle later, with 46% polar cells per embryo after the 32-cell stage. This difference probably is related to the different cell stages for embryo compaction and blastocyst formation in the two species.

451. **Synergistic Effect of Ethanol and Cycloheximide on Activation of Freshly Matured Bovine Oocytes**. X. Yang, G.A. Presicce, L. Moraghan, S. Jiang and R.H. Foote

Bovine follicular oocytes were collected from ovarian antral follicles (2 to 7 mm in diameter) from slaughtered cattle. They were matured in vitro (IVM) for 23 to 24 h and then activated. In Experiment 1, 4 concentrations of ethanol were compared. The activation rates of oocytes were 4, 12, 36 and 27%, respectively, following exposure for 7 min to 0, 5, 7 and 10% ethanol. In Experiment 2, 7% ethanol was tested with exposure times of 0, 5, 7.5 and 10 min, and 6, 32, 27 and 33% of the oocytes were activated, respectively. In Experiment 3 the synergistic effect of ethanol and electric pulse was compared within 4 treatments: A) 7% ethanol alone, B) electric pulse alone, C) ethanol first and then electric pulse treatment, and D) electric pulse first followed by ethanol exposure. Of the oocytes activated, 37, 31, 28 and 51%, respectively, were from Treatments A through D. In Experiments 4 and 5 the possible synergistic effect of ethanol and a protein synthesis inhibitor, cycloheximide, was studied within 4 treatments: A) parthenogenetic control with no activation treatment, B) ethanol alone, C) cycloheximide alone, and D) ethanol treatment followed by cycloheximide. The oocyte activation rates in Experiment 4 in Treatments A through D, respectively, were 9, 44, 43 and 84%. Corresponding values for development of oocytes to the 2 to 8-cell stage after culture for 3 d (Experiment 5) were 9, 20, 14 and 45%, respectively ($P<0.05$). In conclusion, exposure to 7% ethanol for 5 min followed by incubation with cycloheximide was the best activation treatment for bovine IVM oocytes.

452. **Semen Quality and Behavior of Holstein Bulls Exposed to Estradiol-Treated Bulls for Mounts**. C.C. Brockett, G.A. Presicce, R.H. Foote, M.T. Kaproth and H.E. Rycroft

The objectives were to test the effects of treating teaser bull mounts on sexual behavior and quality and quantity of sperm obtained from sires as managed in large commercial AI breeding organizations. In a change-over design the same teasers were either untreated or treated with estradiol. Five semen producing bulls were ejaculated twice per day on Tuesdays and Fridays after epididymal reserves were partially depleted. A 15-min period of continuous sexual preparation with three false mounts allowed was standard before each semen collection. All bulls were attracted to and licked the preputial area of the teaser mounts followed by the Flehmen response

during the period of sexual preparation. Bulls usually completed the false mounts in ≤ 15 min, and all thrusted vigorously with both hind feet moving forward synchronously at this time on 100% of the 80 semen collections. Major differences among bulls and between first and second ejaculates occurred in semen volume, sperm concentration, and total sperm collected. An increase of 10% in total sperm output when bulls were exposed to treated teasers could be of commercial benefit. The correlation between total time to first mount for the two ejaculates per bull each day and total sperm collected per bull per day was -.44. Thus, the shorter time to first mount may be useful as a low level predictor of higher sperm output per bull.

453. **Factors Affecting Preservation and Fertility of Bull Sperm: A Brief Review**. R.H. Foote and J.E. Parks

This paper is a brief review of the factors which contribute to the number of sperm required for insemination to obtain high fertility and ways that sperm viability might be prolonged. Damage to sperm during freezing results in about 6×10^6 motile frozen sperm post-thaw ($>10 \times 10^6$ total) per insemination being required for near maximal fertility, whereas 2.5×10^6 motile fresh sperm result in high nonreturn rates. Multiple inseminations to bracket the time of ovulation usually are not economical excepting in superovulated cows. Earlier unpublished work on sperm packaging for slow release in the cow and methods for stabilizing membranes to increase sperm survival time in the cow are discussed. Current studies are directed toward reducing catabolic metabolism of sperm and studying membrane changes during freezing and thawing and during incubation with bovine oviduct epithelial cells. Studies with bull sperm indicate that the choline and ethanolamine phosphoglyceride components of their membranes represent an unstable configuration. Exposure of sperm to liposomes with the sterol, cholesterol, can alter the phospholipid bilayer and increase capacitation time. Similar approaches may produce sperm with a longer fertilizing life following insemination. New in vitro procedures permit low cost modeling of fertilization, which will facilitate research by reducing the cost of in vivo studies.

454. **Survival of Rabbit Spermatozoa Frozen and Thawed at Different Rates With and Without Seeding.**
Y. Chen and R.H. Foote

The ability to freeze rabbit spermatozoa with resulting high survival and fertility, would be very useful experimentally and have practical application. Experiments reported here were designed to explore different methods of freezing and thawing rabbit spermatozoa to improve their survival. Dutch male rabbits on a regular semen collection schedule provided sufficient spermatozoa per ejaculate that this could be incorporated in factorial arrangements in an egg yolk-acetamide extender processed differently. This extender provides substantial protection during freezing, but previous reports indicated that there was considerable damage to spermatozoa during freezing and thawing. Results of experiments reported here demonstrated consistently that mechanical seeding of extended semen at -6°C, so that it did not supercool to -14°C, improved post-thaw progressive motility of spermatozoa substantially ($P<0.01$). Holding the semen at -6°C to equilibrate before seeding, or slow freezing after seeding had only minor effects. Freezing at -15°C/min from -6°C after seeding to -100°C and then transfer to liquid nitrogen was equal or superior to slower rates of freezing (-5°C/min and -10°C/min). Thawing semen at 25°C was superior to 45°C or 65°C ($P<0.01$). With optimal freezing procedures the post-thaw percentage of progressively motile spermatozoa usually exceeded 60%.

455. **Embryo Transfer in Domestic Animals**. R. H. Foote

This is a chapter providing the general reader with a historical perspective on the development of embryo transfer. The advantages and limitations are discussed along with the applications in cattle, sheep, goats, pigs, horses and laboratory animals. In addition to the general procedure of embryo collection and transfer, laboratory procedures of culture, freezing and micromanipulation are introduced. Cloning also is described briefly.

456. **Surface Alterations of the Bovine Oocyte and Its Investments During and After Maturation and Fertilization In Vitro.**
H. Suzuki, X. Yang and R.H. Foote

Surface characteristics of the bovine oocyte and its investments before, during, and after maturation, and fertilization in vitro were evaluated by scanning electron microscopy (SEM). Oocyte diameters were also measured during SEM analysis of the oocyte. The cumulus cells manifested a compact structure with minimal intercellular spaces among them in the immature oocytes. These became fully expanded with increased intercellular spaces after maturation in vitro, but contracted again after fertilization. The zona pellucida (ZP) showed a fibrous, open mesh-like structure in the maturing and matured oocytes. The size and number of meshes on the ZP decreased dramatically after fertilization. The vitelline surface of immature oocytes was characterized by distribution of tongue-shaped protrusions (TSPs) varying in density. After 10 and 22 hr of maturation incubation, oocyte surface microvilli (MV) increased to become the predominant surface structure, and TSPs decreased substantially. The vitelline surface of fertilized oocytes (at 6 and 20 hr) was similar to that of the matured oocytes, but unfertilized oocytes had less dense MV than did fertilized oocytes (at 20 hr). The diameter of the oocytes decreased from 99 to 80 μm during maturation and increased to 106 μm after insemination ($P<0.05$). Membrane maturation was characterized by surface changes from a TSPs-predominant pattern to a MV-predominant pattern. Thus, bovine oocyte maturation involved cumulus cell expansion and ZP maturation which changed dramatically upon fertilization. Also, volumetric changes occurred following oocyte maturation and insemination.

