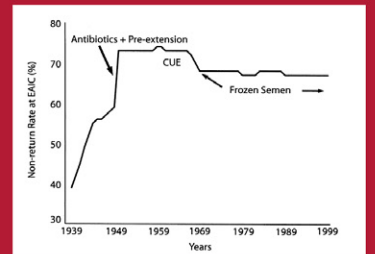
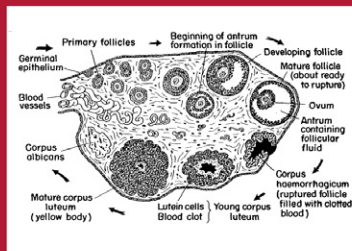
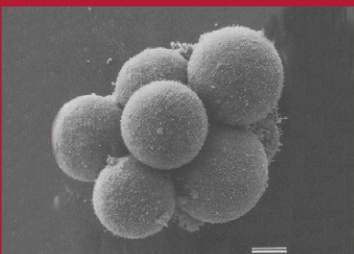
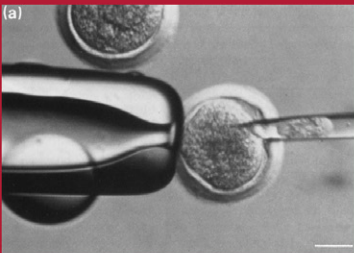
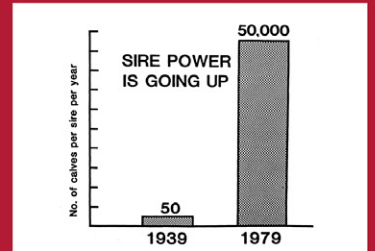
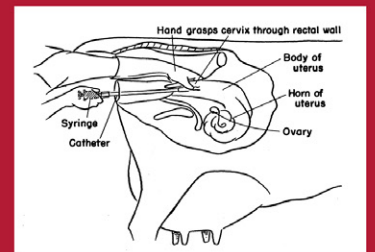
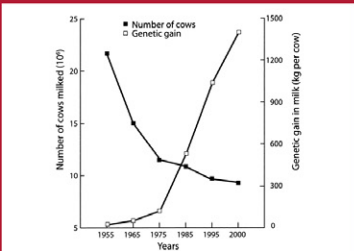
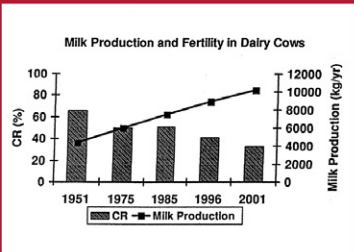
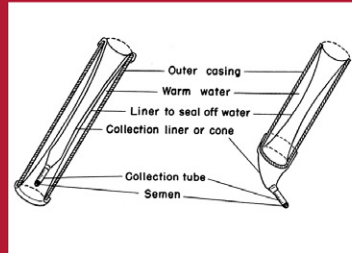
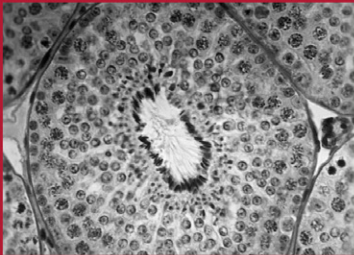


Highlights in Dairy Cattle Reproduction in the Last 100 Years

Robert H. Foote



Cover (clockwise starting at the top left)

1. Cross section of a seminiferous tubule.
2. Scanning electron micrograph of bull spermatozoa.
3. Artificial vagina.
4. Inseminator drawing liquid semen into a catheter about 1941.
5. Diagram of insemination technique.
6. Sire power through artificial insemination.
7. Liquid nitrogen tank with frozen semen.
8. Nonreturn (fertility) rates in A. I.
9. Diagram of the dynamics of an ovary.
10. Imaginary way to catch a cycling cow in estrus.
11. Scanning electron micrograph of an 8-cell embryo.
12. Nuclear transfer of a blastomere into an oocyte.
13. Genetic improvement through artificial breeding.
14. Increase in milk per cow and the decrease in fertility.

The Author and the Review

Robert Hutchinson Foote grew up on a dairy farm in Gilead, Connecticut. Multiple injuries in World War II required him to change his vocational goals from dairying to education. After the war he received his M.S. and Ph.D. degrees from Cornell University where he has spent the last 60 years.

He had the advantage of having mentors and close scientific friends around the globe who contributed directly during the past 75 years to the broad areas of reproductive physiology, nutrition, genetics and veterinary medicine. Leaders in these disciplines worked with the dairy industry to revolutionize productivity of the dairy cow. The author of this review also had the good fortune of working with all aspects of the local artificial breeding cooperative and its dairy farm members from the beginning. Today Genex CRI is one of the few surviving commercial AI organizations in the USA.

During these 60 years the author had an opportunity to share his own research, and that of hundreds of others, with about 8,000 undergraduates and several hundred graduate students and visiting scientists. Some of this information assimilated is shared here. Also, I have had the stimulating experience of meeting with high school students and interested adult citizens, discussing applications for animals and implications for our own species. The Part I, Overview is written with this audience in mind. In Part II, the intention in documenting the incremental increase in knowledge over the past 100 years is twofold. First, it is to enable present and future scientists to find and enjoy in one place a map as they travel the old discovery trail. Second, it should help researchers put their findings in perspective as each one pursues his or her own discovery trail.

Highlights in Dairy Cattle Reproduction in the Last 100 Years

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June 1, 2005

Preamble.

Part I. An Overview

Part II. A Detailed Documented Account

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Preamble

Preamble to Highlights in Dairy Cattle Reproduction in the Last 100 Years

Robert H. Foote

A detailed history of the broad field of dairy cattle reproduction during many decades could fill several books. It is a remarkable history. Dairying moved forward with machinery, computers, and other technology as part of the Industrial Revolution.

Many researchers in many countries working with various species historically contributed knowledge about reproductive function that was applied usefully to dairy cattle. Even before 1900 a large platform of information had been assembled that served as a springboard for the rapid advances during the past 100 years. To keep this essay in manageable proportions, the components that led to changes in reproductive management practices are emphasized.

The essay is divided into two parts. **“Part I. An Overview”**. This is a story of the events as they unfolded in research laboratories and organizations serving dairy farmers, particularly through artificial insemination (AI) and other reproductive biotechnologies. This is written as an overview, avoiding scientific and technical details. If it is appetizing, one can find the main menu in Part II. Each part is subdivided into the male component and the female component.

“Part II. A Detailed Documented Account.” History is exciting when we travel a road where we become increasingly aware of the miracles of reproductive biology, especially when we understand them more fully. The author traveled the roads leading to many places where much research was done. Of course we still “see through a glass darkly”. However, the reader can explore here many essential components of reproductive biology, and take time to contemplate how selected aspects were transformed into doable programs that revolutionized the breeding and reproductive management of dairy cattle. Early references have been chosen frequently for this historical narrative because of their importance and lack of availability on the electronic network. The reader should utilize the electronic network to find thousands of current reports.

As a consequence of the changes and improved nutrition and health, the Holstein cow in the United States today converts the products of the sun’s energy and plant photosynthesis into more than three times the milk output per cow of her predecessors 50 years ago. Simultaneously, dairy cow numbers decreased by 57 percent.

Unless otherwise acknowledged, figures are by the author. Suggestions by Dr. Harold Hafs, staff in Cornell’s Publications Department and assistance with manuscript preparation by Suzanne Bremmer are appreciated.

Part I. An Overview

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Introductory Historical Comments

Reproduction is an old habit, art, and science. So, in reflecting upon the achievements of the past 100 years we should remember that a large platform of information had been established before the last century, which provided a base for the more recent advances.

In 1677 Regner de Graaf described the Graafian follicle of the ovary as the source of the eggs. In 1827 Karl E. Von Baer announced that he had removed a small object from a follicle and stated, “When I placed it under the microscope I was utterly astonished, for I saw an ovule just as I had already seen them in the tubes, and so clearly that a blind mind could hardly deny it.” De Graaf also was interested in the testis. He was the first to describe seminiferous tubules in the testis. An amazing individual, he died at the age of 32. He was a friend of Antoine Leeuwenhoek, a careful grinder of lenses in Amsterdam. The assembly of lenses (a microscope) enabled Leeuwenhoek to see sperm in 1677. His report of “animalculis”, as he called the sperm cells, followed in 1678.

Later anatomists described the structure of the ovary and testis in detail. The fact that the testes were essential for reproductive function was known in ancient times. Eunuchs played an important role in several early cultures and religions. More than 100 years after Leeuwenhoek’s description of sperm, Lazzaro Spallanzani showed unequivocally that semen was essential for fertilization by producing pups in dogs following artificial insemination (1784). Furthermore, by filtering the semen he showed that only the non-filterable components of the semen, now recognized as sperm, were responsible for fertilization.

The late 1800s was a golden age for further elucidation of the functions of the testes and ovaries, sperm and eggs, fertilization, and early cleavage stages of the young embryo. Superb anatomists described reproductive organs accurately in minute detail. The testes and ovaries also were known to exert several effects on different organs of the body as well as influence sexual behavior. However, the identification of specific hormones and their functions was a product of the 20th century. The word hormone was first used by Ernest H. Starling in 1905, taken from the Greek meaning to excite.

In the 20th century, knowledge of reproductive function continued to be advanced by the collective research conducted with laboratory animals such as mice, rats, and rabbits as well as chickens. Then it was the challenge for researchers working with large domestic animals to determine whether their reproductive systems function the same way as they do in laboratory animals, or to identify specific differences. So, in describing briefly a variety of discoveries and applications in dairy cattle reproduction and technology, one should note that much useful knowledge often originated with work done in other species. Knowledge gained, like sexual reproduction, requires cooperation.

Advances in Endocrinology

The two pituitary gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), were originally thought to be one hormone.

The only man alive in 2005 (Samuel L. Leonard, 100 years old) responsible (with coworkers Harry Fevold and Frederick Hisaw) for the discovery of the two hormones in the late 1920s still reminisces clearly about this discovery in his home in Ithaca, New York. Leonard was 25 years old when he carefully extracted pituitary glands in rats and showed that one of the two extracts caused follicular development and the other one caused ovulation. He was at the University of Wisconsin as a graduate student at that time. He presented these results at the American Association for the Advancement of Science meetings in New Orleans in 1931, after driving for four days in a model T Ford from Rochester, New York with Roland K. Meyer, then working in Rochester. Others believed there was only one gonadotropin, so there was considerable controversy at the meeting. Incidentally, Leonard's first task as a graduate student was to determine "the amount of female sex hormone in normal and pregnant cow urine." Senior researchers will remember the aroma in labs where steroid hormones were being extracted from gallons of cow urine.

Discovery by G.W. Harris in the 1940s of the portal vessel pathway, which provided a direct vascular connection from the hypothalamus to the anterior pituitary, opened the way for discovery of the gonadotropic releasing hormone (GnRH) in the hypothalamus of the brain. GnRH controlled the release of pituitary gonadotropic hormones. The hypothalamic-pituitary-gonadal linkage is the principal means by which reproductive function is regulated in most mammals, including dairy cows and bulls.

There were gradual increases in understanding of the chemistry and function of the hormones in the following decades. However, the development of radioimmunoassays (RIAs) in the 1960s, capable of measuring with great specificity and sensitivity hormones in blood and other body fluids and tissues, greatly speeded advances in monitoring low concentrations of reproductive hormones circulating in dairy cattle. The RIA procedure became automated to assay hundreds of samples daily, compared with old tedious methods that required a day or more to obtain a single result. These procedures led to understanding the endocrine control of the estrous cycle. Many applications followed, such as induction of ovulation after parturition in lactating cows, superovulation and embryo transfer, and synchronized ovulation in cows for programmed timed artificial insemination (AI).

The Male Component

Gonadotropins were found to regulate the development of the testes of bulls, involving tubular development where sperm are produced, and the Leydig cells between the tubules, which produce testosterone. The latter hormone affects other organs and partially controls male sexual behavior. However, no major applications have been found to date to enhance spermatogenesis and increase the reproductive capacity of bulls through treatment with hormones.

The greatest impact of the male on reproduction in dairy cattle has been through artificial insemination (AI). Accompanying the development of AI were major advances in knowledge of testis function, sperm anatomy and physiology, and sperm evaluation and preservation. Also, many myths and mysteries

regarding cow reproductive physiology and fertility were resolved, important for the application of AI.

The Beginning of AI

Scattered reports and rumors of the use of AI in individual cases have persisted since the 1300s when Arabs were alleged to have obtained semen from the vagina of prize mares belonging to other tribes and then used the fluid obtained to inseminate their own mares. The first organized use of AI was started in Russia in 1899 by Dr. Ivanovich Ivanov to breed mares for the cavalry. He collected sperm from sponges placed in the mare before mating. Later Ivanov constructed artificial vaginas to improve semen collection, modeled after one made by Dr. G. Amantea for dogs in 1914. The first AI cooperative for dairy cattle in the western world was organized in Denmark in 1936.

Artificial insemination was reported to have been used with fresh semen on a limited basis in herds in New York State, Minnesota, Missouri, and Wisconsin in the 1930s. Professor Enos J. Perry from Rutgers University had seen AI performed in Denmark in 1937. He prevailed upon his friend, Professor Stanley Brownell, who had been inseminating cows regularly in the Cornell herd, to come to New Jersey in December 1937 to demonstrate to New Jersey dairymen semen collection by massage of the seminal vesicles. K.A.F. Larsen from Denmark demonstrated AI techniques, using the Danish method, but his bull artificial vagina did not arrive with him to demonstrate semen collection. Perry organized New Jersey dairymen to form an AI cooperative early in 1938.

Commercial AI Initiated

The first completely farmer-owned cooperative started later in 1938 in New York State. Soon many small AI cooperatives and private organizations sprang up, culminating in about 100 in the US in the 1950s. It was clear from the beginning that if AI was to flourish, the initial rather crude techniques used needed improvement. Much research was needed to increase knowledge of bull management, testis function, semen collection, sperm evaluation, extension, packaging, and preservation, along with better insemination programs in the field. To help meet that need the Pioneer Cooperative started in Dryden, New York, in 1938. It later became the New York Artificial Breeders Cooperative, Inc. and moved to Ithaca in 1945, on the edge of the Cornell University Campus. This unique cooperation between farmers, New York State, and the private university, Cornell, became a world model of harmonious cooperation. Research on some aspects resulted in practical application more rapidly than others. Rather than try to fit all the pieces together chronologically, we will follow the story from the standpoint of the sperm cell as its life begins in the testis and finally how a few out of trillions of sperm produced may encounter an egg in a cow.