457. **Relationships Between Administration of GnRH, Body Condition Score and Fertility in Holstein Dairy Cattle.**
W. Heuwieser, J.D. Ferguson, C.L. Guard, R.H. Foote, L.D. Warnick and L.C. Breickner

The objective of this study was to determine the effect of GnRH treatment in dairy cows differing in body condition score. A total of 2437 primiparous and multiparous cows was allocated randomly to receive either GnRH (100 μg) or placebo at the post partum exam (25 to 35 d) and at the first breeding. Complete records were available from 1906 cows: Group 1 (n = 535) placebo and placebo; Group 2 (n = 489) GnRH and placebo; Group 3 (n = 438) placebo and GnRH; and Group 4 (n = 444) GnRH and GnRH. The change of body condition score during early lactation was determined on a 1 to 5 scale (1 = emaciated to 5 = obese). Cows with a high body condition score (≥ 3.0) at the first breeding had 8.8 fewer days to first service and 6.4 fewer days open but more services per conception (0.16) than cows with low body condition score (< 3.0). Conception rate improved when GnRH was administered at the first breeding to cows with a body condition score of < 3.0 at the first breeding regardless of parity. The administration of GnRH at the post partum exam decreased the conception rate in first lactation cows but was beneficial for cows in second and greater lactations. Thus the efficacy of GnRH may not be consistent in all parities and body condition groups.

458. **Early Development of IVM/IVF Bovine Embryos Cultured with or without Somatic Cells in a Simple Serum-free Medium with Different Concentrations of CO_2 and O_2.**
B.K. Yang, X. Yang and R.H. Foote

The effect of various O_2 and CO_2 mixtures on development of bovine embryos produced by in vitro maturation and in vitro fertilization (IVM/IVF) was examined in a newly formulated simple KSOM medium versus a complex Menezo B_2 serum-free medium and in co-culture. Numbers of cells comprising blastocysts were also counted using Hoechst 33342 stain. Following routine IVM/IVF procedures oocytes and zygotes were cultured for 40 to 44 h in various media. Then 2- to 8-cell embryos had cumulus cells removed and were allotted randomly for continued culture to the same experimental culture media, but with various gas conditions. In Experiment 1, the percentage of embryos developing to and beyond morula stages in 5, 10 and 20% O_2 with 5% CO_2 in humidified air was 22, 15 and 12%, respectively, with the 22 versus 12% being different ($P<0.05$). Blastocyst cell number in the 5 and 10%

O_2 treatments was higher than in 20% O_2 ($P<0.05$). Also, the simple KSOM and complex Menezo B_2 media were compared and more embryos developed into blastocysts (10%) in KSOM medium than in B_2 medium (5%; $P<0.05$), but cell number did not differ ($P>0.05$). In Experiment 2, the proportions of embryos developing into blastocysts in 5 and 10% CO_2 were 15 and 6% ($P<0.05$), respectively, and in 5 and 10% O_2 were 15 and 7%, respectively ($P<0.05$). Mean blastocyst cell number (82) was highest with 5% CO_2 and 10% O_2 even though more blastocysts were obtained with 5% CO_2 and 5% O_2 ($P<0.05$). In Experiment 3, embryos were co-cultured on monolayers of bovine oviduct epithelial cells (BOEC) or buffalo rat liver cells with KSOM medium in 5 and 10% O_2 and 5% CO_2. Treatment effects did not differ except 10% O_2 was superior to 5% O_2 when considering both morulae and blastocysts (41 vs 33%; $P<0.05$). These experiments indicate that the O_2-CO_2 concentrations can affect the development of bovine embryos produced by IVM/IVF when cultured in a simple serum-free medium with and without co-culture and that the simple KSOM medium can serve as a defined medium to study required supplementation for culturing IVM/IVF bovine oocytes.

459. **Pulsatile Release of FSH for Superovulation in Cattle.** A.G. Jimoh, D.L. Wise, J.D. Gresser, R.H. Foote, R.C. Rhodes, L.H. Underhill and D.J. Trantolo

The studies reported here were directed towards the development of an implantable microcapsule which "pulses" release of follicle stimulating hormone, FSH, for application to superovulating cows. Final dose forms were administered using membrane-coated cylinders. The "pulse" of the FSH is achieved by membrane encapsulation of an effervescent/swelling core containing citric acid, sodium bicarbonate, glucose and FSH. Entry of water results in sufficient pressure increase (by gas generation) to rupture ("burst") the membrane. Time to rupture is dependent upon several factors, such as membrane permeability and thickness, and core composition and loading. The final dose forms were implanted by means of a trochar. This system was tested in sheep to substantiate in vivo "burst" times and then tested in cows to determine efficacy. In vivo burst times in sheep varied from 8 to 96 hr, based upon maximal FSH values in blood serum, and generally parallel the planned times resulting from in vitro tests. Multiple capsules designed to release FSH as a pulse or steady state were tested on a limited number of cows plus a control (n = 10). Four of the combinations resulted in 11, 11, 14 and 16 ovulations, indicating that further development has promise of providing a one-injection system using FSH for superovulating cattle.

460. **Beneficial Effects of Culturing Rabbit Zygotes to Blastocysts in 5% Oxygen and 10% Carbon Dioxide.** P.B. Farrell and R.H. Foote

Zygotes were collected from superovulated Dutch rabbits 19 h after injection of luteinizing hormone and insemination. Oocytes that appeared to be unfertilized were discarded and the zygotes were distributed equally within each donor female across all culture treatments. Culture dishes contained 500 µl of macromolecule-free RD medium consisting of equal parts of RPMI 1640 and low glucose Dulbecco's modified Eagle's medium. Embryos were cultured at 39°C in several gas combinations of N_2 plus the following: 1) 1% O_2:10% CO_2 2) 5% O_2:10% CO_2 and 3) 20% O_2:10% CO_2. The control (treatment 4) was 95% air:5% CO_2. The experiment was replicated with embryos from 11 donors providing 295 useable zygotes. After 84 h of culture the percentages of blastocysts formed in treatments 1 to 4, respectively, were 13, 86, 82 and 59 ($P < 0.01$). The corresponding mean cell counts, including all cleaved embryos cultured (excepting degenerate ones), were 55, 183, 118 and 68 ($P < 0.01$). These results indicate that 10% CO_2 combined with 5% O_2 is a more effective gas phase for culturing rabbit zygotes in a synthetic medium than the commonly used 5% CO_2, and that 5% O_2 is superior to either 1% or 20% O_2.

461. **Effect of Inositol and Glycine with Increasing Sodium Chloride and Constant Osmolality on Development of Rabbit Embryos.** J. Li and R.H. Foote

Many commercially available culture media have a high sodium content as sodium chloride and sodium bicarbonate. We examined the effects of

osmolytes, inositol and glycine, on embryos cultured in synthetic RD medium with a lower concentration of NaCl (93 mM) and a higher concentration of NaCl (116 mM) with media held constant at 270 mosmols. There were no significant effects of either glycine or inositol on embryo growth when the embryos were cultured in RD medium with 93 mM NaCl ($P>0.05$). Culture of 1-cell embryos for 72 hr in RD medium with 93 mM NaCl, 116 mM NaCl and 116 mM NaCl containing 0.56 mM inositol resulted in 77, 14 and 55% expanded blastocysts, respectively ($P<0.05$). Corresponding values for 2-cell embryos cultured for 67 hr were 84, 34 and 66% expanded blastocysts ($P<0.05$). When 1-cell and 2-cell embryos were cultured in RD medium with 93 mM NaCl, 116 mM NaCl, 116 mM NaCl plus 1 mM glycine and 116 mM NaCl plus 1 mM glycine and 0.56 mM inositol, expanded blastocysts from 1-cell embryos were 79, 19, 49, and 48%, respectively ($P<0.05$), and expanded blastocysts from 2-cell embryos were 91, 32, 52, and 53%, respectively ($P<0.05$). Inositol and glycine presumably behave as osmolytes in providing substantial protection for rabbit 1-cell and 2-cell embryos cultured in a medium with high NaCl concentration.

462. **Repeatability of Measurements on Human, Rabbit and Bull Sperm by Computer-assisted Sperm Analysis when Comparing Individual Fields and Means of 12 Fields.**
P. Farrell, V. Trouern-Trend, R.H. Foote and D. Douglas-Hamilton

To compare repeatability of measurements of human, rabbit and bull sperm on two Hamilton Thorne IVOS units, software version 10. Semen samples from seven normal human subjects, six rabbits and eight bulls were obtained at regular intervals. The samples were diluted, two chambers filled and 12 fields were recorded, using high resolution recorders. Computer-assisted sperm analysis (CASA) was performed nearly simultaneously with two Hamilton Thorne IVOS units. Reproduction Research Laboratories, Cornell University and Hamilton-Thorne Research, Beverly, Massachusetts. Optimal settings were established for evaluating by CASA sperm from three species. Fifteen variables were analyzed. The correlation coefficients for most variables characterizing sperm motion and concentration, when means of 12 fields were calculated, were 0.95 to 1.00. There were too few hyperactive sperm to obtain a reliable correlation for human sperm ($r=.63$) and repeatability of elongation was low only for human sperm ($r=.75$). Two units of Hamilton Thorne IVOS, software version 10, were capable of providing nearly identical estimates of many CASA variables of human, rabbit and bull sperm. Correlations for the paired estimates of many sperm characteristics usually exceeded 0.97.

463. **Rabbit Blastocyst: Allocation of Cells to the Inner Cell Mass and Trophectoderm**. J.R. Giles and R.H. Foote

The proportion of total cells in the blastocyst allocated to the inner cell mass (ICM) and trophectoderm (TE) is important for future development and may be a sensitive indicator to evaluate culture conditions. The number of cells and their distribution within the two primary cell lineages were determined for the rabbit embryo developing in vivo after superovulation or nonsuperovulation or embryo transfer and compared with embryos developing in vitro. Comparisons were made with cultured embryos or embryos grown in vivo until 3.5, 4.0 and 4.5 days of age. Embryos from superovulated rabbits developed in vivo for 3.5, 4.0 and 4.5 days, respectively, had 361, 758 and 902 total cells ($P<0.05$) and in nonsuperovulated rabbits had 130, 414 and 905 total cells ($P<0.05$), with increasing proportions of ICM cells over time ($P<0.05$). One-cell embryos recovered from superovulated females and transferred to nonsuperovulated recipients developed more slowly with 70, 299 and 550 total cells after 3.5, 4.0 and 4.5 days of culture ($P<0.05$). The proportion of ICM cells increased with age of the embryo. Corresponding values for one-cell embryos cultured in vitro resulted in 70, 299 and 550 total cells ($P<0.05$). However, in vitro culture of morula stage embryos in the presence of fetal bovine serum for 24 hr did not delay growth. In addition, the proportion of ICM/total cells were 0.17, 0.25 and 0.29 for embryos developing in vitro at 3.5, 4.0 and 4.5 days, respectively, and were similar to those for embryos developing in vivo at each of the three recovery times. These data establish for the first time the number and proportion of cells allocated to the ICM of the rabbit embryo developing in vivo or under defined conditions in vitro.

464. Bovine Oviductal Epithelial Cells (BOEC) and Oviducts: I. For Embryo Culture. II. Using SEM for Studying Interactions With Spermatozoa.

H. Suzuki and R.H. Foote

The oviduct (uterine tube) plays a major role in reproduction. It is a dynamic organ which selectively permits a few sperm to undergo capacitation and reach the oocyte which has continued to undergo maturation following ovulation. Then following fertilization the embryo undergoes cleavage before arriving in the uterus. Extensive information has become available from in vitro studies on oocyte as well as spermatozoal interactions with oviductal cells. Bovine oviduct epithelial cell (BOEC) monolayers with simple media provide an environment in which zygotes can be cultured to blastocysts in 6 days with cell numbers essentially equivalent to blastocysts grown totally in the donor animal. These yield normal pregnancy rates upon transfer. The simple protein-free media currently under test hold promise for elucidating specific requirements of the preimplantation embryo and these defined conditions facilitate many related studies on in vitro fertilization and genetic engineering of embryos. The second part of this paper is an extensive study on the interaction of fresh and frozen-thawed bull spermatozoa with BOEC and segments of intact oviducts as viewed by SEM. Both types of oviductal cells were incubated at 39°C for 0, 3, 6 and 9 hr, using material obtained from periovulatory cows. Sperm attached immediately to both types of epithelium and reached a peak at 3 hr. They were found primarily in the furrows of the intact oviducts. Secretory droplets appeared rapidly on the anterior portion of the sperm head and acrosomal changes were evident in 3 hr, similar to those reported in vivo. Changes were more rapid with frozen-thawed sperm.

465. Nuclear Transfer of Putative Rabbit Embryonic Stem Cells Leads to Normal Blastocyst Development.

F. Du, J.R. Giles, R.H. Foote,
K.H. Graves, X. Yang and R.W. Moreadith

Rabbit embryonic stem-like cells, characterized by embryoid body formation and differentiation into cell types representative of all three germ layers, were studied for their ability to promote early embryonic development after nuclear transfer. After culture of the reconstructed embryos, 23% (n = 35) developed successfully into morulae or blastocysts, compared with 34% (n = 62) for cloned embryos derived from nuclear transfer with embryonic blastomeres. The cloned embryos from the embryonic stem-like cells appeared normal, with an average of 26% inner cell mass cells, similar to that of control non-manipulated embryos (25%) or cloned embryos from blastomeres (25%). Thus, nuclear transfer of rabbit embryonic stem-like cells leads to early embryonic development that is indistinguishable from blastomere fusion. These results have implications for the development of gene targeting in a species (rabbit) that may be a more suitable model for studying certain human diseases. In addition, this technique may be applicable to other species from which putative embryonic stem cells have been derived, particularly agriculturally important animals.

466. Development of Bovine Embryos in KSOM with Added Superoxide Dismutase and Taurine and with Five and Twenty Percent O_2.

Z. Liu and R.H. Foote

To further define requirements for embryo development without the assistance of complex media, serum or co-culture, the effects of 5% and 20% O_2 concentrations, superoxide dismutase (Experiment 1) and taurine (Experiment 2) were tested in a simple culture medium (KSOM modified with 2.5 mM HEPES). Bovine embryos were produced by in vitro maturation and in vitro fertilization of oocytes. After fertilization, embryos were cultured in 5% O_2:5% CO_2:90% N_2 at 39°C for the first 40-44 h, and then embryos with 4- to 8-cells were cultured in different treatments for another 6 days. Percentage development subsequently was based on this number. In Experiment 1, more blastocysts were produced in 5% O_2:5% CO_2:90% N_2 (37%) than in 20% O_2:5% CO_2:75% N_2 (18%, $P < 0.01$). Blastocyst development with 0, 300 and 600 units/ml of superoxide dismutase (SOD), respectively, was 26, 26 and 30% ($P > 0.05$). In Experiment 2, more

blastocysts were formed in 5% O_2 (39%) than in 95% air (30%, $P < 0.05$). Also, with 20% O_2 and O, 7 and 14 mM taurine in the medium, blastocyst formation was 18, 35 and 36% ($P < 0.05$). However, in 5% O_2, 7 and 14 mM taurine was not beneficial ($P = 0.99$), as 39% blastocysts were formed with no taurine. In conclusion, results from both experiments demonstrated that 5% O_2 was superior to 20% O_2 for development of IVM/IVF bovine embryos. Superoxide dismutase had no effect in either 5% or 20% O_2. However, 7 mM and 14 mM taurine counteracted the negative effect of 20% O_2. The best culture system tested here is simple and resulted in sufficient blastocyst formation that it can serve as an excellent base for further studies of embryo requirements.

467. **Development of Early Bovine Embryos in Co-culture with KSOM and Taurine, Superoxide Dismutase or Insulin.** Z. Liu, R.H. Foote and X. Yang

Three experiments, utilizing 2578 embryos, were designed to test the effects of media, taurine, superoxide dismutase and insulin on the development of embryos produced by in vitro maturation and in vitro fertilization (IVM/IVF). Embryos showing at least 1 cleavage during culture for 40 to 44 h after IVM/IVF were selected for further culture under various conditions for 6 d at 39°C in 5% CO_2:95% air. A Buffalo rat liver (BRL) cell co-culture was used in all 3 experiments. Experiment 1 was a 3 × 2 factorial arrangement with KSOM (a high potassium simplex optimization-derived medium containing only 12 ingredients), Menezo B_2 and TCM-199 media with or without 7 mM taurine. Blastocyst production in the 3 media, respectively, was 48, 36 and 29% ($P<0.05$). Addition of 7 mM taurine increased the percentage of blastocysts from 34 to 42 ($P<0.05$). In Experiment 2, superoxide dismutase (SOD) did not improve blastocyst development ($P>0.05$). In Experiment 3, insulin (75 ng/ml) added to KSOM resulted in 46% morulae plus blastocysts compared with 35% for the control ($P<0.05$). These results indicate that the co-culture of embryos in KSOM with taurine or insulin added is superior to commonly used complex media for efficient production of blastocysts following IVM/IVF.