The Testis

The bull testis, capable of producing billions of sperm per day, made it biologically possible to develop extensive AI programs. The general idea that the testis produced lots of sperm was recognized when commercial AI started, but little objective qualitative or quantitative information was available. Anatomists had described the almost endless seminiferous tubules of the testis, in which spermatogonial

cells underwent mitotic divisions to form more cells that underwent two divisions of meiosis to form spermatocytes and finally spermatids. Each spermatid contained only one set of chromosomes, potentially to be combined with a set of chromosomes from the female at the time of fertilization.

Now we know how each round spermatid undergoes spermiogenesis. During this period the round cell develops a long flagellum, the tail of the sperm cell, and loses most of its cytoplasm. This process is coordinated by nurse cells within the seminiferous tubules, the Sertoli cells. By this time young sperm cells have taken about two months to develop, and they are ready to be released from the testis. Then the sperm cells are pushed out of the paired testes through efferent ducts, the paired epididymides, and they move into the common urethra. Sperm continually pass out of the urethra of the penis with urine, or they are expelled in large numbers at the time of ejaculation.

The testis is composed mostly of seminiferous tubules with supporting connective tissue, but the extratubular space contains many Leydig cells where testosterone is produced. Production and secretion of testosterone is stimulated by the pituitary gonadotropin LH. Testosterone and other androgens stimulate development of male accessory sex organs and development of male muscular features such as the huge neck of the bull. Sexual behavior also may be affected by small quantities of estradiol.

The events just described occur in the sexually mature postpubertal bull after being established during the first year of life. At puberty active spermatogenesis starts, triggered by pulses of gonadotropic hormones, FSH and LH. Intratesticular testosterone also is important in maintaining spermatogenesis as well as maintaining sex drive, or libido, in the bull after sexual maturity.

Growth and Maintenance of the Bulls

In the beginning of commercial AI, adult bulls with natural service proofs (records on cows in the owner's herd) were recruited. These bulls were already seniors by the time of purchase. What were the nutritional requirements to maintain high sperm production, high libido, and a long physically active life? Most studies with dairy animals had been conducted with cows requiring high energy intakes for milk production and reproduction. Recommendations were not suitable for bulls. Studies at Cornell in the 1940s demonstrated that a 12% protein concentrate supplementing a mixed grass hay diet fully met the needs of mature bulls. No animal proteins, wheat germ, or vitamin A supplements were needed, contrary to some beliefs and clever merchandizing by salesmen.

One fault with these early rations was that the concentrate fed was formulated to supply adequate calcium utilized by the cow to nourish the growing fetus and produce calcium-rich milk. Bulls fed this ration accumulated too much calcium with over-ossification leading to fractured vertebrae. The concentrate supplement was reformulated with appropriately reduced calcium.

As the AI program grew, selected bull calves were brought to AI centers for rearing. It was important that they grow rapidly to reach puberty and produce good quality semen at an early age to use for insemination to establish their genetic transmitting ability. Balanced rations promoting rapid growth, and early

onset of spermatogenesis were desirable, provided that the feed was scaled back as animals matured to avoid overcondition (fat). Each selected bull represented an investment of tens of thousands of dollars, so a long reproductive life after selection was necessary to recover the costs.

Sexual Behavior and Frequency of Semen Collection

In the early stages of AI only a few cows were enrolled in the program. Once every one or two weeks a bull was brought out and allowed to mount a cow and semen was collected into an artificial vagina. Other bulls also were ejaculated on a similar schedule assuring that enough sperm were collected to meet the needs.

As the AI programs grew more sperm per sire were needed to meet the demand. Studies at Pennsylvania State University, Cornell University, the University of Illinois and Michigan State University demonstrated that the simple procedure of collecting a sample of semen occasionally harvested only a small fraction of the sperm produced. The paired testes of a mature bull manufactured billions of sperm each day. This is a true miracle of life.

With appropriate teaser (mount) animals and manipulation of the bulls in a favorable sexual preparation setting most bulls increased their libido. The number of sperm per semen collection increased. It was found that bulls could be ejaculated two or three times a day twice per week. Under these conditions bulls ejaculated about 30 billion sperm per week. Experimental attempts to stimulate greater sperm production or increase libido through a variety of hormonal, pheromonal, or other treatments have been unsuccessful.

Semen Characteristics and Evaluation

In the foregoing section it was established that large numbers of sperm can be harvested. Equally important is their quality and potential fertility, and how this potential can be estimated? This has been the focus of thousands of studies, a few of which are discussed in Part II.

Sperm numbers in a semen sample were determined by measuring the volume (ml) of the ejaculate and the sperm concentration per ml. The simplest estimate of a sperm cell's viability was determined by evaluating sperm motility with the aid of a microscope. Methods of preparing wet semen smears for microscopic observation varied, as did subjective estimates of the rate of swimming speed and percentage of swimming cells. Although these estimates were positively correlated with fertility, the correlations were low. More recently computer-assisted sperm analysis (CASA) has improved the quantification of motion characteristics of sperm.

Under certain conditions, such as a cool temperature, sperm are immobilized, and the question arose, what proportion of sperm cells are alive? Supravital stains such as nigrosin-eosin, aniline blue-eosin, and fast green-eosin showed that live cells with intact plasma membranes do not allow the eosin stain to penetrate the cells. They remained unstained. This proportion of cells usually was higher than the percentage of motile cells.

Staining of cells of many types, including sperm cells, to determine their general shape had been well developed when AI started. So sperm morphology was determined, especially as some of the bulls used in the beginning of AI produced a substantial proportion of abnormal forms of sperm cells. After

multiple generations of selection, bulls used today produce a high proportion of normally shaped sperm. The foregoing tests were the most widely used initially to evaluate sperm quality. In the 1960s researchers at Virginia Polytechnic Institute used advanced microscopy to evaluate the acrosome. This cap on the sperm cell plays a fundamental role in fertilization, so abnormal acrosomes and loss of the acrosome were predictors of reduced fertility.

Tests such as pH of semen, oxygen consumption by sperm, and other tests of sperm metabolism did not add more useful information than was obtained by the previous tests. DNA variability among sperm was reported in the 1950s, but the tests were very complicated and not used. More recently new stains and flow cytometry permit objective and rapid evaluation of variation in sperm DNA content and integrity, characteristics that have been positively correlated with fertility.

Preservation of Sperm

For many years the AI industry depended upon being able to maintain the fertility of sperm at 5°C (40°F) for at least 2 days after semen collection, until it could be delivered to inseminate cows. A great help for preserving liquid bull semen was the discovery by Dr. Paul Phillips at Wisconsin in 1939 that egg yolk in an egg yolk-phosphate buffer protected sperm. Glenn Salisbury and colleagues at Cornell soon improved the medium with a yolk-citrate combination in 1941. Also it was discovered that the lipoproteins of egg yolk, in addition to protecting sperm at a convenient storage temperature of 5°C, protected sperm from coldshock during cooling from body temperature to 5°C. Later, heated milk in various forms was developed to preserve bull sperm.

Venereal diseases, particularly vibriosis caused by *Vibrio fetus* (later called *Campylobacter fetus*), were a problem in natural service. Some infected bulls with natural service proofs were bought for AI service as the disease was not detected. Work at Cornell with sulfanilamide, then with penicillin, streptomycin and polymyxin D, and at Pennsylvania State University with penicillin and streptomycin, showed that semen treatment prevented *Vibrio* infection. This finding gave a major boost to fertility as herds using only AI soon were free from this disease. Then AI expanded rapidly. Later, staff at the New York State Veterinary College at Cornell eliminated *Vibrio fetus* in the bull stud.

A remarkable, somewhat accidental discovery by British workers in the late 1940s was that bull sperm could be frozen successfully provided that glycerol was added to the medium for preserving sperm. Before this discovery could be used practically, methods of freezing, new packaging, and low-temperature storage units had to be developed. Methods for proper thawing and handling of the thawed semen also had to be developed (see Part II). By the mid 1950s frozen bull semen was being used commercially. J. Rockefeller Prentice, owner of American Breeders Service, gave a substantial sum of money to Union Carbide, and its Linde Division to improve the insulation of tanks to store frozen semen in liquid nitrogen at -196°C. The whole cryobiology industry flourished after that development.

Sperm Numbers and Sire Power

Now all the conditions were in place for most individual bulls to produce enough sperm to meet the demands. Several of these conditions had been established before they became critical in AI. In the late 1930s Danish veterinarians established that sperm could be deposited with a pipette through the cervix of the cow into the uterus, guided by one gloved hand inserted into the rectum to the point where the cervix could be grasped and manipulated. Many fewer sperm were needed for insemination when they did not have to navigate through the tortuous cervix. Then Salisbury and colleagues in the 1940s and 1950s reported that fresh bull semen diluted (later called extended) with preserving media to 5 to 10 million sperm per insemination resulted in maximal fertility.

With proper sexual preparation a bull could ejaculate 30 billion sperm per week, enough to inseminate 3,000 cows with 10 million sperm per cow. However, not all the liquid semen could be used within 2 days of collection. In contrast, nearly all the semen frozen can be stored until used, but about 20 million frozen sperm per insemination are required for optimal fertility.

Fertility (Conception Rate)

High conception rates were important for AI to succeed. A reliable method for measuring conception rates simply and on a wide sample or population of inseminated cows was very important. Bulls, semen quality, processing procedures, and inseminator skills all required evaluation to improve fertility. Pregnancy diagnosis by veterinarians was available only sporadically and on a limited basis. A.W. Thompson, at the local AI cooperative, and Glenn Salisbury at Cornell, in 1947 developed the highly successful nonreturn-rate method. Because all cows inseminated were reported to the cooperative, the date of every return or repeat service also was reported. These data provided the information necessary to tabulate the nonreturn cows and calculate the overall rate at specified intervals by bull, inseminator, herd, etc. Today many other methods are being researched, particularly as frozen semen from multiple sources used within a herd results in little nonreturn data being reported.

Current Technologies Under Development

Sexed Sperm

It is possible to sort sperm by gender potential quite accurately. This is done by staining the sperm DNA, and detecting the difference in DNA content between “male” and “female” sperm containing the sex chromosomes. Flow cytometry is used to separate sperm very rapidly. However, billions of sperm must be sexed and only a few million sperm per hour can be sexed. Only a small fraction of sperm separated most completely are used. Therefore sexed sperm are not widely available, despite the fact that producing mostly female calves in a dairy herd would be very valuable. Special uses of sexed sperm such as for IVF with the production of female embryos could be practical. Relatively few sperm are required for IVF.

Freeze-Dried Sperm

Mouse sperm can be freeze-dried with the result that microinjection of freeze-dried sperm into oocytes can result in viable embryos. As sexed freeze-dried sperm presumably can be stored cheaply and successfully for many years, this may be an inexpensive method for preserving special germ plasm. It will have little application in commercial AI because the sperm are killed in the process. The DNA remains functional but requires microinjection into an oocyte for fertilization and development to be initiated.

The Female Component

In contrast with the male, the female is born with all the oocytes (eggs) it will ever have. Up to several million oogonia may be produced by mitotic divisions in the ovary of the fetus. Surviving oogonia undergo the first division of meiosis. Then primary oocytes produced are arrested before completing meiosis I, and they are stored in that condition. A majority of oocytes will regress continuously and be lost. Other oocytes are stored in primary follicles, and later in secondary and tertiary follicles. Only a few of the original millions of oocytes will reach the tertiary follicle stage and be ovulated once the estrous cycle begins in the sexually mature female (see Part II).

The Estrous Cycle

Estrous cycles herald the initiation of puberty. The hypothalamus becomes more active at puberty. Episodic surges of gonadotropins, particularly LH, act on the ovary causing one follicle to become dominant over other large tertiary follicles. Estrogen (E2) secretion by the follicle increases, and surges of LH stimulate ovulation.

The E2 surge causes the cow to display behavioral signs of estrus before the LH peak occurs. The interval from the start of estrus to the LH peak averages about 9 hours, but it is highly variable. Ovulation occurs about 22 to 29 hours later, which is after the end of estrus. A corpus luteum (CL) grows in the ruptured follicle area, producing progesterone (P4). The secretion of P4 prevents estrus and further ovulation from occurring again for about 21 days. The young CL grows rapidly, maintains P4 secretion, and then regresses, so that the decline in P4, accompanied by an increase in E2 results in estrus on the average about every 21 days. However, there is considerable variation in length of various estrous cycles.

Detection of Estrus

Accurate detection of estrus by the farm manager or by designated individuals was and is extremely important for insemination to occur at the proper time. It is fortunate that cows ovulate after the end of estrus, and sperm survive for several hours in the female tract, providing a substantial window of opportunity for successful insemination. The optimal time of insemination is about 5 to 17 hours after the beginning of estrus.

It is important that cows be observed frequently enough so that the first detection of estrus coincides rather closely with the true beginning of estrus. Great emphasis in cooperative extension programs was placed on helping dairy

farmers to implement a standard plan for detecting estrus. The sign of the cow standing when mounted was supplemented with other signs: the behavior of riding others, increased activity, and vaginal mucus discharge. Many aids were produced commercially that showed that the cow had been mounted but did not require continuous visual observation. Electronic equipment was developed to monitor each cow's activity and the data were transmitted to a computer.

Time to Inseminate Cows During Estrus

Inseminators planned their routes, where possible, to inseminate first in the morning those cows seen in estrus (heat) the night before. Those seen in estrus for the first time in the morning were inseminated later in the day. This was known as the "A.M. – P.M., and P.M. – A.M. Rule." It was developed in the 1940s by Dr. George Trimberger, then at Nebraska, after careful observation of cows and palpation of their ovaries every 2 hours to detect ovulation. Inseminations were performed at different times during estrus to determine when the highest conception rate occurred. Based upon this research the practical rule was established.