468. **Ethylene Glycol Monomethyl Ether Effects on Health and Reproduction in Male Rabbits.** R.H. Foote, P.B. Farrell, D.H. Schlafer, M.M. McArdle, V. Trouern-Trend, M.E. Simkin, C.C. Brockett, J.R. Giles and J. Li

Male Dutch rabbits were weighed and randomly assigned within each weight group to five groups of six animals each (plus one more in the highest dose group). They received 0, 12.5, 25.0, 37.5 or 50.0 mg of ethylene glycol monomethyl ether (EGME) per kg of body weight in the drinking water 5 days/week for 12 weeks. Feed and water consumption were monitored daily and body weight weekly. All animals consumed the water and feed, maintained body weight and were in good health throughout the experiment. Semen was collected twice weekly for 12 weeks and 96% of the ejaculates were obtained. By weeks 6 and 9 most males in groups receiving 50.0 or 37.5 mg of EGME per kg were oligospermic. Only minor changes in other characteristics of sperm obtained from treated animals were found, as measured by computer assisted sperm analysis. Fertility of the males still producing sufficient sperm during week 12 to use for insemination was tested with 96 does producing 2839 oocytes, and fertility of treated males (41%) was not lower ($P > 0.05$) than 47% in controls. At necropsy all vital organs were grossly normal with no notable histopathology. However, the groups of animals receiving 37.5 and 50 mg of EGME per kg of body weight produced fewer sperm and had smaller testes than controls ($P < 0.05$). Although, all rabbits appeared grossly normal, there was a marked disruption of spermatogenesis as ingestion of EGME increased above 25 mg/kg of body weight. Rabbit testes appear to be more sensitive to EGME than testes of rats or mice.

469. **Development of In Vitro Matured/In Vitro Fertilized Bovine Oocytes In A Simple Defined (KSOM) Medium.** B.K. Yang, J.R. Giles, X. Yang and R.H. Foote

The experiments reported here take advantage of the large number of in vitro matured and in vitro

fertilized (IVM/IVF) bovine oocytes which can be produced, permitting the design of controlled experiments to establish a simple defined medium for the study of early embryo requirements and to further test this medium as a component of co-culture. A total of 1386 IVM/IVF oocytes were used to compare a simple medium (KSOM) with complex culture conditions used successfully for culture of bovine embryos. All experiments were extensively replicated factorials. In Experiment 1, KSOM was equivalent to the complex Menezo B_2 medium in producing blastocysts from IVM/IVF produced embryos and was superior to Menezo B_2 medium when both were used with buffalo rat liver cells (BRLC), yielding 25% vs 8% blastocysts, respectively ($P<0.05$). In Experiment 2a, KSOM was tested with 0 or 1 ng/ml of platelet derived growth factor (PDGF) and in Experiment 2b, 0, 1 and 5 ng/ml of PDGF were tested. In Experiment 2a blastocyst formation was higher ($P<0.05$) when PDGF was added to the co-culture and also was higher ($P<0.05$) for morulae plus blastocysts in Experiment 2b when PDGF treatments were combined and compared to no PDGF. The results with the simple KSOM medium are sufficiently promising to indicate that growth factors, amino acids and other specific requirements of the embryo may be examined in future studies with KSOM as a base. Additionally, KSOM appears to be superior to Menezo B_2 medium often used commercially for co-culturing bovine embryos with BRLC.

470. **Effects of Amino Acids on Development of IVM/IVF Bovine Embryos in a Simple Protein-free Medium.**

Z. Liu and R.H. Foote

Effects of commercially available amino acids, referred to as Eagle's non-essential amino acids (NEAA) and essential amino acids (EAA), on the development of bovine embryos produced by in vitro maturation (IVM) and in vitro fertilization (IVF) in a simple modified protein-free KSOM medium containing polyvinyl alcohol (PVA) were studied. Embryos produced by IVF were cultured in 5% O_2:5% CO_2:90% N_2 at 39°C for the first 40-44 h in modified KSOM. Then embryos with ≥ 4 cells were cultured in modified KSOM-PVA with different amino acids for another 6 days in experiments 1 and 2. In Experiment 3, to further study embryo hatching and degeneration as criteria of media suitability, the culture time was 8 days. In Experiment 1 more blastocysts were produced ($P < 0.05$) in the medium with 0.5X NEAA (37%) than with 0.25X or 0.5X EAA (both 15%). In Experiment 2, when 0, 0.5 and 1X concentrations of NEAA were combined with 0 and 0.5X concentrations of EAA, the 1X NEAA with 0.5X EAA resulted in the most blastocysts (39%) with 11% hatching ($P < 0.05$). Without NEAA, 1X EAA resulted in 6% blastocysts ($P < 0.05$). In Experiment 3, KSOM-PVA with 1X NEAA plus 0.5X EAA resulted in 41% blastocysts, similar to 40% blastocysts in KSOM-BSA with the same amino acids added. When culture was continued for 2 more days, more embryos hatched in these two treatments. These results indicate that the combination of 1X NEAA with 0.5X EAA promoted blastocyst formation and hatching, but EAA alone was detrimental. With PVA successfully replacing BSA, a completely defined medium resulted which was equal or superior to complex media, and is potentially useful for embryos from other species, such as humans.

471. **Media and Dilution Procedures Tested to Minimize Handling Effects on Human, Rabbit and Bull Sperm for Computer-Assisted Sperm Analysis (CASA).**

P.B. Farrell, R.H. Foote, M.M. McArdle, V.L. Trouern-Trend and A.L. Tardif

Proper handling of semen prior to CASA is critical, if the analysis is to be representative of the fresh sample. The effects of diluting medium or dilution and holding time before CASA on multiple sperm characteristics were studied. Four replicates of unselected semen samples from each of eight human donors were diluted with PBS-glucose plus BSA, with TALP and with high potassium TALP to a concentration of approximately 25×10^6 sperm/ml. The diluted semen was held for 0, 1 and 2 hours at approximately 30°C before CASA, with little difference between the three diluters in all 12 variables measured. There was a decline of 3 to 6% in the proportion of motile sperm during 2 hours ($P<0.05$). Donors were the largest source of differences ($P<0.05$). Rabbit sperm (5 bucks × 4 ejaculates per buck) were processed similar to the human sperm. There was a major effect of media. The

average percentages of motile sperm over 2 hours in TALP, K-TALP and PBS were 76, 42 and 29, respectively ($P<0.05$), with a decline of only 3% in TALP during 2 hours. Hyperactivity and other characteristics were affected by treatment. Donors were a large source of variation. Bull semen (10 bulls × 2 ejaculates per bull) either was not diluted or diluted 2-fold or 4-fold with TALP, held for 0, 1 and 2 hours at 30°C and then diluted to 25×10^6 sperm/ml with TALP. There was little change in most sperm characteristics in any treatment during the first hour, although many were statistically significant. The percentage of motile sperm in undiluted semen declined from 87 to 82% during 2 hours. Modified TALP was a suitable medium for sperm from all three species, and a simple PBS-glucose-BSA medium can be used for human sperm.

472. **Methods for Assessing Sperm Motility, Morphology, and Counts in the Rat, Rabbit, and Dog: A Consensus Report.** J. Seed, R.E. Chapin, E.D. Clegg, L.A. Dostal, R.H. Foote, M.E. Hurtt, G.R. Klinefelter, S.L. Makris, S.D. Perreault, S. Schrader, D. Seyler, R. Sprando, K.A. Treinen, D.N.R. Veeramachaneni and L.D. Wise

Reproductive toxicity studies are increasingly including assessments of sperm parameters, including motility, morphology, and counts. While these assessments can provide valuable information for the determination of potential reproductive toxicity, the methods for conducting the assessments have not been well developed in all laboratories and are continually evolving. The use of different methods in different laboratories makes comparison of data among laboratories difficult. To address the differences in methods, a working group was convened to discuss methods currently in use, share data, and try to reach consensus about optimal methods for assessment sperm parameters in rats, rabbits, and dogs. This article presents the consensus report, as well as future research needs, with the hope that optimized common methods will aid in the detection of reproductive effects and enhance interlaboratory comparisons.

473. **Dairy Cattle Reproductive Physiology Research and Management: Past Progress and Future Prospects.** R.H. Foote

Artificial insemination developed as the solution for two important problems in the dairy cattle industry during the past 50 yr: 1) the need for genetic improvement and 2) the elimination of costly venereal diseases. Cooperation of researchers, extension workers, veterinarians, dairy producers and emerging AI organizations in pooling their expertise, was instrumental in the remarkably rapid development of AI. The cooperation of universities, government, and producers to fund reproductive specialists to collaborate and transfer findings quickly to potential users was a major component of this successful venture. Money invested in these experiments was estimated to have returned about $100 for each $1 invested. Successful freezing of sperm led to the development of the field of cryobiology, and AI paved the way for embryo transfer. The development of ultrasound equipment; various types of rapid hormone assays; prostaglandins, progestogens, and GnRH; and computerization made various alternative management plans for controlling reproduction viable. Multidisciplinary, multigeographical teams to gather basic needed information have potential for making excellent progress. As herd size increases, new programs for efficient reproductive management and for identifying needed research through computer modeling are a must. Sexed embryos from elite cows and bulls will be used selectively. When embryonic stem cell technology becomes practical, it will revolutionize cattle breeding.

474. **Effect of Inert Particles in Rabbit and Bull Semen on Sperm Cell Concentration and Motility Measured by CASA, and Variability Due to Chamber Differences.** P.B. Farrell, R.H. Foote and D.H. Douglas-Hamilton

The precision, accuracy and repeatability of computer assisted sperm analysis (CASA) procedures developed to discriminate between spermatozoa and granules in rabbit semen were tested. Also,

to explore the general applicability of the system, bull semen, essentially free of nonsperm particles, was used as a model system to test the effects of variable-sized granules, uniform latex spheres and chamber depth on counts of spermatozoa. The CASA estimates were obtained on spermatozoa diluted with TALP to approximately 25×10^6 spermatozoa per milliliter and evaluated at 37°C. In replicated factorial experiments the estimated concentration of rabbit spermatozoa in semen did not differ between counts obtained by CASA with a Hamilton Thorne unit and with the hemacytometer. Repeatability between paired counts by CASA was +0.98. Granules did not interfere with estimates of concentration or motility of spermatozoa unless the numbers exceeded 20% of the sperm cell population. Counts on bull spermatozoa by CASA were correlated with estimates obtained spectrophotometrically ($P=0.91$, $P<0.05$). Extensive additions of granules or 3.9 µm diameter latex spheres to bull spermatozoa were required before there was any detectable effect on sperm numbers or motility. Variation in chamber depth was a substantial source of variation and a novel method of calibration is described. Also, known numbers of latex spheres can be added to spermatozoa to increase reproducibility of results with CASA. These procedures are applicable to spermatozoa of other species.

475. **Sodium Chloride, Osmolyte and Osmolarity Effects on Blastocyst Formation in Bovine Embryos Produced by in Vitro Fertilization (IVF) and Cultured in Simple Serum-free Media.** Z. Liu and R.H. Foote

A simple serum-free medium (KSOM), relatively low in NaCl concentration, has been developed for culturing mouse zygotes produced in vivo. The present studies were designed to test modifications of this medium to establish optimal conditions for culture of bovine embryos produced in vitro. Embryos were produced by standard in vitro fertilization procedures. They were cultured for 6 days in media varying in NaCl concentration and osmolarity with and without osmolytes, betaine or inositol. Increasing NaCl concentration from 95 mM to 108.5 and 122 mM decreased blastocyst formation ($P<0.05$). Partial substitution of NaCl by sorbitol indicated that both NaCl concentration and osmolarity were important. Neither betaine nor inositol protected the embryos against high concentrations of NaCl. The concentration of NaCl should not be higher than 95 mM and total osmolarity of the medium should be between 250 and 270 mosmols.

476. **Motility and Other Characteristics of Human Sperm Can Be Measured by Computer Assisted Sperm Analysis of Samples Stained with Hoechst 33342.** P.B. Farrell, R.H. Foote and M.J. Zinaman

To develop methods for using a DNA-specific dye to discriminate between motile and nonmotile sperm and static particulate matter in fresh and diluted semen, using computer-assisted sperm analysis (CASA). Donor semen was divided for treatment as fixed stained sperm (Hoechst 33342 stain, Sigma Chemical Company, St. Louis, MO), and fresh motile and nonmotile stained sperm, along with unstained control sperm. Normal human volunteers in an academic research and medical environment were used. The semen was delivered to the laboratory within one hour of collection. Semen quality was measured by CASA. Fixed or fresh human sperm stained with Hoechst 33342 dye should be diluted to $\leq 50 \times 10^6$ sperm/mL to count sperm accurately. Motile and nonmotile sperm were suitably stained with 5 to 10 µg/mL of dye when diluted with a simple diluent, but the dye concentration should be increased to 40 µg/mL when egg yolk is in the diluent. The DNA-specific dye, Hoechst 33342, can be used to discriminate between motile and nonmotile sperm and other particulate matter when evaluated by CASA with instrumentation equipped with suitable optics.

477. **Effect of Amino Acids and α-Amanitin on the Development of Rabbit Embryos in Modified Protein-free KSOM with HEPES.** Z. Liu, R.H. Foote and M.E. Simkin

Four experiments were conducted to test the effects of Eagle's non-essential amino acids (NEAA) and essential amino acids (EAA), glycine and the RNA polymerase inhibitor, α-amanitin, on the development of preimplantation rabbit embryos in

modified protein-free KSOM medium. Embryos were distributed randomly into different treatments and cultured in 5% O_2:5% CO_2:90% N_2. In Experiment 1, 100% of the embryos became blastocysts in the medium with Eagle's 1X NEAA and 0.5X EAA, but 100% stopped development at the morula stage in KSOM without amino acids. These morulae failed to develop further when transferred to amino acid supplemented medium after 72 hr of culture. Glycine alone in modified KSOM (Experiment 2) was ineffective in supporting development of 8-16-cell stage embryos past the morula stage. In Experiment 3, the addition of 1X NEAA and 0.5X EAA at 0, 12, 24, 36 and 48 hr of culture resulted, respectively, in 57, 65, 65, 44 and 14% blastocysts on Day 3 ($P<$ 0.05) and 86, 77, 77, 78 and 69% on Day 5 ($P >$ 0.05). Omission of Eagle's amino acids until 48 hr clearly delayed embryo development. In Experiment 4, when α-amanitin (20 μM) was added to the medium containing Eagle's amino acids after 0, 12, 24, 36 and 48 hr of culture most embryos cleaved only once or twice after adding the α-amanitin. Without the inhibitor, 94% of the zygotes developed into blastocysts. These results indicate that modified KSOM or KSOM plus glycine could not support rabbit embryo development past the morula stage, but this block was overcome by adding Eagle's amino acids. An exogenous source of amino acids was not critical for embryo development during the first 24 hr of culture, but was required after that for development to equal controls. Addition of α-amanitin at multiple pre-blastocyst stages limited further embryo development to 1 or 2 cleavage divisions, with no blastocyst development.