In large herds many herd owners now keep banks of frozen semen on the farm. They attend training schools to learn proper techniques for thawing frozen semen and to practice proper insemination techniques. Good conception rates can be obtained by experienced farm personnel. As these inseminations are not reported to the semen supplier, little information is available to the supplier on conception rates. Special programs involving cooperating herds often must be organized to obtain fertility information on semen used.

Fixed-Time Insemination with Controlled Ovulation Time

Detection of estrus by frequent observation of cows is time consuming, and time is a precious commodity on a dairy farm. As herds became larger, identification of specific cows in estrus in large groups became more difficult. Could cows be managed so that a group of cows at a desirable period after calving have their estrous cycle controlled? Then all could be inseminated conveniently at a fixed time. This idea also had the potential for avoiding weekend inseminations. Research on controlling ovulation time in heifers by administering progesterone for a preset time started with work by Lester Ulberg and Lester Casida at Wisconsin in 1951. Other progestagens became available for use along with estrogens. By the 1970s prostaglandin F_{2α} (PGF_{2α}) and analogs became available to control ovulation time. Administration of PGF_{2α} was used to control destruction of the corpus luteum (CL). Later, synthetic gonadotropic-releasing factors (natural GnRH and analogs) became available. Many agricultural colleges and pharmaceutical companies began conducting hundreds of trials using various combinations of progestagens or sequential treatment with GnRH and PGF_{2α} (see Part II for details). Systems for controlled ovulation with fixed-time insemination were developed. They are used in many large herds. The systems are convenient and save labor, but the cost of drugs and administration must be balanced against the convenience.

Superovulation and Embryo Transfer

Methods were developed in the 1960s to superovulate cows, inseminate them, and collect embryos from the oviducts by surgical methods. Thus, a superior cow could produce more offspring following transfer of the embryos to recipients. Surgery was eliminated in the 1970s following Japanese and English research that demonstrated how the entire procedure could be conducted nonsurgically by collecting older embryos from the uterus and transferring them to uteri of recipients.

A problem still existed. A large number of recipients at the correct stage of the estrous cycle (same stage as the donor) was needed. Methods were developed for synchronizing donor and recipients, but one could not predict in advance how many good embryos would be obtained and, therefore, how many recipients to synchronize.

This problem was overcome in the 1970s with the discovery that mouse embryos could be frozen in a medium containing glycerol by use of a procedure modified from the one used for bull sperm. Soon this embryo freezing procedure was modified for successful cryopreservation of bovine embryos. Frozen embryos could be stored until suitable recipients, either undergoing natural or controlled estrous cycles, were available. These major biotechnology advances made it practical to increase bull power tremendously through AI and cow power somewhat through superovulation and embryo transfer.

Embryo culture methods also improved greatly in the 1960s, 70s, and 80s. Good culture methods made it possible to use oocytes aspirated from ovarian follicles at any time of the estrous cycle, during pregnancy, or from juveniles approaching puberty. These oocytes were matured and fertilized in vitro (IVF). Then they were cultured to the blastocyst stage and transferred to recipients or frozen the same way in which blastocysts produced naturally were processed.

Genetic Improvement: Sire and Cow Sampling

A method of sampling and testing young sires for their genetic ability to sire high-producing offspring had been developed by Charles Henderson at Cornell by 1950. This genetic research was essential because sires proved in natural service reflected primarily the type of management that daughters received in one herd. This obscured their genetic value. Their proofs were not repeatable in AI. So the system of sampling many young bulls in many herds using AI and selecting a few superior bulls was developed and adopted by AI organizations in the 1950s.

In the 1980s multiple ovulation-embryo transfer (MOET) programs were established by a few AI organizations. These programs were designed to make possible more intensive genetic selection on both the sires and dams to accelerate genetic progress. However, despite the intensive selection of dams the major contribution to genetic progress was through the bull.

Modern Cow Milk Production and Reproduction

Dairy cows in 2005 are individually fed a ration balanced for their milk production and stage of lactation, all possible with computers, transponders, and feeding concentrates in the milking parlor. The administration of growth hormone (bovine somatotropin, bST) has increased metabolic efficiency and total production. Milking 3 times per day also is required to empty the storage container (udder) for a refill to obtain maximal milk output of high-producing cows.

The result of all of the changes in reproductive biotechnology plus improved feeding and management is that the average Holstein cow produces three times as much milk as her forebearers 50 years ago. Today 9,000,000 cows produce more milk than 21 to 22 million cows did 50 years ago. With the cow diverting so much energy into milk production, the cow is in a negative energy balance for about 2 months after calving. During this time reproductive fitness is reduced because the demand for milk production takes priority. The result is pregnancy rates resulting from a single insemination during the first 2 to 3 months after calving are reduced to about 35 to 45 percent compared with 60 to 65 percent 50 years ago.

Future

Many changes in dairy cattle reproductive management occurring during the past 100 years have been described. More of the science and technical details are documented in Part II.

What does the future hold? Experts in reproduction, nutrition, and other aspects of management are using mathematical skills and computers to mine the published data to construct models. These models will reveal components where important data are lacking, which will lead to design of new experiments. Refined models will indicate better ways of optimally managing dairy animals. The optimal system may not be the one designed for maximal production. It will consider a variety of inputs and outputs relative to the health of the cow, the economy of the farm, the environment and society.

Genetic selection of animals also may be modified. Genomic analysis of animals at birth may be of value in making initial selections of animals to raise for further testing. Will cloning and genetic engineering play roles in breeding and reproduction? It is likely that a few of the most outstanding bulls will be cloned as insurance against injury and possibly for more widespread distribution of the bull's genes. Genetic engineering to produce hornless bulls siring hornless females also would be of considerable economic value. Engineering a bull so that only females would be reproduced would be of great value. There are numerous other possibilities. However, it is unlikely that cloning of cattle will become commonplace because of the complexity and cost compared with the simple powerful technology of AI.

Note: A more complete technical text follows as Part II.

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Part II. A Detailed Documented Account

Introduction

Domestic mammals have played a major role in the survival and development of various cultures of *Homo sapiens*. Ruminants, such as cattle, goats and sheep have provided human beings with nutritious protein and calcium (milk and meat), clothing (hide and hair), power, fuel (dried dung), organic fertilizer and companionship. Of great importance is the ability of ruminants to convert energy from the sun that has been utilized by plants through photosynthesis to produce huge quantities of feed for animals. While some of the plant materials fed to animals compete for food for human consumption, the most abundant plant material, cellulose, is a major part of the diet of ruminants, and it is of little food value for humans.

In many areas of the world the chief source of milk is from the family goats, scrounging on grass, leaves, garbage and other inedibles for humans. Even in rapidly developing economic powers, such as China, currently there are 100,000,000 goats and only 3,000,000 dairy cows. It is estimated that 25,000,000 dairy cows are needed to improve the diets of children, many of the sick and elderly in China. However, in most of the industrial nations milk is produced on large dairy farms and processed into many kinds of dairy products. On these farms efficient high producing cows convert forages, supplemented with mixtures of grain, into highly digestible nutritious milk.

The greatest advances in both the reproductive and genetic aspects of breeding dairy cattle have occurred in the 20th century, the focus for this article. However, an overview of the state of knowledge and art of breeding prior to 1900 is included as a starting point for describing the advances made subsequently. Suggested sources for more information include Marshall (1910), Asdell (1964), Mann (1964), Salisbury et al. (1978), van Tienhoven (1983), Setchell (1984), Cupps (1991) and Knobil and Neill (1998).

Early History

The process of sexual reproduction has fascinated human beings throughout history. Many great thinkers in early recorded history, such as Aristotle, stated views on the subject, based on assumptions and myths current at the time. Few facts were established until the 1600s when de Graaf published the details of testis structure in 1668 and described the Graafian follicle in the ovary in 1672. He was the first person to describe the seminiferous tubules of the testis. He was a remarkable individual whose career was cut short by death at 32 years of age.

The Netherlands was the center of other fundamental discoveries, partly because of the superb magnifying glasses made there. The most famous maker of lenses to form simple microscopes was van Leeuwenhoek (1632 to 1723). Totally untrained in science he began at the age of 39 to experiment with grinding lenses. An interesting side of van Leeuwenhoek was his global view of the world. He predicted that the planet could not sustain more than 13 billion people. The very best of his 550 lenses remaining today have a resolving power of 1.4 microns. His method of grinding lenses was secret, and no one produced better lenses until

the 19th century. With this equipment van Leeuwenhoek and a medical student, Johan Ham, observed sperm swimming in seminal plasma. Van Leeuwenhoek reported this observation of “animalculis” in 1678, with an accurate description of the gross morphology of a sperm cell. These discoveries led to a new concept of fertilization in which it was assumed that the sperm cell was the main source of new life. Others believed that the egg was the chief source of new life. In any event, the discovery of sperm in the seminal plasma added to the concept that the male contributed a vital component to reproduction because it was also widely known that castration produced sterility. Castration was associated with various religious rites and the production of eunuchs was common in various early civilizations. Also, castration was used as a form of punishment or revenge in some cultures (Setchell, 1984).

It remained for Spallanzani in the 1780s to explore the nature of fertilization in mammals. In 1784 he performed the first documented artificial insemination in dogs. Following insemination of a bitch three pups were born 62 days later, a normal period of gestation for the dog. Later he filtered semen and found that it was only the residue on the filter and not the fluid filtrate that could produce a pregnancy.

The 1800s became the golden age for further elucidation of the function of the testes and ovaries (Marshall, 1910). In 1841 Kölliker established that sperm came from the testis and in 1871 Von Ebner described different stages of spermatogenesis in cross-sections of the seminiferous tubules. In 1865 Sertoli was the first one to clearly describe the Sertoli cells (Setchell, 1984) that play a major role in coordinating the process of spermatogenesis within the seminiferous tubules. The interstitial cells (Leydig cells) that produce testosterone were described earlier by Leydig in 1850.

In 1827 Von Baer described a mammalian ovum. He was astonished after removing an ovum from the ovarian follicle to recognize that it was just as he had seen ova or eggs in the Fallopian tubes.

The main features of a mammalian ovary were described by Waldeyer in 1870 (see Marshall, 1910), along with the process of ovum formation. He noted the rare occurrence of two ova in one follicle.

The ovaries and testes were known to be organs of secretion affecting other parts of the body. This was based on observations that when the primary sex organs were removed, or gonadal injuries or deficiencies occurred prepubertally, sexual development was altered. Also, in the late 1800s and early 1900s several reports indicated that sexual function was restored when sex organs were transplanted to other areas of the body. An often cited case is that of Brown-Séquard who in 1889 reported that injections of ovarian or testicular extracts affected reproductive vigor. No active principles responsible for these changes had been isolated, but in 1905 Starling proposed the term “hormone”, from the Greek (indicating arousal) to identify this class of chemical substances.

The placenta was recognized by Harvey in 1651 as an organ bringing nutrients to the fetus. Little more was known about the placenta until the 1800s.

Marshall’s book “The Physiology of Reproduction” (1910) is a gem as a source of information up to the early 1900s. It is a critical comprehensive compilation of

information that had been published primarily in English, German and French. The book is dedicated to Marshall's friend, Walter Heape. Heape (1891) collected young embryos in rabbits, transferred them to recipient rabbits and produced young. Reproductive technology has never been the same since.

The Twentieth Century

So the foundations were laid for many remarkable discoveries in the 20th century. Basic discoveries made with laboratory animals, such as rats and rabbits, usually preceded those with domestic animals. Because of the similarity in reproductive function among mammals, advances in knowledge gained in one species usually furthered understanding of reproductive function in other species. Therefore, discoveries in several species will be relevant in discussing the remarkable development of reproductive biotechnologies which have had great impact in the practical breeding of dairy cattle worldwide in the 20th century. Emphasis in this treatise is on the adult animal, excluding fetal development (Wells, 1959).

The Male Component

The Testis and Spermatogenesis

The testis (Albert, 1961), with its prodigious production of sperm cells, is the most important organ that made possible the tremendous application (Foote, 1981) of artificial insemination (AI). This potential is one of the major reasons why animal scientists have focused much attention on the mechanism by which billions of sperm are produced by the testis. Studies at Cornell University were facilitated by the location of a large AI cooperative on the edge of the university campus (Fig. 1)



Fig. 1 Inspection in 1945 of the new artificial breeding center (NYABC) under construction on the edge of the Cornell University campus. In the front row (L to R) are Professor Glenn Salisbury, Governor Thomas Dewey, and H.E. Babcock, chairman of the C.U. Board of Trustees. In the center, 2nd row, is Cornell president Edmund Day and on his left is Professor F.B. Morrison. Behind President Day is Harold "Cap" Creal, a leading dairyman in NY State. Others are unidentified. This unique harmonious cooperation between farmers, the state and the private university yielded enormous benefits to agriculture. Photograph loaned by Mrs. Dorothy Salisbury.

Large scale AI was practiced in Russia, particularly in horses, starting in 1899 (see Ivanoff, 1922). It spread to Japan and western Europe subsequently (Foote, 1999). In the United States AI was reported to have been tried in a few herds in several states, but commercial AI was initiated in the US in 1938 (Perry, 1968; Herman, 1981; Sipher, 1991) after several American scientists visited Denmark where AI cooperatives were operating.

Modern investigation of the details of the kinetics and duration of spermatogenesis became intensive in the late 1940s. Roosen-Runge and Giesel (1950), LeBlond and Clermont (1952) and Clermont and LeBlond (1953) published detailed descriptions of the stages of the cycle of the seminiferous epithelium in the rat, Ortavant in the ram (1954) and Swierstra and Foote (1963, 1965) in the rabbit. Amann (1962) described spermatogenesis in the bull, and Berndtson and Desjardins added a detailed description in 1974.

The organization of the testis to produce trillions of sperm cells yearly, and maintain stem cells as a source of continuous supply of sperm cells is one of the biologic wonders of the world. Not only is the number of cells produced almost beyond comprehension, but the differentiation of the spermatogonia type A stem cells into sperm cells requires enormous changes to produce a cell with more than 2000 parts. This process of spermatogenesis goes on continuously in the seminiferous tubules coiled throughout the testis in assembly lines that total about one mile in length. A portion of a seminiferous tubule is shown in Fig. 2 (Amann and Schanbacher, 1983).

The initial step in the events to convert a spermatogonium, type A, into a sperm cell involves mitotic cell divisions to produce many spermatogonia. Then a sequence of two highly specialized divisions of meiosis result first in primary spermatocytes and then in secondary spermatocytes with only one set of chromosomes in each cell. The original spermatogonia have a paired set of chromosomes, typical of all body cells. Only in the testis of the male and in the ovary of the female does meiosis occur. It is necessary for each gamete (sperm and oocyte) to contain only one complete set of chromosomes because the paired set of chromosomes will be restored by fertilization. Another amazing complex event occurs during meiosis, and that is each homologous pair of chromosomes synapse (come together) and exchange some parts. This assures that there will be great genetic diversity, as each member of a chromosome pair is randomly distributed to progeny cells.

Accompanying the mitotic spermatogonial divisions and the two divisions of meiosis is an enormous synthesis of the hereditary material of life, deoxyribonucleic acid (DNA). Each cell contains several billion chemical bases that must be assembled in the correct sequence to produce new chromosomes, locking in the correct genetic information.

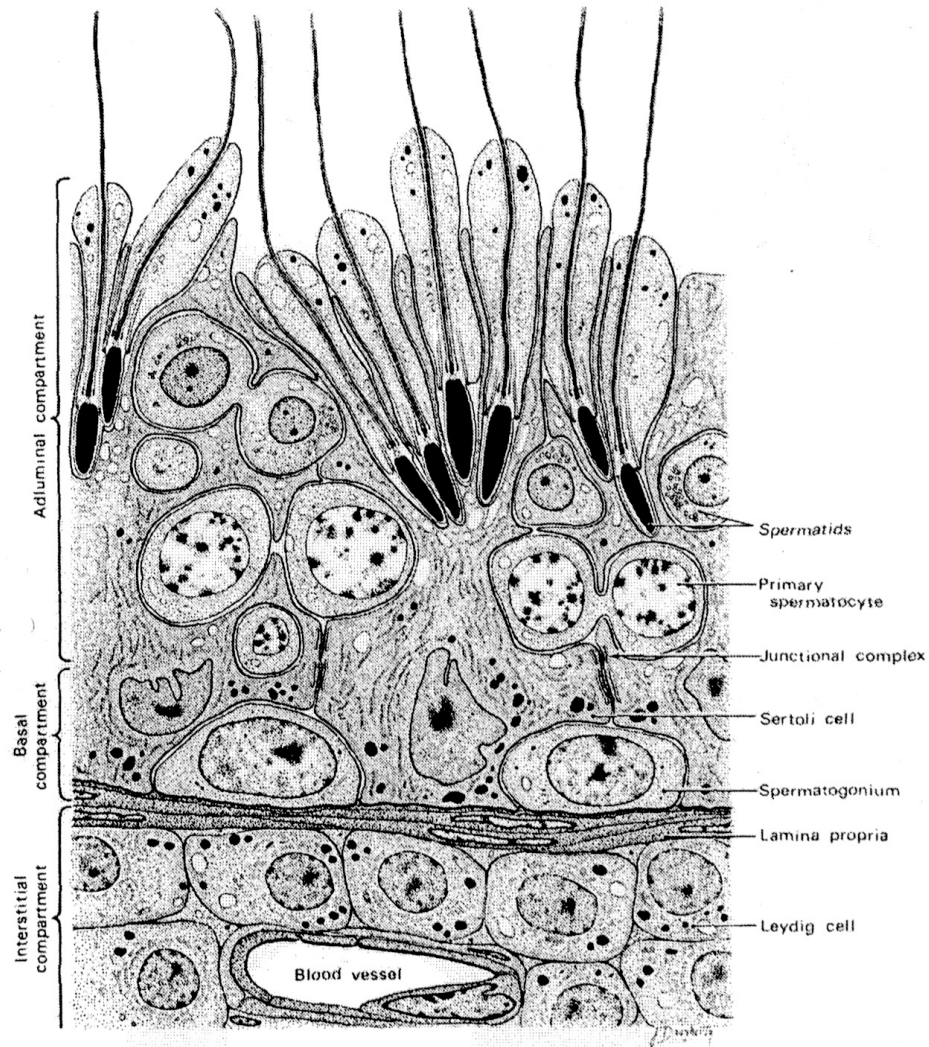


Fig. 2 Drawing of a portion of a seminiferous tubule containing the spermatogenic elements in the basal and adluminal compartments and the interstitial compartment outside of the tubules where androgens are secreted. Secondary spermatocytes are not shown (see text). From Amann and Schanbacher, *J. Anim. Sci.* 57(Suppl. 2):380-403. 1983. By permission.

The next phase in the production of sperm is to transform these approximately round secondary spermatocytes with one full set of chromosomes into the typical mammalian sperm cell. This process is called spermiogenesis. Many genes that have been inactive previously are turned on here to direct formation of sperm. For example, actin filaments gradually form bundles of fibers that produced the tail (flagellum) of the sperm cell. The nucleus of the secondary spermatocyte becomes the principal component of the head of the sperm cell to which the flagellum is attached. Wrapped around the flagellum adjacent to the head is a mitochondrial sheath that provides the energy for the tail to propel the sperm. Golgi bodies are transformed into an acrosomal cap overlying the apical portion of the sperm head. The plasma membrane and other membranes retained are important also for proper functioning of the sperm cell. Much of the cytoplasmic material is discarded, as the fully formed sperm cell basically is a package of

genetic material in the head with a motor and propeller (flagellum). The sole function of this cell is to deliver one set of chromosomes, the paternal genome, to the egg to initiate the beginning of a new discrete individual.

Much of this whole process of spermatogenesis is coordinated by Sertoli cells, amazing flexible cells to which the germ cells are attached within the seminiferous tubules until they are mature and released into the lumen of the seminiferous tubules and leave the testis. The whole process to form a sperm cell in the bull requires about 61 days, so changes in quality of ejaculated semen may reflect testicular insults two months earlier. The whole process of sperm formation is amazing. Many more details than are given here can be found in Amann (1962), Foote (1969), Johnson, Gomes and VanDemark (1970), Clermont (1972), Amann and Schanbacher (1983), Foote and Berndtson (1992) and Knobil and Neill (1998).

A practical application of the testicular studies was the recognition that the larger the testes the longer was the total sperm production line formed by the seminiferous tubules. Early histologists had noted this in the 1800s. Consequently, many studies (see references in Foote, 1969, 1984, 1998; Amann, 1970) revealed that there was a high correlation between testis size and sperm output. This feature was important for AI organizations. In the 1960s Foote and coworkers undertook a multiple year study to measure several thousand testes of bulls in seven AI organizations. The heritability of testicular size was 0.67 (Coulter et al., 1976). This high heritability indicates that important progress can be made in selecting lines of bulls not only with superior genetics for milk production, but also bulls with large testes to produce more sperm to transfer genes to tens of thousands of progeny. A positive correlation was found between testicular size of bulls and milk production of their daughters (Coulter and Foote, 1979).

So taking simple measurements of testicular size (Foote, 1969) is important. Veterinary theriogenologists include testis size as an important part of the breeding soundness examination of bulls (Cates, 1975). Other important noninvasive measurements of testis quality and function include testicular consistency (Hahn et al., 1969), ultrasonic profiles (Coulter and Bailey, 1988), and evaluation by thermography (Kastelic et al., 1997).

Endocrine Regulation of Testicular Function and Sexual Behavior

The major endocrine component of the testis is composed of clusters of cells, called Leydig cells, distributed throughout the interstitial areas among the seminiferous tubules (Fig. 2).

These cells secrete the male hormone testosterone (T_4) and small quantities of other steroid hormones. The production of T_4 is stimulated by the pituitary release of the gonadotropic hormone LH (luteinizing hormone), and a hormonal balance is maintained as T_4 exerts a negative effect on the hypothalamus and anterior pituitary gland (Amann and Schanbacher, 1983). The intratesticular concentration of testosterone is much higher than in the peripheral blood due to the venous-arterial transfer of the hormone in the vascular bed, the pampiniform plexus. Thus T_4 secreted by the testis is locally recycled through the testis. This high concentration of testosterone is important in maintaining spermatogenesis, as was shown in elegant studies by Dvoskin (1944), that were overlooked by

subsequent researchers. The secretion of FSH in the male is modulated by a weak feedback of T_4 on the hypothalamus and anterior pituitary gland, a similar effect of inhibin produced by Sertoli cells, and by estradiol converted from T_4 by aromatase activity. Sertoli cells also secrete androgen binding protein that carries T_4 through excurrent ducts to the epididymis.

Development of spermatogenesis in prepubertal animals also is dependent upon the secretion of the gonadotropic hormones, FSH and LH, with LH playing a dominant role acting on the Leydig cells stimulating secretion of testosterone. The tonic release of LH becomes more pulsatile stimulated by surges in GnRH as puberty approaches (Schanbacher, 1981; Amann, 1983). The endocrine pattern for bulls is similar to other species. For references to the interaction of hormones in the hypothalamic-pituitary-testicular axis excellent reviews are available (Johnson, Gomes and VanDemark, 1970; Hafs and McCarthy, 1979; Amann and Schanbacher, 1983; Cupps, 1991).

Attempts to alter permanently the sexual behavior and sperm output of bulls through injections of compounds influencing hormone secretions in bulls have been ineffective (Hafs and McCarthy, 1979). Studies with castrated males (steers) by McDonald et al., 1976), Sawyer and Fulkerson (1981) and Dykeman et al. (1982) demonstrated that T_4 and especially estradiol (E_2) were powerful stimulators of libido in steers. Estradiol was approximately 1000 times as potent as T_4 in eliciting many of the sexual behavioral characteristics of mature bulls (Dykeman et al., 1982), but responses to E_2 and T_4 were not completely identical. This indicates that both hormones may play a role in promoting male sexual behavior. The fact that T_4 can be aromatized to E_2 suggests that the conversion of small quantities of T_4 to E_2 by the male and/or secretion of small quantities of E_2 by Sertoli cells may be necessary for the full expression of the complement of sexual behaviors in the bull. This concept is supported by the failure of dihydrotestosterone to promote male sexual behavior (Dykeman et al., 1982). Dihydrotestosterone cannot be converted to E_2 , although doses given were sufficient to influence penile development (Sawyer and Fulkerson, 1981).

Bulls vary considerably in circulating concentrations of T_4 . However, no correlation was established in a large bull stud between the concentration of blood plasma T_4 and sexual behavior (Foote et al., 1976). From this result it is concluded that bulls with low libido have problems other than low circulating T_4 concentrations which affect their sexual behavior. A summary of the Cornell studies on hormones and sexual behavior can be found in Foote (1998).

Nutrition of Young and Mature Bulls

When centers were formed to initiate commercial AI there was little information on how bulls should be fed to produce the best quality of semen. It was often stated that bulls should be in "good working condition", but what was that condition? Bulls brought into AI stations from private herds often appeared to be in show ring condition, with excess fat.

Research by Branton et al. (1947) resulted in establishing simple feeding standards to maintain bodyweight and high quality semen production. In practical terms the ration recommended consisted of 0.46 lb of a simple grain concentrate supplement and 0.9 lbs of a mixed grass hay per 100 lbs of bodyweight.

A concentrate mixture containing 12% protein was adequate to maintain a healthy body condition.

Some believed that animal protein was necessary in the diet, but studies conducted by Branton et al. (1949), comparing animal versus plant proteins in the concentrate mixture, demonstrated that semen quality was equally well maintained by plant versus animal proteins. Others believed that low fertility of bulls could be cured by feeding supplemental vitamin E. However, Salisbury (1944) found no beneficial effects of supplementing the diet of bulls with wheat germ. Vitamin A deficiency was known to impair testicular function in laboratory animals and also possibly bulls pastured under extremely dry range conditions. However, bulls consuming good quality hay stored large amounts of carotene in the liver. Feeding bulls a diet with minimal carotene had no effect on semen quality up to the time that the bulls showed other characteristics of vitamin A deficiency, such as visual impairment (Bratton et al., 1948). So, the feeding requirements of mature bulls were met easily by the sound management of providing good quality hay and a limited concentrate supplement. Too liberal feeding of adult bulls led to excess fatness and sluggishness (Almquist, 1982).

Unfortunately, for several years many adult bulls were fed a concentrate supplement readily available commercially for maintaining dry cows. The low protein content was satisfactory, but the calcium content was high. This concentrate feed had been formulated to provide calcium for fetal growth and the onset of lactation in cows. This high calcium intake in mature bulls resulted in excessive calcification of the bones, especially of the vertebrae leading to osteopetrosis (Krook et al., 1969). The loss in flexibility in the backbone in older bulls resulted in difficulty in mounting and also in injuries to the vertebral column. The condition was not correctible in bulls having this problem, but was avoided subsequently by feeding new bulls a maintenance ration with appropriate concentrations of calcium.

Studies of genetic progress, and the initial lack of it in the AI industry, soon focused attention on the need to progeny test many young bulls and select only the few best bulls for extensive use. Raising the bull calves in the AI center reduced the problem of possible spread of disease and provided an opportunity to feed them properly. A new question arose. What is the best nutrition program to grow bulls rapidly so that they can produce semen at an early age and have a long and fertile life if selected for use after progeny testing? Extensive studies for 15 years were conducted by Bratton et al. (1961). These studies demonstrated that bulls fed on low, medium (control), and high planes of nutrition for the first 80 weeks of life came into semen production at 58, 46 and 38 weeks of age. Underfeeding also was shown by VanDemark et al. (1964) to delay puberty, whereas acceleration was economically valuable in being able to test the genetic ability of bulls at a young age by obtaining daughters sooner with milk production records. All bulls were placed on a normal diet thereafter and performed equally well, avoiding the problems of overcondition (Almquist, 1982). So providing excellent nutrition of young bulls was an important component of bull management.

Management of Semen Collection

When AI of dairy cattle began to be widely accepted in the US by the late 1940s, demand for sperm from the top bulls sometimes exceeded the supply. It was clear from the basic studies on the kinetics of spermatogenesis that bulls produced many more sperm than were harvested by the semen collection and scheduling procedures then being practiced by AI organizations. This prompted researchers to study procedures that might stimulate bulls sexually so as to ejaculate more sperm at the time of semen collection, and to examine the potential of collecting semen more frequently. Studies to examine both procedures were initiated at Cornell University by Bratton and colleagues (Collins et al., 1951; Bratton and Foote, 1954; Hafs et al., 1959), by Almquist et al. at Pennsylvania State University (Hale and Almquist, 1960; Amann, 1970; Almquist, 1973, 1982) and at Michigan State University (Hafs et al., 1962).