478. **Computer-Assisted Sperm Analysis (CASA) of Human, Rabbit and Bull Sperm with the Hamilton Thorne Unit Equipped to Detect Hoechst 33342 Stain.** R.H. Foote

Computer-assisted Sperm Analysis (CASA) offers the potential of simultaneously characterizing many parameters of large numbers of spermatozoa rapidly and objectively. Results can be video taped for review and reanalysis. To maximize the probability of detecting true differences among samples of semen or males producing the semen, all random or systematic errors in processing the semen should be kept to a minimum. These potential sources of variability include the following: 1) initial sampling of the biologic material, 2) method of processing the semen for CASA, 3) time elapsing between initial sampling and analysis, 4) instrument settings and gates used in analyzing the specimen, 5) accuracy of the specimen chambers used, and 6) the number of chambers, fields, and sperm examined to provide adequate statistical sampling of the material being analyzed. Different instrument settings on the instrument are required for valid evaluation of sperm from different species. Also, when comparing values among laboratories, equipment made by different manufacturers likely will yield somewhat different results. However, different machines of the same make and model should give equivalent results when the same settings are used. Our research in recent years has all been done with Hamilton Thorne equipment. When two instruments of the same model line are compared with the same settings, they yield almost identical results provided a sufficient number of sperm are evaluated ($r \geq .98$). A sufficient number of sperm to analyze for human, rabbit and bull, we have found is at least 200 per sample. We dilute semen to about 20 to 25 $\times$ 10^6 sperm per ml, so that there are about 25 sperm per field. Then we usually count two chambers with six fields per chamber at 37°C. This yields approximately 300 total sperm, but the number in each category (such as the percentage of motile sperm) is less than 300. The initial collection of semen must be done under optimal conditions, following brief sexual rest to obtain a representative sample. These samples should be collected on more than one occasion. They should be preserved (diluted) with the proper medium, temperature shock should be avoided and the CASA analysis should begin relatively soon after semen collection. We found that with a modified Tyrode's solution semen from rabbit, human and bull can be preserved well for at least 1 hour at 30°C before using CASA, although the analysis should start as soon as possible. Use of PBS for dilution was less satisfactory. Specimen chambers vary somewhat and either the same glass chambers should be used or use disposable chambers from a reliable manufacturer. We minimize variation among chambers by not counting the first two or last two fields in each chamber. The settings used to evaluate sperm of different species by CASA obviously are very important. The playback on the Hamilton Thorne unit allows one to check many fields on the TV

monitor visually and ascertain that the imaging equipment is properly identifying and classifying each sperm cell. This is especially important when nonsperm particulate matter might erroneously be identified as dead sperm. When there is extensive foreign material in the semen, or with semen extenders such as whole milk, which virtually obscure sperm, with phase contrast, the use of Hoechst dye 33342 can be used. The Hamilton Thorne IVOS unit is equipped with ultraviolet (UV) illumination to detect sperm heads stained with this DNA-specific dye.

479. Differential Sensitivity of One-cell and Two-cell Rabbit Embryos to Sodium Chloride and Total Osmolarity During Culture into Blastocysts. J. Li and R.H. Foote

One-cell or two-cell rabbit embryos were cultured in protein-free media varying in NaCl concentration and osmolarity to determine relative sensitivity of embryos to changes in media composition. Embryos from replicates of donor rabbits were distributed randomly across treatments and cultured at 39°C. Zygotes were cultured in Experiments 1, 2A and B, and 3, and 2-cell embryos were cultured in Experiments 4A and 4B. In Experiment 1, blastocyst formation and number of cells were highest ($P<0.05$) in the control medium with 93 mM NaCl (270 mosmols) compared with media containing 63 and 116 mM NaCl (220 and 316 mosmols). In Experiment 2, embryos were cultured in media with 70 or 93 mM NaCl, varying in osmolarity from 250 to 320 mosmols by adding sorbitol. In media with 70 mM NaCl and osmolarities of 250, 280 and 300 mosmols, there were 41, 56 and 50% expanded blastocysts, respectively ($P<0.05$). With 93 mM NaCl and osmolarities of 270, 293 and 320 mosmols, embryos developed into 37, 53 and 27% expanded blastocysts, respectively, ($P<0.05$). In Experiments 3A and 3B and 4A and 4B, the osmolarity of the medium was maintained at 270 mosmols by adding sorbitol to media containing 40 or 60 mM NaCl, and other ingredients were reduced in media containing 100 and 116 mM NaCl to compensate for the higher NaCl. Zygote development into blastocysts was greatly suppressed ($P<0.05$) in media with 40, 60, 100 and 116 mM NaCl, compared with the control (93 mM NaCl), whereas development of 2-cell embryos into blastocysts was much less affected. These results appear to reflect a direct sodium chloride as well as osmolarity effect on embryo development.

480. Effects of Amino Acids and α-Amanitin on Bovine Embryo Development in A Simple Protein-Free Medium. Z. Liu and R.H. Foote

Five experiments, utilizing 3741 embryos produced in vitro, were designed to test the effects of Eagle's non-essential amino acids, and combinations of Eagle's essential amino acids and the RNA polymerase inhibitor, α-amanitin, on the development of preimplantation bovine embryos in a modified protein-free KSOM medium. Embryos were cultured in 5% O_2:5% CO_2:90% N_2 at 39°C for the first 40-44 hr in modified KSOM and embryos with ≥ 4 cells were cultured in modified KSOM-PVA with different amino acids in Experiments 1 to 4, and with the addition of α-amanitin in Experiment 5. In Experiment 1, addition of 0.5X of the essential amino acids, with different concentrations of nonessential amino acids significantly increased hatching of blastocysts and decreased blastocyst degeneration, but increasing the non-essential amino acids from 1X to 5X did not stimulate embryo development. In Experiments 2 to 4, increasing only the glycine concentration, or adding each of the 12 essential amino acids singly or several in combination to the medium containing nonessential amino acids, did not significantly improve embryo development. Taurine (0.4 mM) in the modified KSOM medium reduced blastocyst degeneration. In Experiment 5, α-amanitin (20 μM) completely inhibited further embryo development when it was added at several stages from 4-cell embryos to morulae. The study with protein-free KSOM plus amino acids provided a completely defined simple medium for culturing bovine embryos, with evidence that continuous mRNA activity and presumed protein synthesis was obligatory to meet the complex and continuous requirements for proteins by the developing blastocyst.

481. **Disruption of Spermatogenesis in Rabbits Consuming Ethylene Glycol Monomethyl Ether.**
W.E. Berndtson and R.H. Foote

Effects of ethylene glycol monomethyl ether (EGME) on spermatogenesis were examined using groups of six to seven Dutch rabbits that received 0, 12.5, 25.0, 37.5, or 50.0 mg of EGME/kg body weight, respectively, in their drinking water 5 d/week. After 12 weeks, animals were euthanized and their testes were removed, weighed, and processed to permit germ cell numbers to be quantified. Spermatogenesis was depressed by EGME in a dose-dependent manner; numbers of round spermatids per Sertoli cell (a measure of spermatogenic efficiency) averaged 8.86, 8.87, 6.20, 2.38, and 7.42 for the 0, 12.5, 25.0, 37.5, and 50.0 mg/kg dosages, respectively. The latter value of 7.42 represents an overestimation of sperm production because it is based on only two unexpected outlier rabbits. Nearly complete destruction of spermatogenesis occurred in the other five animals in this highest dosage group, precluding evaluation by the histometric method. Numbers of homogenization-resistant elongated spermatids per testis, measurable on all animals, averaged 231, 256, 195, 52, and 67 $\times$ 10^6, respectively. The correlation between the predicted sperm production, based on the elongated spermatids at necropsy, and the number of sperm ejaculated by the males during week 12 was 0.92. Thus, EGME impaired rabbit spermatogenesis in a dose-dependent manner. Generally, rabbit spermatogenesis was at least 10 times more sensitive to EGME than previously reported for rats and mice.

482. **Development of Rabbit Zygotes into Blastocysts in Defined Protein-Free Medium and Offspring Born Following Culture and Embryo Transfer.**
J. Li, R.H. Foote, Z. Liu and J.R. Giles

Requirements for optimal culture of embryos are not known. Most commercially used systems include complex media, serum and co-culture. Some components may be unnecessary and even inhibitory. In the present report, completely defined protein-free media, previously found to be useful in culturing rabbit or bovine embryos, was modified and production of young following culture of rabbit embryos in these media was tested for the first time. Zygotes were obtained from superovulated rabbits. The effect of a novel antioxidant, tempol, was tested (20 to 0.001 mM), but it was either toxic or ineffective. Either 600 units of superoxide dismutase or 2.5 mM of taurine increased embryos hatching after 72 h of culture in RD medium from 46% in the control to 75 and 76%, respectively ($P<0.05$), in the presence of 20% O_2. Smaller effects were observed with 5% O_2. Transfer of 60 embryos cultured for 48 h to the early blastocyst stage resulted in 30 young (50%) compared with 35/60 (58%) young from uncultured control transfers. Only 12% young were obtained from slower developing morulae. In modified KSOM medium, pregnancy rate from transferred morulae was low, but results were equivalent to embryos transferred without culture or cultured in RD medium. Thus, high viability was established for rapidly growing embryos, but fewer slow growing embryos were rescued.