The conditions surrounding each bull at the time of semen collection were studied. What were the novel conditions that would stimulate a bull to ejaculate the maximal number of sperm after a relatively short time of sexual preparation? It was found that several minutes of active stimulation were required, including a) false mounting of a teaser mount animal several times without allowing the bull to ejaculate, b) moving the bull and/or the teaser back and forth a few feet as needed for each bull, c) bringing the bull to another nearby location with a new teaser animal, or some combination of these variables. Expert bull handlers learned what conditions were most appropriate for each bull.

Another factor studied was what kind of animal should be selected as a mount animal. Originally cows were selected because they were the mount animals in natural service. However, cows could be a source of infection if a bull accidentally was allowed to copulate with the mount animal. Furthermore, they were not physically sturdy enough to be used for repeated mountings. Fortunately, cattle are homosexual. Other bulls or steers could be used for mount animals.

There was a steady supply of bulls rejected following the extensive genetic testing of young sires. Bulls with a quiet temperament, and those that appeared to sexually stimulate the service sires were selected as mount animals. While under some conditions the presence of an estrogenized animal may have been a slight added attraction (Brockett et al., 1994), having a variety of mount bulls available filled the needs for sexual preparation of most bulls.

Comfortable housing and good footing during semen collection to avoid slipping and injury were essential. Warm temperature and suitable pressure of the artificial vagina (AV) also were important positive variables necessary to provide the optimal conditions for collecting semen from each bull (MacMillan et al., 1966; Seidel and Foote, 1969a). Protection against coldshocking the sperm when semen was collected under cold environmental conditions was required (Fig. 3). These conditions were carefully researched and evaluated under practical conditions using bulls in AI organizations (Salisbury and Willett, 1940; Saacke et al., 1982). Size of the artificial vagina was especially important to insure that most of the sperm ejaculated were deposited in the AV at the entrance to the semen collection tube. Otherwise millions of sperm could be lost adhering to the walls of the AV (Foote and Heath, 1963). Loss of these sperm meant loss

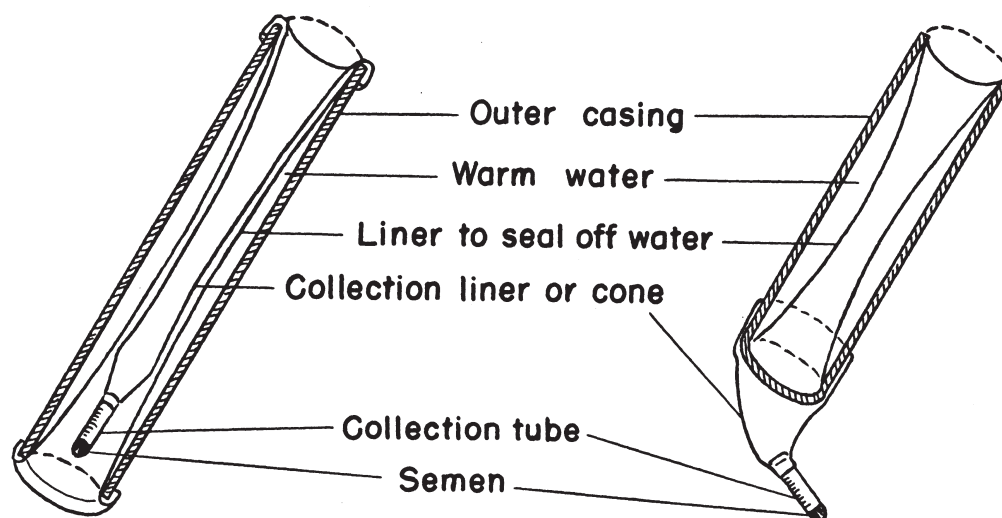


Fig.

Fig. 3 The artificial vagina (AV) designed to protect the semen from coldshock during cold weather (Salisbury and Willett, 1940) is shown on the left. The AV shown on the right is typical of those used commercially, with an insulated jacket (not shown) slipped over the AV for protection.

of the potential to inseminate the maximal number of cows. These studies on semen collection equipment were aided by high speed motion photography and subsequent analysis frame by frame (Seidel and Foote, 1969b).

The earliest method of collecting semen was by massaging the vesicular glands per rectum. Later electroejaculation of bulls (Dziuk et al., 1954) was studied as an alternative method of collecting semen. The electrical method has found application with beef bulls on ranches and at auctions for breeding soundness examinations. However, training bulls to serve the AV in the AI industry was superior, except for an occasional injured sire unable to mount.

Frequency of Semen Collection and Sperm Output

The general concept held initially by the AI industry that frequent semen collection resulted in lowered sperm quality and fertility was challenged initially by modest increases in frequency of semen collection from one time per week to two ejaculations within an 8-day period (Bratton and Foote, 1954). The more frequent semen collections increased sperm output with no loss in fertility. Hafs et al. (1959) collected semen on a daily basis versus a group of bulls ejaculated on a weekly basis for 32 weeks. Only the semen collectors became tired. The bulls ejaculated daily greatly increased sperm output per week, and their fertility following a total of 49,244 inseminations was as high as that for the bulls ejaculated weekly. Elegant studies carried out at Penn State (Amann, 1970; Almquist, 1973, 1982) gave similar results. As a consequence of this research AI organizations usually collect two or three ejaculates of semen any one day for large batch processing, and repeat this procedure at 3 to 4-day intervals. Under these conditions Holstein bulls with large testes collected throughout the year could yield up to two trillion sperm per year.

Semen Characteristics and Evaluation

This section will be presented in some detail because the explosion of research connected with the development of dairy cattle AI had application in other species. For example, increased knowledge of function and fertilizing characteristics of bull sperm was adapted in the medical field for evaluating and preserving human sperm. The great advantage of analyzing extensive data on bull semen characteristics was because much associated fertility data are available (Everett and Bean, 1986) and Grossman et al. (1996).

Ejaculates of semen contain essentially two components, the sperm from the testis and the seminal fluid mainly secreted by the accessory sex glands (Mann, 1964). Extensive research on the biochemistry of semen by Mann and others has been summarized in a book by Mann (1964) with approximately 2,000 references. Knowing the typical concentration of many components in semen was important for several reasons. First, was the interest in fertility. What differences in certain constituents of semen from different males, if any, were associated with differences in fertility? Second, what differences among ejaculates within bulls might be related to fertility? What were the effects of different handling and semen collection procedures on the different constituents of semen? What was the source of origin of these constituents in semen? Finally, what characteristics of semen reflected pathologic conditions in the bull?

Research on sexually transmitted diseases had been ongoing in veterinary colleges before AI became a commercial enterprise. Faculty in the New York State College of Veterinary Medicine at Cornell, such as Williams, Olafson, Roberts, Gilman, McEntee, Lein, and others (Roberts, 1986) played significant roles in studying the detection, etiology and treatment of various reproductive diseases. Major studies on bull reproductive pathology in Europe include those by Lagerlof (1934) and Blom and Christensen (1947). Clumps of leukocytes in semen were the most notable grossly visible sign of semen pathology. However, pathogenic organisms could be present in normally appearing semen, and they required numerous laboratory tests to detect them. Eventually infected bulls in AI centers either were treated successfully (Lein et al., 1968) or eliminated. Later all new bulls entering the AI centers as young calves were raised in isolation. These bulls were specific-pathogen free. The following discussion of semen characteristics is restricted to semen from healthy animals.

Seminal plasma.

Knowing the composition of the fluid portion of semen could be important from the standpoint of fertility, either because it reflects some component present that enhances the fertility potential of the ejaculated sperm, or it reflects the fertilizing capacity inherent in the sperm (Killian, 1992). With AI the semen is highly extended with media during semen processing. This reduces the role in AI that any seminal plasma component might have in natural service. However, such a component could be added to the extended sperm used for insemination (Amann et al., 1999). Other constituents of seminal plasma (Sprott et al., 2000) associated with bull fertility might lead to identification of a genetic marker useful in identifying young bulls with a potential for high fertility. These discoveries

and many others may lead to ways of improving the fertilizing potential of semen. However, cryopreservation can complicate application of a technology if it involves a component attached to the surface membrane. Freezing can remove surface components of sperm important in fertilization (Lessard et al., 2000).

Sperm cells.

The sperm cell has been studied extensively (Hancock, 1952; Bishop, 1962; Maule, 1962; Mann, 1964; Beatty, 1970; Salisbury et al., 1978; Fawcett and Bedford, 1979; Barth and Oko, 1989). Only selected examples of the literature are cited here. Initially we will focus on the characteristics of semen usually evaluated by commercial AI organizations (Table 1). The characteristics measured are driven by the objective of AI organizations to harvest as many high quality sperm as possible from their genetically superior sires.

Table 1. Typical Characteristics of Ejaculated Semen by Mature Holsteins: Bulls Under Different Conditions of Semen Collection

Seminal Characteristics ^a	Ejaculated 1X/week		Four ejaculates/week Sexual preparation
	No. prep. ^b	Sexual prep. ^b	
Volume, ml	5.5	8.0	7.0
Concentration, 10 ⁶	1200	1700	1400
Motility, %	70	75	70
Sperm/ejaculate, 10 ⁹	6.6	13.6	9.8
Sperm/week, 10 ⁹	6.6	13.6	39.2
Abnormal sperm,%	<10	<10	<10
^a From the literature.			
^b No sexual preparation before semen collection versus sexual preparation described in the text.			

The volume of semen collected was the easiest trait to measure, and it is one of the first to be recorded. Volume originally was estimated by eye, viewing the semen collected in a graduated test tube. Bubbles on the top of the semen and parallax introduced considerable error in the estimated volume. With the introduction of simple top-loading balances Foote (1998) developed the more accurate method of estimating volume by weight. Later the author learned from his friend, Professor Nishikawa in Japan that the latter, for many years, had estimated the volume of ejaculates of stallion semen by weighing them.

Volume of semen collected in each ejaculate is decreased as the frequency of ejaculation is increased (Hafs et al., 1959; Amann, 1970, 1981). Table 1 also shows that increased sexual preparation increased the volume of semen collected. Using appropriate semen collection equipment also increases the volume of semen collected (Seidel and Foote, 1969a,b).

Of great importance is the concentration of ejaculated sperm. This characteristic is one of the most variable of the seminal traits. Clinically, bulls with firm testes generally produce semen with a higher concentration of sperm (Hahn et al., 1969). It is extremely important to measure sperm concentration

accurately so that the total sperm per ejaculate (volume times concentration) is known. This allows the AI organization to determine how many sperm are available for processing into individual breeding units. Likewise it is important for researchers and bull stud managers to have reliable quantitative information when evaluating effects of treatment and/or management procedures on sperm output. Considerable advances have been made in counting sperm from the original slow inaccurate hematocytometric method to the faster and more accurate photometric method (Comstock and Green, 1939; Salisbury et al., 1943b; Foote, 1972b) or the use of particle image counters (Parks et al., 1985), and flow cytometry (Parks, 1992).

The motility of the sperm cell reflects sperm cell viability. This measurement has been demonstrated in thousands of reports to be related to fertility (Bratton et al., 1956; Maule, 1962; Mann, 1964; Salisbury et al., 1978; Amann, 1989; Malmgren, 1997; Larsson and Rodriguez-Martinez, 2000; Foote, 2003a). The most common simple method of estimating motility is to dilute the sperm to a standard concentration that allows the number of moving and nonmoving sperm to be assessed when viewed microscopically. This assessment of the percentage of motile sperm should be done by a trained observer. It is a simple procedure, but it is subject to many variables. With fresh sperm how much has it been diluted and with what diluent (Farrell et al., 1996a)? At what temperature are the sperm when they are observed? What is the training of the observer? What kind of microscope is used? Is the motility estimate made immediately after semen collection, after incubation or some period of storage, or after freezing and thawing? The rate of forward motion as well as the percentage of motile sperm is related to fertility (Bratton et al., 1956).

Rothschild (1953) and Rikmenspoel et al. (1960) were pioneers in studying sperm motion with imaging and electronic equipment. This led to the development of equipment capable of measuring a variety of sperm motion characteristics, referred to as computer-assisted sperm analysis (CASA). The equipment underwent many improvements. Eventually it was capable of monitoring sperm in complex fluids such as milk by utilizing wavelengths compatible with the DNA stain Hoechst 33342 (Farrell et al., 1996b; Tardif et al., 1998). The multiple sperm characteristics measured by CASA can be combined into an index highly correlated with the fertility of sperm (Farrell et al., 1998). However, because of equipment cost and technical requirements, CASA has not been adopted widely by the cattle AI industry. Rather it is used by researchers measuring effects of experimental treatments, the impact of noxious environments, and by large medical clinical facilities (Amann and Katz, 2004).

Gross morphology of sperm, particularly head shape, has been studied since the development of the simple microscope by van Leeuwenhoek in 1678, with multiple advances in instrumentation (Fawcett and Bedford, 1979). Multiple references on bull sperm morphology and the classification of different characteristics are available (Mercier and Salisbury, 1947; Blom, 1950b; Maule, 1962; Salisbury et al., 1978; Barth and Oko, 1989). In the early days of AI there was substantial variability among bulls in the morphology of their sperm. Eventually most dairy bulls selected for testing came from pedigrees that contained several

generations of breeding by AI where bulls have been selected for high semen quality and fertility. The proportion of abnormal sperm usually is low, with rare exceptions (Hough et al., 2002), as is shown in Fig. 4.