483. **Effects of Gas Atmosphere, Platelet Derived Growth Factor and Leukemia Inhibitory Factor on Cell Numbers of Rabbit Embryos Cultured in a Protein-free Medium.**
J.R. Giles and R.H. Foote

While culture conditions for embryos has improved, development in vitro is not equivalent to in vivo growth. Platelet-derived growth factor (PDGF), mouse or human leukemia inhibitory factor (mLIF, hLIF) and 10% fetal bovine serum (FBS) added to protein-free culture medium, as well as two gas atmospheres were evaluated for effects on rabbit embryo development. Adding PDGF, mLIF or hLIF to the culture medium did not result in detectable differences in total cell number for blastocysts. The culture of rabbit embryos under a gas atmosphere of 10% CO_2:5% O_2:85% N_2 resulted in improved total cell numbers ($P<0.01$) for blastocysts compared to those developing under 5% CO_2:95% air (230 vs. 159, respectively). Supplementing RD medium with 10% FBS improved the number of total cells (264 vs. 155) and inner cell mass cells (71 vs. 47). These

results indicate that when defined culture conditions promote a high proportion of 2-cell embryos developing into blastocysts, the addition of certain growth factors may not have a detectable beneficial effect, although 10% FBS improved culture conditions.

484. Computerized Imaging and Scanning Electron Microscope (SEM) Analysis of Co-Cultured Fresh and Frozen Bovine Sperm.

H. Suzuki, R.H. Foote and P.B. Farrell

Fresh and frozen-thawed bull sperm were incubated with bovine oviductal epithelial cells and segments from the oviducts to examine the usefulness of these culture systems to model sperm changes in vivo. Changes in sperm motion characteristics (CASA) and surface morphology (SEM) were evaluated. In Experiment 1, fresh and frozen sperm were suspended in the Brackett and Oliphant medium or modified Tyrode's medium (mTALP) and incubated for 0, 3, 6 and 9 hours in direct contact with bovine oviductal epithelial cell (BOEC) monolayers prepared from oviducts of cows in the periovulatory phase of estrus. The percentage of motile sperm decreased gradually in mTALP, but decreased rapidly in Brackett's defined medium after 3 hours of incubation, with overall averages of 55 and 32%, respectively. The percentage of motile fresh sperm exceeded frozen-thawed sperm under all conditions. In Experiment 2, sperm suspended with mTALP were incubated in dishes without monolayers (control), with monolayers, and within the segments of the oviduct for 0, 3 and 6 hours. In the epithelial cell monolayers the percentage of motile sperm was similar to the controls throughout incubation, but after 3 hours in the oviductal segments, a decrease, partly associated with more rapid rupture of acrosomal membranes occurred. Sperm velocity was higher (100 μm/seconds) in fresh sperm than in frozen sperm (85 μm/seconds). Acrosomal changes, discernible with SEM after 3 hours of incubation, increased with time and were always found more often in frozen than in fresh sperm. The BOEC monolayer system provided a useful in vitro model to study pre-fertilization changes in sperm.

485. Computer Assisted Sperm Analysis for Assessing Initial Semen Quality and Changes During Storage at 5°C.

A.L. Tardif, P.B. Farrell, V. Trouern-Trend and R.H. Foote

Computer-assisted sperm analysis equipment was used to evaluate bull sperm initially in a modified Tyrode's solution, in Cornell University extender and in egg yolk-glycerol-tris extender, and following cooling and storage in the latter two extenders. Two ejaculates of semen were collected from each of eight bulls. Semen was aliquotted in a factorial arrangement. The semen, diluted to approximately 20×10^6 sperm/ml, was loaded into two 20-μm chambers, and six microscope fields from each chamber were videotaped for each treatment of each ejaculate of semen. Eight sperm characteristics analyzed with the Hamilton Thorne IVOS unit are reported and bulls differed significantly in several of these. The initial percentages of motile sperm in modified Tyrode's solution, Cornell University extender, and egg yolk-glycerol-tris extender were 87, 79 and 66; little change followed after cooling and storage at 5°C in the latter two extenders. Also, there was a small but significant decline in sperm velocity during 3 d of storage. Hyperactive sperm increased slightly during storage. The procedures used can rapidly and accurately measure many sperm characteristics in fresh semen and in semen stored in egg yolk extenders, and detect differences among bulls.

486. Sperm Numbers Inseminated in Dairy Cattle and Nonreturn Rates Revisited.

R.H. Foote and M.T. Kaproth

Three experiments were conducted to test fertility when sperm numbers per insemination ranged from 10×10^6 to 40×10^6 total sperm. All semen was from Holstein bulls that were on a regular schedule of semen collections. The semen was extended with heated homogenized whole milk, cooled, glycerolated, and frozen according to standard procedures. Data resulted from semen distributed to a large group of inseminators to

minimize differential field effects on treatment. All experiments were a randomized block design, including a split plot in Experiment 2. In Experiment 1, data for 31,399 first inseminations distributed among treatments of 20×10^6, 25×10^6, 30×10^6, and 40×10^6 total sperm resulted in 69.8, 70.0, 70.1, and 70.1% nonreturns at 59 d, respectively. In Experiment 2, data for 18,197 first inseminations divided over treatments of 12×10^6, 16×10^6, and 20×10^6 total sperm resulted in 70.2, 72.4, and 70.8% nonreturns at 59 d, respectively. In Experiment 3, 38,890 first inseminations distributed over treatments of 10×10^6, 13×10^6, 16×10^6, and 20×10^6 total sperm resulted in 70.5, 72.2, 73.1 and 71.5% nonreturns at 59 d, respectively. Bull nonreturns ranged from 64 to 76% in the three trials. These results indicate that, under good conditions, total sperm numbers per straw can be reduced to 10×10^6 total sperm with a reduction of nonreturn rates at 59 d, for most bulls, of about 1 percentage unit from the maximum when professional inseminators are used.

487. Effect of Repeated Testicular Biopsy On Testis Function and Semen Quality in Normal and Triethylenemelamine Treated Dogs.

W.L. Hunt and R.H. Foote

The study was designed to quantitatively evaluate the effect of repeated testicular biopsy of avascular areas by the open method upon spermatogenesis, semen quality and sperm output in normal dogs and those with depressed spermatogenesis following triethylenemelamine (TEM) treatment. After a 5-week standardization period of semen collections three times per week, 20 sexually mature Beagle dogs were divided into four groups, approximately equalized based on previous sperm output. Semen collections were continued for 20 weeks. One group was controls without treatment until unilaterally orchidectomized after weeks 16 and 20; a second group had one testis biopsied on weeks 1, 5, and 9, and removed after week 16, and the second testis removed after week 20. Groups three and four received 0.2 or 0.4 mg/kg of body weight of TEM at week 0, with surgery the same as for group 2. Treatment had little or no effect on the percentage of motile sperm. Sperm output per week during the summer declined in the control group from 851×10^6 to 725×10^6 (15%). Thus, part of the decline in the biopsied control group (22%) and decreases in sperm output for the 0.2 mg and 0.4 mg of TEM groups of 56% and 83%, respectively, may reflect seasonal effects. At 16 weeks the testes removed from the controls averaged 7.1 g and the testes biopsied three times in the second group averaged 6.4 g ($P > 0.05$). Scrotal-testicular measurements taken in live animals were correlated with testicular weights at orchidectomy ($r = 0.81$). An average of 36.7 mg of tissue was removed at each biopsy, sufficient to evaluate 100 to more than 200 seminiferous tubules in cross-section. Frequency of stages of the cycle of the seminiferous epithelium was similar and normal in the control and biopsied control dogs. The biopsy procedure was equally applicable to testes with suppressed spermatogenesis, but stages were not quantified because of stage disruption. It is concluded that repeated testicular biopsy of avascular areas can provide sufficient tissue for histopathologic evaluation without significant interference with spermatogenesis.

488. Quantification of Bull Sperm Characteristics Measured by CASA and the Relationship to Fertility.

P.B. Farrell, G.A. Presicce, C.C. Brockett and R.H. Foote

Two experiments were conducted to evaluate semen quality of bulls housed under controlled conditions at a large AI facility and relate results to fertility. In Experiment 1 semen was collected from six 6-yr-old bulls twice daily at 3- to 4-d intervals for 3 d. In Experiment 2 eleven 6- to 11-yr-old bulls were used. Extensive breeding information was available and semen was collected as in Experiment 1 but replicated 4 times. Standard semen analysis and computer-assisted sperm analysis (CASA) with the Hamilton Thorne IVOS, model 10 unit, were performed on 36 first and second ejaculates in Experiment 1 and on 44 first ejaculates in Experiment 2. Sixteen fields (2 chambers with 8 fields per chamber) were examined per sample. In Experiment 1 the correlation between estimated sperm concentration by spectrophotometry and CASA was 0.91 ($P < 0.01$). Among bulls the range in the percentage of

motile spermatozoa was 52 to 82 for CASA versus 62 to 69 for subjective measurements made by highly experienced technicians. Thus, CASA, with high repeatability, provided a more discriminating estimate of the percentage of motile sperm cells than did the subjective procedure. Bull effect was much greater than any other variable in the experiments. Chamber differences were small and so the results for the 2 chambers with 8 fields each were combined. One to five CASA values were correlated with bull fertility, defined as 59-day nonreturn rates corrected for cow and herd effects. The percentage of motile spermatozoa accounted for a small fraction of the total variation in fertility ($r^2 = 0.34$). However higher r^2 values (0.68 to 0.98) were obtained for 2 to 5 variables used in the multiple regression equations. The results are promising, and further testing will determine more precisely which of these CASA variables are most useful in estimating bull fertility potential.

489. Use of Hoechst 33342 Stain to Evaluate Live Fresh and Frozen Bull Sperm by Computer Assisted Analysis.

A.L. Tardif, P.B. Farrell, V. Trouern-Trend, M.E. Simkin and R.H. Foote

The objective of this research was to investigate possible procedures for evaluating living bull sperm stained with Hoechst 33342 while in a simple medium and in commonly used complex egg yolk-glycerol-tris, and whole milk-glycerol extenders. The two semen extenders provide good cryoprotection, but whole milk-glycerol virtually obscures the sperm. In order to evaluate sperm motion characteristics when static nonsperm particles are present, a new Hamilton-Thorne epifluorescent optical system (UV) with a strobe light was developed for potential use with DNA-stained sperm. This permitted exam-ination for the first time of sperm motion characteristics in milk. In Experiment 1 (4 bull semen replicates with 5 dye concentrations $\times$ 3 incubation times), 2.5 μg/ml of Hoechst 33342 stained live and dead sperm sufficiently in a modified Tyrode's solution to measure all sperm characteristics without depressing motility, validated by using phase contrast to analyze stained and unstained controls. In Experiments 2a and 2b, each using semen from 4 bulls, with a 5 $\times$ 5 factorial, it was determined that 40 μg/ml to 60 μg/ml of dye in egg yolk-glycerol-tris or whole milk-glycerol, with UV illumination for 20 minutes, was optimal. There was no detrimental effect on sperm motility. In Experiment 3, analyses of two ejaculates, from each of eight bulls, confirmed that motion characteristics of sperm in egg yolk-glycerol-tris and while milk-glycerol were not depressed when stained with Hoechst 33342. These experiments demonstrate that the dye concentrations and exposure times developed for use with the new epifluorescent optics facilitate evaluating bull sperm frozen in particle-filled whole milk, and should be useful for sperm evaluation of a variety of species when nonsperm particulate matter may otherwise interfere.

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APPENDIX

This short list of references contains thousands of references covering more than 50 years of research. Also, The National Association of Animal Breeders publishes much on AI in their biennial Proceedings of Technical Conferences through their headquarters at Columbia, MO. Each January issue of the journal Theriogenology is packed with reproductive biotechnologies.

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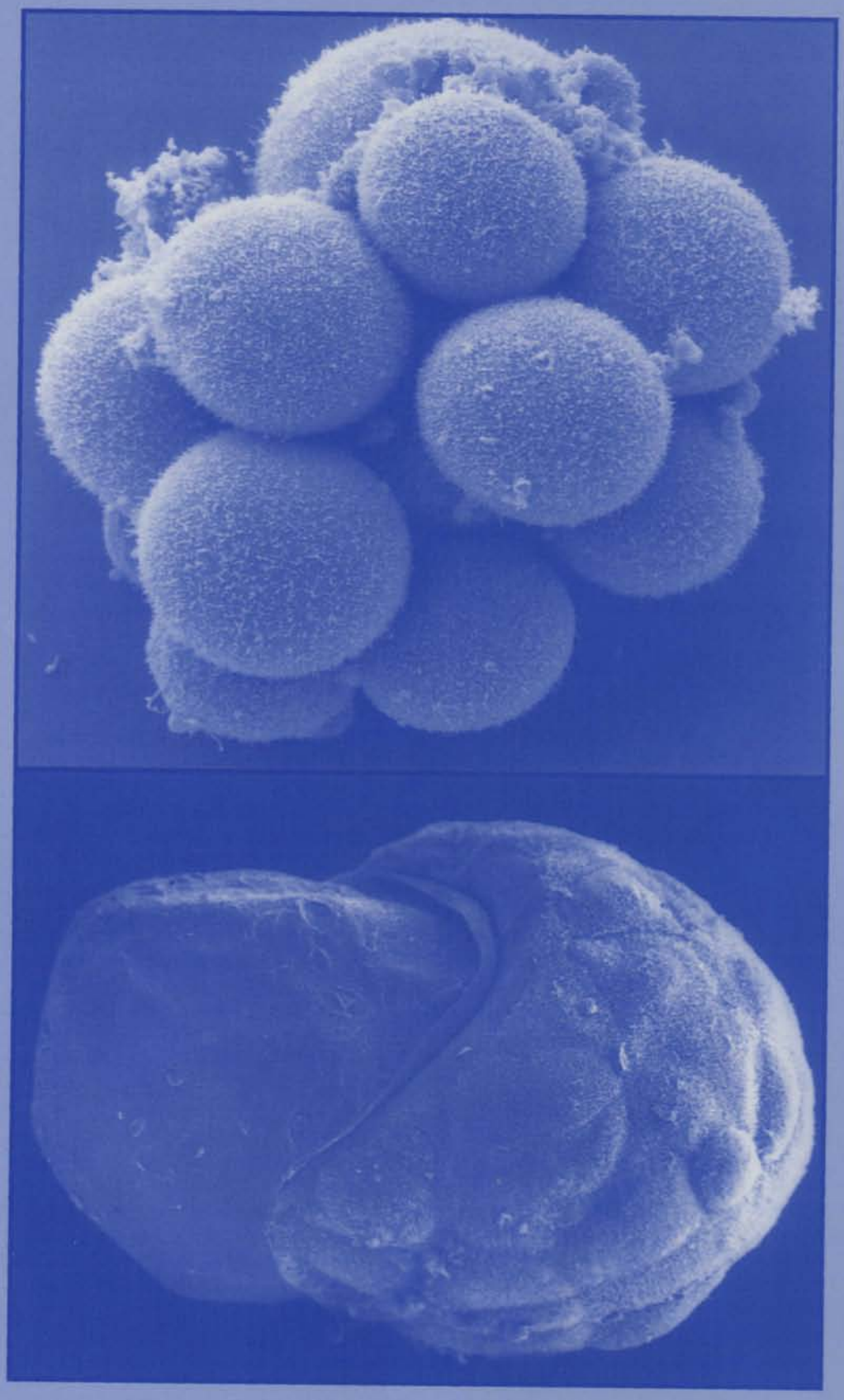

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