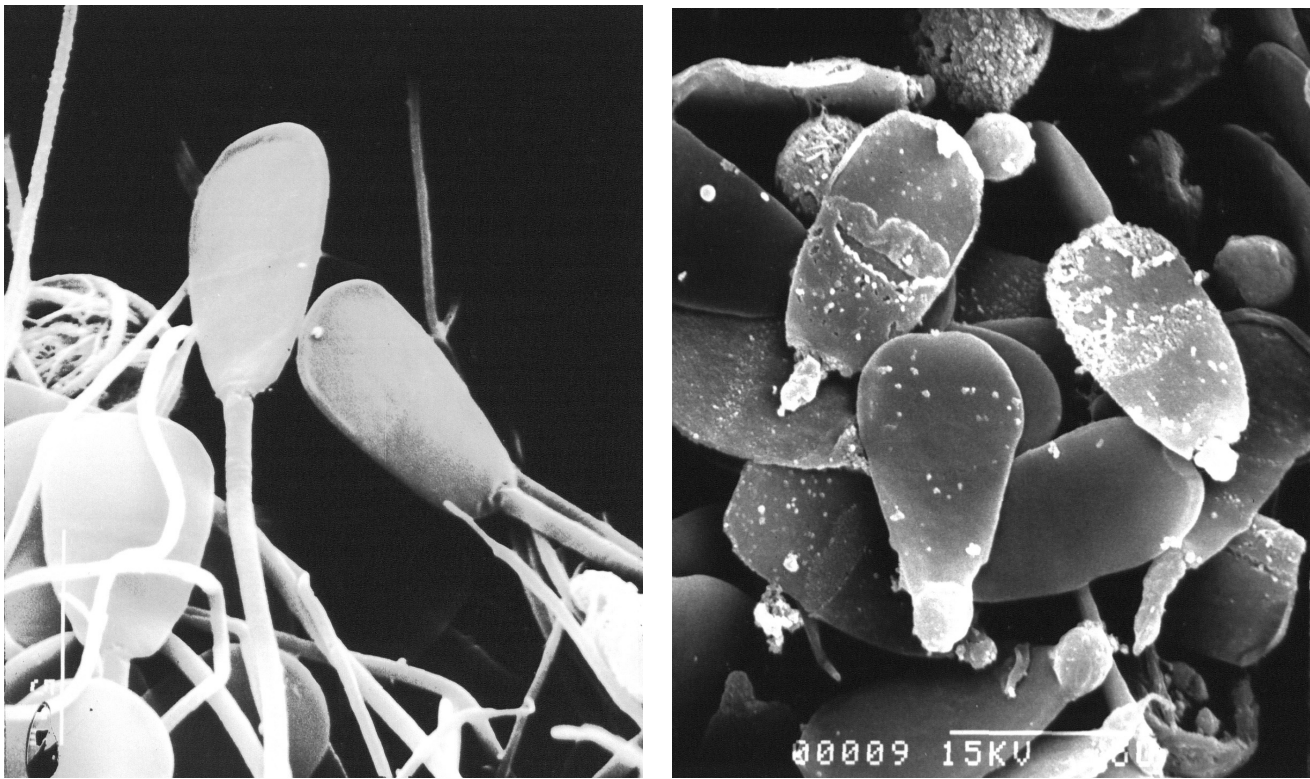


Fig. 4 Normal bull sperm on the left, and abnormal sperm showing extreme teratospermia on the right. The two bulls producing these sperm were half brothers. This inherited condition is rare. For details see Hough et al., 2002.

One specific characteristic that has found considerable usefulness is the morphology of the acrosome. This sperm cell component must undergo a reaction (acrosome reaction, AR) prior to fertilization, and fertility is impaired when abnormal acrosomes are formed or acrosomes are damaged by some environmental impact. Saacke and Marshall (1968) described a simple procedure for characterizing the acrosome on unfixed cells, thus avoiding fixation and processing artifacts.

Following insemination many changes in sperm must precede the AR which is not fully understood after thousands of published studies the past 50 years. One change is referred to as capacitation of sperm. There are many biochemical events comprising capacitation causing sperm to swim in a hyperactive pattern (Suarez, 1996). One biophysical change is cholesterol loss from sperm membranes (Ehrenwald et al., 1988). Cryopreservation of sperm can initiate capacitation of bull sperm (Cormier et al., 1997). Attempts to alter these events in vitro (Way and Killian, 2002), including the improvement in fertility of cryopreserved sperm, are being investigated (Purdy and Graham, 2004).

Likewise flow cytometry combined with various staining procedures has provided a wealth of information about sperm membrane integrity, osmotic behavior, live-dead sperm proportions, sperm chromatin (DNA) structure,

mitochondrial function, and acrosomal integrity (Graham et al., 1990; Parks, 1992; Garner et al., 1997a,b; Thomas et al., 1997; Evenson et al., 2002). The integrity of sperm cell membranes were explored in the early days of AI by the use of supravital stains (Blom, 1950a; Salisbury et al., 1978; Barth and Oko, 1989). Later studies of the volume of sperm exposed to solutions varying in solute concentration demonstrated that sperm with intact membranes behaved as osmometers (Lindahl and Drevious, 1964). The ability to swell was positively related to the proportion of unstained sperm (Bredderman and Foote, 1971; Liu and Foote, 1998a, b) and to fertility (Bredderman and Foote, 1969). The principle of viable sperm having the capacity to swell in hypoosmotic solutions was adapted to evaluate human sperm by a hypoosmotic swelling test (Jeyendran et al., 1984; Smikle and Turek, 1997). Much of the research with bull sperm was applied in medical clinics, partly because many of the technical people in medical laboratories dealing with sperm had been trained in animal and dairy science departments.

The DNA content of bull sperm and its variability in bulls of low fertility was studied in the 1950s (Leuchtenberger et al., 1956; Leuchtenberger, 1960), and later by Gledhill (1966, 1970). Paufler and Foote (1967) reported that the DNA of bull sperm exposed to light was damaged. However, all these early studies required tedious work to evaluate individual sperm. This changed when Evenson et al. (1980) demonstrated that sperm DNA could be evaluated by high speed flow cytometers using fluorometric procedures. Much has been learned about the genetic integrity of sperm DNA since then (Evenson et al., 2002). However, the technical characteristics of the flow cytometers and skill of the technicians required have limited their application primarily to researchers studying infertility in men and the effect of environmental toxins on the testes and sperm produced. Nevertheless, it is likely that in the future, as gene mapping combined with genetic expression and genetic engineering advances, DNA analysis in several forms will play a role in breeding programs for dairy and beef cattle.

Size of sperm heads has been investigated (van Duijn, 1960; Beatty, 1970), partly testing the assumption that the difference in DNA content between sperm containing the X versus Y chromosome might lead to a bimodal distribution (Chandler et al., 2002). Foote (2003a) cautioned that processing procedures could affect head size. With the ease of measuring large populations of sperm electronically, any artifacts produced could become statistically significant and wrongly interpreted.

In the reviews of literature previously cited are many other tests of sperm quality, such as electrical activity, oxygen consumption measured directly or as a methylene blue or resazurin dye reduction test, migration of sperm through mucus or synthetic gels, and vertical swimup of sperm in liquid medium. More recently, binding to zona pellucida (Zhang et al., 1998; Braundmeier et al., 2002), penetration of hamster or bovine oocytes (Gordon, 1994; Foote, 2003b), changes in sperm activity associated with capacitation (Suarez, 1996; Lefebvre and Suarez, 1996), and IVF (Rodriquez-Martinez, 1998) have been studied as indicators of the fertilizing ability of sperm. While many of these studies have contributed to understanding sperm function, none of the tests has been sufficiently correlated with fertility, or rapid and simple enough, or of additional value beyond the

classical tests of sperm motility, to be used routinely in AI programs.

The most likely immediate improvement in being able to predict the fertility of sperm with currently available information may be by combining several tests of semen quality (Farrell et al., 1998; Rodriguez-Martinez, 1998; Zhang et al., 1998). This would require equipment that could measure several characteristics precisely with high repeatability under standardized conditions (Amann, 1989). Also, it should be recognized that prediction of future relationships is subject to greater error than correlations obtained based upon past performance.

Semen Processing and Preservation

The initial development and rapid expansion of AI in the 1940s and 1950s was made possible by several factors. First was the discovery of the protective effect of egg yolk on sperm (Phillips and Lardy, 1940; Salisbury et al., 1941), and later, heated milk (O'Dell and Almquist, 1957) for preserving liquid semen. The Salisbury medium contained sodium citrate as a buffer with egg yolk. This became much more popular than the phosphate buffer used by Phillips and Lardy because citrate dispersed the fat globules in egg yolk, making a clearer medium in which to examine the sperm microscopically. Davis et al. (1963) obtained excellent results with an egg yolk-Tris extender that was widely used. Both egg yolk and milk contain lipoproteins and other macromolecules that protect sperm against coldshock when they are cooled from body temperature to 5°C (Foote and Bratton, 1949). These semen extenders made possible delivery of semen into the field at 5°C where it could be used for insemination for up to 3 days.

Another medium that gave the highest fertility with liquid semen in the 1960s was a complex buffer combined with egg yolk. It was called CUE as a Cornell University Extender (Foote, 1998). It was not useful for frozen semen, so it was discontinued eventually. However, researchers in New Zealand (Shannon et al., 1984) modified it for use at 5°C to inseminate large numbers of cows with only 2 million unfrozen sperm per cow during the intensive short breeding season in New Zealand.

CUE also was used by Nebel et al. (1985) for microencapsulation of liquid sperm. However, they used egg yolk-citrate-glycerol extender for freezing sperm. This novel system for packaging semen is being researched with the objective of prolonged survival and possible sustained release of sperm in the female (Vishwanath et al., 1997).

A note on terminology should be mentioned because the early literature refers to media used for conserving sperm as “diluters”. Many media were simply buffers used for dilution. However, buffered egg yolk extended the useful life of sperm. Foote and Bratton (1949) coined the word “extender” to replace “diluter”. Soon extender terminology was adopted worldwide. The authors had also thought of a snappy term “suspender” but discarded the idea.

An early improvement in semen extenders was their formulation to control infectious agents in semen by adding antibacterial agents (Foote and Salisbury, 1948a; Almquist et al., 1949; Foote and Bratton, 1950). Many of the bulls brought into the AI organizations with natural service proofs also had *Vibrio fetus* (later renamed *Campylobacter fetus*). This disease was not well-studied at the time because the organism was difficult to culture.

After antibiotics were added to semen extenders it was apparent that all early aborted feti brought to the State Diagnostic Laboratory at the Veterinary College at Cornell University came from herds using their own bulls in natural service. Antibiotics added to semen prevented infection by *Vibrio fetus* (McEntee et al., 1959) and other organisms (Fig. 5).

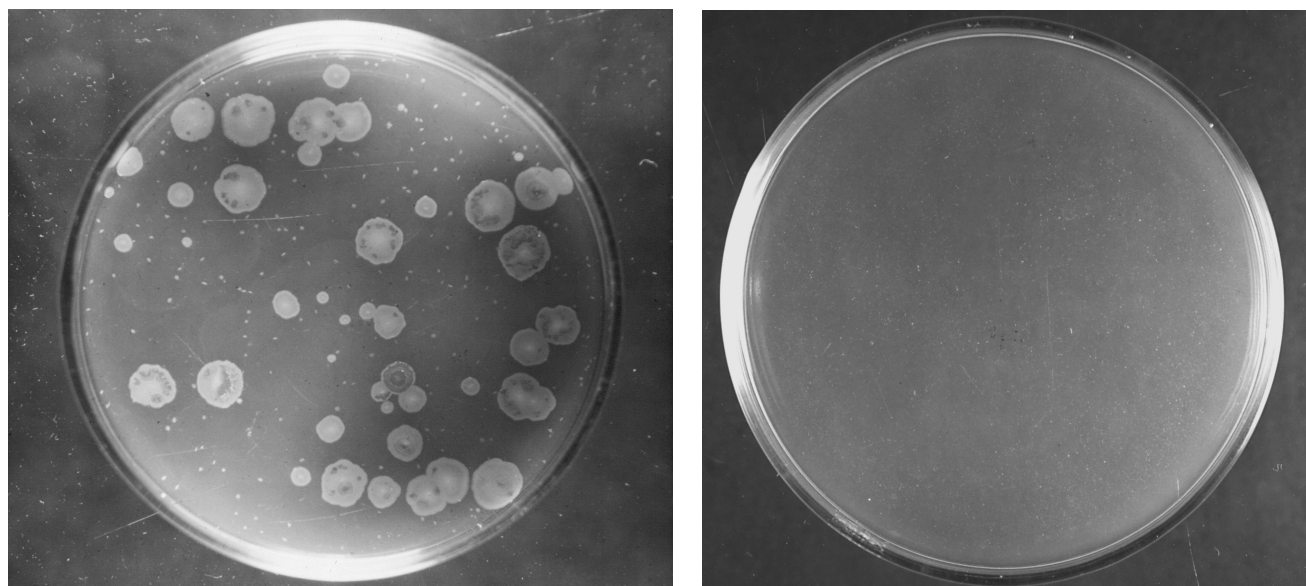


Fig. 5 *Pseudomonas pyocyaneus* and other organisms in bull semen growing on agar medium (left photo). The inclusion of penicillin, streptomycin and polymyxin D in the semen extender controlled the growth of all organisms in the treated semen (right photo). R.H. Foote, unpublished, 1947. See text

Veterinarians recommended AI to dairymen to control this disease and animal scientists and extension personnel recommended AI also for improving herd genetics and breeding records. With the improved semen cooling procedures and antibacterial agents in semen extenders, the nonreturn rate increased about

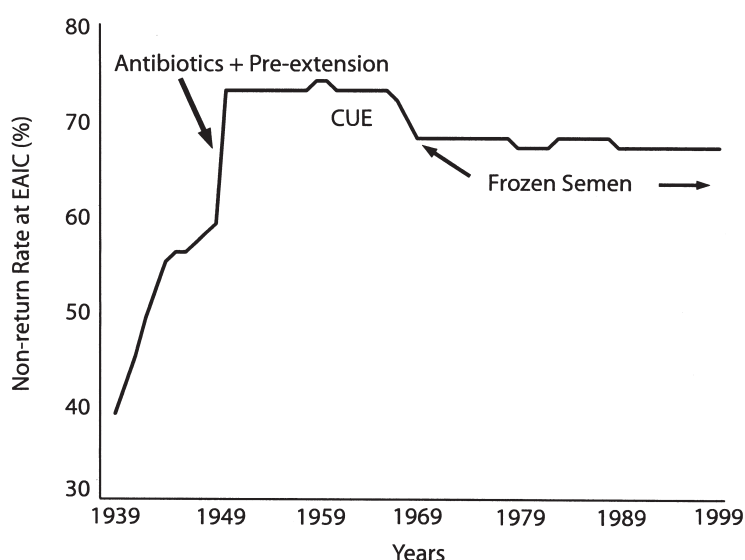


Fig.6 Many improvements in AI technology contributed to the improvement in non-return rate. Noteworthy were the addition of antibiotics to semen and the pre-extension of semen with egg yolk medium before cooling, as well as the introduction of Cornell University Extender (CUE). Adoption of frozen semen resulted in a decline in fertility. See text for details.

15 percentage units (Fig. 6). Conception rates in herds using AI started to exceed those in herds using bulls. Later Dr. Lein and colleagues (1968) at the Veterinary College at Cornell successfully treated infected bulls.

This was a golden age of growth for AI. Neither Cornell nor Pennsylvania State University received any remuneration for their achievements with semen extenders. No patents were filed. The cooperating AI organizations were essentially partners in the process. We all could have used money from patents effectively to support new research, as funds for agricultural research were minimal. But the world benefited both agriculturally, and from all the knowledge and technical skills that workers trained in this field brought to the higher paying jobs in the medical field. Often there were ten-fold differences in wages between PhDs who did research on National Institutes of Health (NIH) grants and the clinical investigators in medical schools who signed the proposals submitted to NIH.

Sperm Numbers Per Insemination

Simultaneous with the research on better extenders for bull semen was the important research on extension rates and sperm numbers needed per insemination to achieve high fertility. The pioneering studies by Salisbury et al (1943a) and later by others have been summarized (Salisbury et al., 1978). In conducting experiments in the 1940s with “diluted” semen Salisbury was criticized by some who declared that the dilution was like “watering the milk”. However, the results showed that fertility improved as one increased the dilution of semen with preserving medium. Why? It is likely that the dilution of semen was beneficial because many of the bulls, unknown at that time, were infected with *Campylobacter fetus*. Dilution of this organism would tend to reduce the incidence of infection, as the dilution effect has been demonstrated with many organisms, and there were more than enough sperm used for insemination. Subsequent studies (Bratton et al., 1954, 1956; Foote, 1970a) demonstrated that 4×10^6 motile sperm per insemination dose resulted in high nonreturn rates (Fig. 7). Actual conception rates also were determined to be excellent (Foote, 1978).

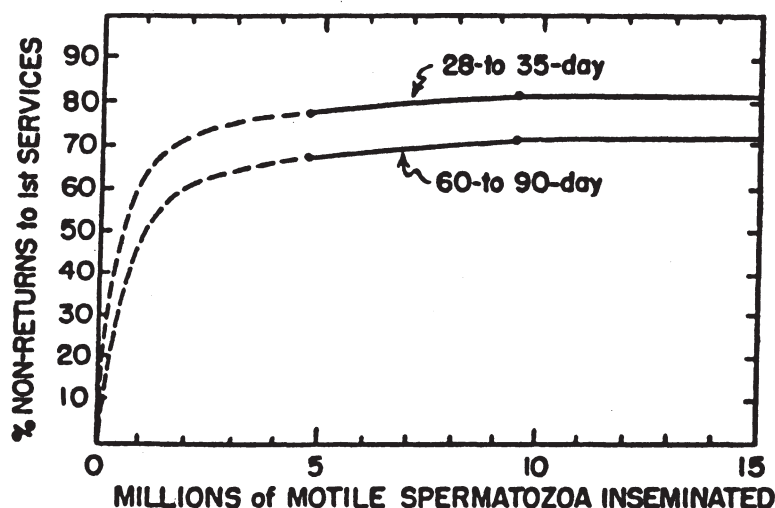


Fig 7. The relationship between the number of motile sperm inseminated and fertility (nonreturn rate). Bratton et al., J. Dairy Sci. 37:1353-1356. 1954.

Frozen Semen

A major change in breeding cattle occurred when it was discovered that bull sperm preservation could be prolonged by freezing. More sperm per breeding unit were required and the whole system of handling semen was revised. This change began after Polge et al. (1949) found that glycerol protected chicken sperm during freezing. Polge's research was aimed at finding sugars that might dehydrate the sperm and protect them. Polge relates that after failure with the sugars he returned 6 months later to repeat the experiments. Presumably he used the same bottle of fructose, and it worked. However, the label had fallen off the bottle. What was in the bottle? A chemical analysis revealed that the bottle contained glycerol and proteins similar to Meyer's albumin used for histology. So bottles stored for 6 months appeared to have been mislabeled.

Serendipity was displayed with this accidental discovery (Polge, 1968). About 1754 Horace Walpole coined the word serendipity, representing accidental discovery combined with sagacity. He conceived the idea from an old Persian tale called "The Three Princes of Serendip". The princes noted that whenever the horse-drawn carriage stopped the horse always ate grass on the same side of the road, despite the fact that the grass was at least as lush on the opposite side. They concluded that the horse must be blind in one eye, and therein lies the sagacity. Unfortunately, the dictionaries do not put emphasis on sagacity in defining serendipity.

Fortunately, the bull semen extenders used for liquid semen such as egg yolk-citrate (Salisbury et al., 1941), milk (O'Dell and Almquist, 1957; Almquist and Wickersham, 1962) and later Tris-buffered egg yolk (Davis et al., 1963), proved to be excellent media for cryopreserving bull sperm when glycerol was included. The 50% egg yolk component originally used in extenders for liquid semen was reduced to 20% by volume for best results with frozen semen (Salisbury et al., 1978).

Packaging of semen for freezing was a problem initially. Whereas extended liquid semen had been shipped to inseminators in test tubes, and inseminators withdrew about 1 ml of semen for each cow inseminated, frozen semen had to be packaged in individual breeding doses. Various types of plastic tubes and glass ampules were used for packaging. Storage initially was in mechanical freezers with solid carbon dioxide (Dry Ice®). Only one dose was thawed at one time. Glass ampules often broke during freezing or thawing. Cassou (1964) modified the system developed by Sørensen (1938), with a method for sealing plastic straws and a gun for insemination (Pickett and Berndtson, 1974). Originally 0.5 ml-capacity straws were used, but 0.25 ml straws are popular because they require less storage space. Soon the Cassou straws were used worldwide.

At the same time that different methods of packaging semen were explored researchers were comparing different freezing protocols and temperatures of storage (Maule, 1962; Salisbury et al., 1978; Herman, 1981). Crystals grew during storage at -79°C with Dry Ice® that damaged sperm. At -196°C, with liquid nitrogen, it appeared that sperm could be stored in a stable condition for a long time (Foote, 1972a). However, liquid nitrogen storage also was a problem, because insulation of the glass vacuum containers available in the early 1950s

was inefficient. Frequent refilling was required to maintain a safe temperature of about -196°C . Manufacturers of tanks were not interested in developing improved containers because the perceived market was small and the cost of development was high. J. Rockefeller Prentice, owner of American Breeders Service, approached the Linde Air Products group and reportedly said, "If I give you a million dollars to develop tanks with more efficient insulation will you be interested?" The rest is an amazing story. Sturdy stainless steel liquid nitrogen storage containers were developed that could hold liquid nitrogen for weeks instead of days, and retain some liquid nitrogen for months. A large cryopreservation industry for the preservation of multiple types of cells, tissues and other biologicals was born. With the adoption of frozen semen the genetics of bulls located in multiple countries became available worldwide. Once the appropriate infrastructure was established semen could be made available wherever needed to inseminate cows at the proper time of their reproductive cycle. This was democracy in action, as the ordinary farmer and "gentleman farmer" had equal access to superior bulls.

There is at least one disadvantage of frozen semen. Despite thousands of studies on methods and media for freezing sperm, no protocol has been developed that does not damage the sperm. Consequently, the conception rate with frozen semen is consistently lower than when comparable unfrozen semen is used (Shannon and Vishwanath, 1995). Possibly additions of fertility enhancers (Amann et al., 1999) or alteration of the sperm cholesterol content could help to counteract this disadvantage. Another potential disadvantage is the possible mishandling of frozen semen as it is passed on through distributors and stored in the field. The major AI organizations follow highly controlled and standardized procedures to assure that only high quality sperm are in the straws or other packaging containers that leave their premises. Our research (Foote, 1975b, 1998) has shown that semen held at the central storage unit under liquid nitrogen for many months does not change in fertility potential, whereas comparable semen held by inseminators decreases in fertility over time.

Insemination Procedures and Timing

The original method of inseminating cattle was called the "Speculum" method. By inserting a speculum into the vagina to expose the cow's cervix, a pipette with the semen contained in it was expelled into the entrance (os) of the cervix. Sperm had to work their way through the tortuous cervix against an outflow of mucous. This required many sperm in order for a sufficient number to enter the uterus and eventually reach the egg.

Danish veterinarians (Hendrikse and van der Kaay, 1950; Perry, 1968; Herman, 1981) developed the rectovaginal method of insemination. This allowed skilled inseminators to insert the catheter through the cervix and deposit the semen into the body of the uterus. This technique is shown later in the text when the structure of the cow's reproductive tract is illustrated. Because sperm no longer had to navigate through the cervix against an outflow of cervical mucous, many fewer sperm were required for insemination per cow and the conception rates were higher (Hendriske and van der Kaay, 1950; VanDemark, 1952).

So from the early stages of AI all conditions were established to make it possible for one superior bull to sire thousands of calves per year (Fig. 8). Not only

do bulls produce billions of sperm per week, but also the semen can be extended successfully to provide a few million live sperm per insemination which, properly placed in the cow, can lead to a high conception rate.

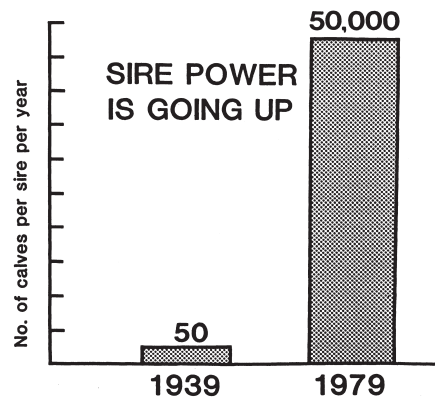


Fig 8. The development of various procedures in AI soon resulted in one bull being capable of siring 1000 times as many progeny as a bull in natural service in 1939.

Hundreds of studies have been published on the optimal place to deposit the extended semen in the cow. These places include deposition of sperm 1) in the cervix, 2) in the body of the uterus, 3) in one uterine horn, 4) both uterine horns or some combination of these locations (VanDemark, 1952; Maule, 1962; Salisbury et al., 1978). Results have varied. Over all studies it appears that depositing the sperm just through the cervix has become the preferred procedure (McKenna et al., 1990). Studies with individual inseminators show that they vary greatly on where sperm are deposited. Retraining of inseminators using dyes deposited in reproductive tracts obtained at slaughterhouses has been useful. Peters and Senger (1983) developed a method with radiopaque material added to extender. This technique permitted evaluation of site of deposition in cows as well as in isolated reproductive tracts. Conception rates improved following retraining.

Timing of insemination relative to ovulation also is critical to success of AI, so programs properly designed to detect onset of estrus accurately are important in determining the correct time for insemination. The best behavioral sign that a cow is in estrus is when the cow stands to be mounted by another cow or detector animal. There are many other signs suggestive that a cow is in estrus and many systems for detecting estrus (Foote, 1975a). Trimberger (1948) was one of the first to study time of ovulation relative to estrus and to recommend a practical system for timing insemination. His system, still used today, is called the “AM-PM” rule. Cows first detected in estrus in the morning should be inseminated in the afternoon the same day. Cows first detected in estrus in the evening should be inseminated in the morning the next day. Fertility results were higher when 44,707 cows in commercial AI were inseminated following the AM-PM rule, although there was no sharp peak (Foote, 1978, 1979). This requires skillful observation of cows both morning and evening. However, Nebel et al. (1994) reported that insemination of cows once per day, particularly in midmorning, gave nonreturn rates as high as when the AM-PM rule was followed.

By incorporating radiotelemetry into a system for detecting estrus (Dransfield et al., 1998; Nebel et al., 2000), the onset of estrus was determined more precisely. Under these conditions higher conception rates resulted from AI 5 to 14 hours after the cows first stood to be mounted. This wide window provides a leeway for inseminating most cows in a herd at one convenient time. More details on the physiology of the estrous cycle, estrus, and synchronization of estrus and ovulation are discussed in a later section on The Female Component.

A major change in who inseminates cows has taken place in the past few decades, as herd size grew and frozen semen replaced liquid semen. Originally, liquid semen was shipped to professional inseminators who performed the inseminations in many herds, usually as a full-time job. After intensive training and daily practice they became very skillful. With the availability of frozen semen large herd owners often purchased their own containers for storing frozen semen and obtained frozen semen from more than one supplier. Herd managers often were trained to perform within-herd inseminations, avoiding costs of travel and medical coverage provided for professional inseminators. Also, herd owner-inseminators could conveniently selectively inseminate some cows two times during one estrus. Various reports and our own unpublished studies do not indicate that this substantially improved the conception rate, however.

Estimating Fertility

In the complex phenomenon of mammalian reproduction, differences in fertility can result from many causes and be evaluated with many endpoints (Foote, 2003a). Consequently, the term fertility has been used in various ways in the preceding sections, and will be an important component in subsequent sections. Therefore, discussion of the term fertility should be helpful.

Here we are dealing with fertility in the context of fertilizing an egg (oocyte) and establishing a pregnancy. We are not dealing with the total cycle of reproduction, i.e. producing successive generations of live animals. The female either conceives or does not conceive, producing the typical binomial of 0 or 1. A sire in artificial insemination is tested across many females, so a proportion of successes and failures can be calculated with the binomial variation being a major source of the total variance. Recognition and measurement of the various sources of variation affecting fertility have been important in studying the biological and technological components affecting the probability of conception and in applying advanced biotechnology. The genetic component of fertility differences appears to be small (Foote, 1970b).

With the development of AI, a simple measurement of fertility was needed on all cows inseminated to compare the fertility of bulls, evaluate semen processing and insemination procedures, and to examine herd reproductive performance under practical farm conditions. Salisbury at Cornell and Thompson at the new New York Artificial Breeders Cooperative, Inc. developed the nonreturn rate to service as an estimate of the conception rate (Thompson and Salisbury, 1947). Inseminators filled out a breeding receipt in triplicate. One copy was given to the farmer, the inseminator kept one and the third copy was sent to the central unit producing the semen. The receipts were tabulated daily as they were received at the central unit. Every cow that returned for a second service was charged to that

cow so that, over a population of tens of thousands of cows, data were accumulated on the proportion of cows that returned or did not return for reinsemination. These results were tabulated at various intervals of time after service so that the percentage of nonreturns for service were calculated at 1 month, 2 months, 3 months, etc. This system was invaluable for researchers conducting hundreds of field trials involving semen from bulls managed differently, and comparing tests of semen quality and semen processing procedures (Foote, 1998).

With liquid semen the nonreturn rate was more accurate in estimating conception rate than it was with frozen semen. With liquid semen the rate was somewhat inflated because some nonpregnant cows were sold or bred to a bull on the farm, and consequently there was no return recorded for the insemination (Foote, 1978). With frozen semen additional problems exist because a herd may stock frozen semen from several suppliers and do their own inseminating. Semen from different suppliers may be used on first and repeat inseminations. Furthermore, many inseminations are not reported, except where the breeding is done by AI professional technicians working for one AI organization. Therefore, the nonreturn rate no longer is reliable as an estimate of conception rate under most present conditions.

Employment of inseminators to perform all of the inseminations within a few large herds is an option that has been used by AI organizations to gather fertility information (Kaproth et al., 2002). In this latter study, pregnancy was determined by palpation of the reproductive organs per rectum. This method of detecting pregnancy is accurate, but it is not a useful method of determining conception rates routinely by AI organizations because many herds do not have pregnancy checks performed. Furthermore, when pregnancy checks are done, the results generally are not available to the AI organization. Ultrasound also can be used effectively (Ginther, 1998). Early cowside pregnancy tests have not proved to be highly reliable (Cordoba et al., 2001).

A weakness in most studies designed to detect differences in fertility in AI is the fact that AI organizations put more sperm in each breeding unit than are needed for optimal conception rates. This really is an insurance procedure. If a procedure truly results in an increased number of highly fertile sperm surviving, this beneficial effect could go undetected because of the excessive number of sperm used. Such a beneficial effect, if detected with fewer sperm, could then be utilized to make sperm from the best sires more widely available by reducing the number of sperm inseminated.

Other methods of estimating fertility have been suggested. For example, competitive fertilization following the insemination of mixed semen from two bulls with different color markings is a very efficient system for ranking bulls on fertility (Saacke et al., 1980; Dziuk, 1996; Foote, 2003b). This system is not practical in routine AI. However, competitive fertilization can be conducted in vitro with mixed semen containing sperm appropriately marked with different colored nontoxic dyes. The mixed sperm can be added to groups of hamster (Davis and Foote, 1987) or bovine oocytes (Gordon, 1994), and the proportion of oocytes fertilized by the differently colored sperm counted. This test can be useful for ranking bulls, but it is not suitable for estimating the actual fertilizing

potential of ejaculates of semen as used for AI.

National tests of cow fertility have been developed using DHI records, but they have been controversial relative to their usefulness for evaluating sire fertility. However, they may be useful in genetic selection of sires with potential for producing daughters that are highly fertile (Clay et al., 2004; VanRaden et al., 2004).

New novel tests that can be combined with currently used tests of semen quality would be beneficial if they are highly correlated with fertility. Before tests are applied routinely, repeated experiments are desirable to check the reliability of initial results and to test them under different conditions.

Experimental Design

An important consequence of the accumulation of large amounts of data on semen quality under standardized conditions was the opportunity to study multiple sources of variation, quantify these, and utilize this information to design efficient experiments. One would like to be able to estimate how many bulls and how many ejaculates per bull, in an optimal experimental design, are needed to detect an expected (specified) difference among treatments at a specified level of significance (often arbitrarily selected at $P < 0.05$ or $P < 0.01$), with a specified probability of detecting the difference. One should include enough replicates in the experimental design to achieve this objective without wasting resources in conducting experiments that are larger than necessary (Hafs et al., 1958; Seidel and Foote, 1973). Similar considerations apply when designing experiments to test fertility of semen from different bulls processed multiple ways (Dunn, 1961). Results should then be applied to sets of data obtained independently to test their repeatability.

Other Developments in Male Reproduction

Separating Sperm by Sex Potential

Separation of sperm so as to produce the desired sex among the offspring (commonly referred to as sexing sperm) has been the subject of dreams, theories and conflicting results for centuries. However, a system to separate sperm based on their DNA content due to the presence of either the X or Y chromosome has been thoroughly tested (Amann and Seidel, 1982; Gledhill, 1985; Johnson, 1992; Cran et al., 1993). Studies by Morrell and Dresser (1989) demonstrated that the staining of sperm with Hoechst dye 33342 to detect differences in DNA did not have a mutagenic effect, and was safe for the sperm. Recent studies (Tubman et al., 2004) demonstrated that calves produced with separated sperm were normal.

The development of equipment to align sperm in a laminar flow so as to minimize errors in detection (accuracy of detection exceeds 90%) and process several thousand sperm per second in discrete droplets is a marvelous achievement in biomedical engineering. Sheer forces that could damage sperm as they are projected through the detector likely put an upper limit on the rate of sorting. Although thousands of sperm can be counted per second, millions of bull sperm

must be separated to inseminate a few cows. As each ejaculate contains billions of sperm, it could take 10 days to sort one ejaculate of bull semen. Only the sperm that are most effectively separated are used, so this has limited the commercial application to date, but multidetector systems may be developed.

Sperm numbers per insemination can be reduced considerably (Seidel et al., 1997) with a moderate decline in conception rate. Therefore under special situations the sexing of limited numbers of sperm could be cost effective. For example, one could use a few million sexed sperm with the X chromosome to fertilize large numbers of oocytes in vitro (IVF). The female embryos produced could be frozen for later ET (Wilson et al., 2005).

For dairy cattle and for endangered species, the production of female embryos would be desired generally. A limited application of sperm sexed to produce males would be to produce sons for sampling in AI. Also, for production of beef cattle, males are desired as they grow faster and more efficiently. So the economic value of sexing sperm is enormous if an inexpensive way of accomplishing this rapidly could be found. For several years this author has expected that any of several laboratories working with genomics would transfer genes or an artificial chromosome designed so that only the sperm cells capable of producing females could fertilize an oocyte. Dairy sires producing only female progeny would be worth many millions of dollars. Clones of these bulls could be made. Several lines should be produced to maintain some genetic diversity, along with conventional AI on a considerable scale to assure that a genetically diverse pool is sustained.

The ultimate in efficiency of sperm utilization is achieved with microinjection. Single sperm can be injected into oocytes by a highly skilled person using excellent equipment. This system produced normal progeny in cattle (Goto et al., 1990), but it is too costly to be practical.

Freeze-dried Sperm

Meryman (1960) reported that bull sperm could be revived after drying, but he could not repeat the results (J. Reprod. Fertil. 5:87. 1963). The first repeatable study claiming that normal progeny could be obtained following microinjection of freeze-dried mouse sperm into oocytes was reported by Wakayama and Yanagimachi (1998). At Cornell we had tried to freeze-dry bull sperm in the 1950s in a marvelous unit designed by Dr. David Lee for studying behavior of microparticles at temperatures approaching absolute zero. A few years ago Dr. Lee shared the Nobel Prize for his research. We were unable to obtain any survival of bull sperm when dried extremely rapidly in the Lee system. We stored the sperm in sealed ampules until we moved into Morrison Hall in 1961. When the techniques for microinjection became available it would have been fascinating to determine what these old dried sperm could do, but, alas, they had been discarded. Many laboratories have notebooks of negative results.

Genetic Benefits

The major objective of developing the AI system was genetic improvement for milk production. Initially it was hoped that bringing sires into AI with good natural service proofs in high producing herds would accomplish this

objective. Unfortunately the high milk production records on daughters of sires purchased for AI resulted primarily from good herd management these daughters received. Their proofs were not repeatable in AI. Little genetic gain was made until a system of selecting young bulls and progeny testing them was designed (Henderson, 1954; Van Vleck, 1981).

This system required testing a group of superior sires with records on daughters in a random sample of herds. The best of these sires were selected to inseminate genetically superior cows with their semen to produce many progeny. The sons produced were raised to sexual maturity, and as rapidly as possible semen was collected and distributed across a random sample of herds that were in the DHIA milk testing program. Milk production of the daughters of these young bulls was compared. Only a few of the genetically superior sons were saved for AI. This program took many years to establish. Genetic progress has been steadily upward ever since.

In addition to the genetic gain from sampling many sons and selecting a few, Henderson and associates (Van Vleck, 1981) devised methods of more accurately evaluating females that were selected to produce young bulls for sampling. This improved the precision of evaluating the genetic merit of the young bulls also.

Reproductive benefits also accompanied sire selection with information on thousands of progeny per sire. Although measures of fertility have low heritability (Foote, 1970b), it is clear that selection of highly fertile sires and elimination of lethal genes has had a beneficial genetic effect.

Summary of the Components and Benefits of AI

The objectives of the AI program in dairy cattle are to 1) control venereal diseases, 2) improve the genetic potential for high milk production, 3) improve body strength and milking characteristics, and 4) reduce genes that are lethal or caused serious defects. The requirements to achieve these objectives are 1) development of knowledge necessary to implement the technology, 2) development of trained providers and users of the technology, 3) development of reliable equipment needed, and 4) development of a suitable infrastructure to assure long-term stability. The major components of the system that determine the output quantitatively are given in the following equations.

Equation 1.

$$\begin{array}{c} \text{Number of} \\ \text{progeny} \\ \text{per sire} \\ \text{per year} \end{array} = \begin{array}{c} \text{Number of sperm} \\ \text{harvested/sire} \\ \text{Number of sperm} \\ \text{planned/cow} \end{array} \times \begin{array}{c} \text{Collected} \\ \text{sperm (\%)} \\ \text{used for} \\ \text{insemination} \end{array} \times \begin{array}{c} \text{Percentage of} \\ \text{of cows} \\ \text{pregnant} \\ \text{per service} \end{array}$$

The components of equation 1, with the exception of the fraction of breeding units produced that are actually used for insemination, were discussed in previous sections. Most bulls with intensive semen collection can ejaculate more than 1.5 trillion sperm per year. However, an occasional ejaculate is lost or culled. When liquid semen was used extra amounts of semen on each of several bulls were sent to each technician to provide choice for the dairy farmer. One could only estimate in advance how many requests for service would be made over a period of 1 to 2 days when most of the liquid semen was used before fertility declined. The percentage of semen used for insemination was highest in the major breed, Holsteins, especially during the peak breeding season (50 to 70%), and lower with semen from other breeds of bulls, especially in the summer low breeding season (10 to 30%). Consequently, much semen was discarded.

With frozen semen more sperm are used per insemination and fertility is slightly reduced compared with liquid semen (Table 2). However, more frozen semen distributed into the field can be used than liquid semen because it can be stored indefinitely until there is a demand for its use. Some ejaculates of semen are culled if prefreeze or postfreeze quality is low, and some breeding units stored for potential use are discarded when semen becomes available from bulls with higher genetic merit. These variables are illustrated in Table 2.

The potential genetic impact per sire is illustrated in equation 2. This equation considers the direct genetic impact of the sire through the production of daughters. Only about 49% of the calves normally will be females. Some calves will not survive to maturity. The best sires will have an indirect impact through their selected sons.

Equation 2.

**Genetic
impact
per sire**

=

**Genetic
superiority
of the sire**

X

**Number of
female progeny
per sire**

The increase in genetic potential of dairy cows has allowed management strategies (see later section on females) to be applied to greatly increase milk production per cow. Because milk production per cow has tripled in the past 50 years the total milk supply in the United States has increased despite the dramatic decrease in number of cows. The decrease in metabolic requirements to maintain the fewer and more efficient cows is equivalent to at least 1,000,000,000 bushels of corn annually. With a few hundred bulls in AI replacing tens of thousands of bulls on farms, additional feed is saved for other uses. Less methane is produced.

Incidentally, farming is safer without a bull on the farm. No case has been reported of a farmer being killed by an inseminator, and that's no joke.

Table 2. Interrelationship of Factors Affecting the Number of Progeny Per Proven Mature Holstein Dairy Sire Per Year ^a				
Sperm output per bull per year (Billions)	÷ Sperm used per cow (Millions)	X Collected sperm used (%)	X Cows pregnant per service (%)	= Number of progeny per year
	Liquid semen			
1500	2	40	50	150,000
1500	2	40	60	180,000
1500	2	80	50	300,000
1500	2	80	60	360,000
1500	5	40	55	66,000
1500	5	40	65	78,000
1500	5	80	55	132,000
1500	5	80	65	156,000
	Frozen semen			
1500	15	70	40	28,000
1500	15	70	50	35,000
1500	15	90	40	36,000
1500	15	90	50	45,000
1500	20	70	40	21,000
1500	20	70	50	26,250
1500	20	90	40	27,000
1500	20	90	50	33,750
1500	25	70	40	16,850
1500	25	70	50	21,000
1500	25	90	40	21,600
1500	25	90	50	27,000
^a This table covers a range of historical conditions. Some bulls could ejaculate up to 3 trillion sperm per year and some much less, but in actual practice 1.5 trillion is a reasonable average. Pregnancy rates with high milk production and frozen semen are considerably lower than in the 1950s when liquid semen was used. Assistance with AI records from Genex CRI is appreciated.				

The Female Component

Gross Anatomy

The general morphology of the cow's reproductive organs are thoroughly described in both old and new texts on veterinary anatomy, or on veterinary obstetrics (Roberts, 1986) and in texts on reproduction (Cole and Cupps, 1959; Salisbury et al., 1978). The single vagina and cervix lead to the short body of the uterus, two horns of the uterus, and two oviducts, each with a funnel-shaped infundibulum ending at the ovary. Of special significance for AI is the muscular cervix with several annular rings protruding posteriorly, making passage of a catheter through the cervix difficult. The Danish procedure, developed in the 1930s, overcame this problem by manipulating the cervix with one gloved hand in the rectum while the insemination pipette was gently inserted through the cervix with the other hand. A simple diagram of the procedure is shown in Fig. 9. In Fig. 10 the cow reproductive tract is opened to show the annular folds of the cervix. These folds form tightly closed rings in the live animal providing a protective barrier to the uterus. Anatomy texts did not provide a detailed description of the arterial system of the bovine uterus until this was supplied by Hansel and Asdell (1951).

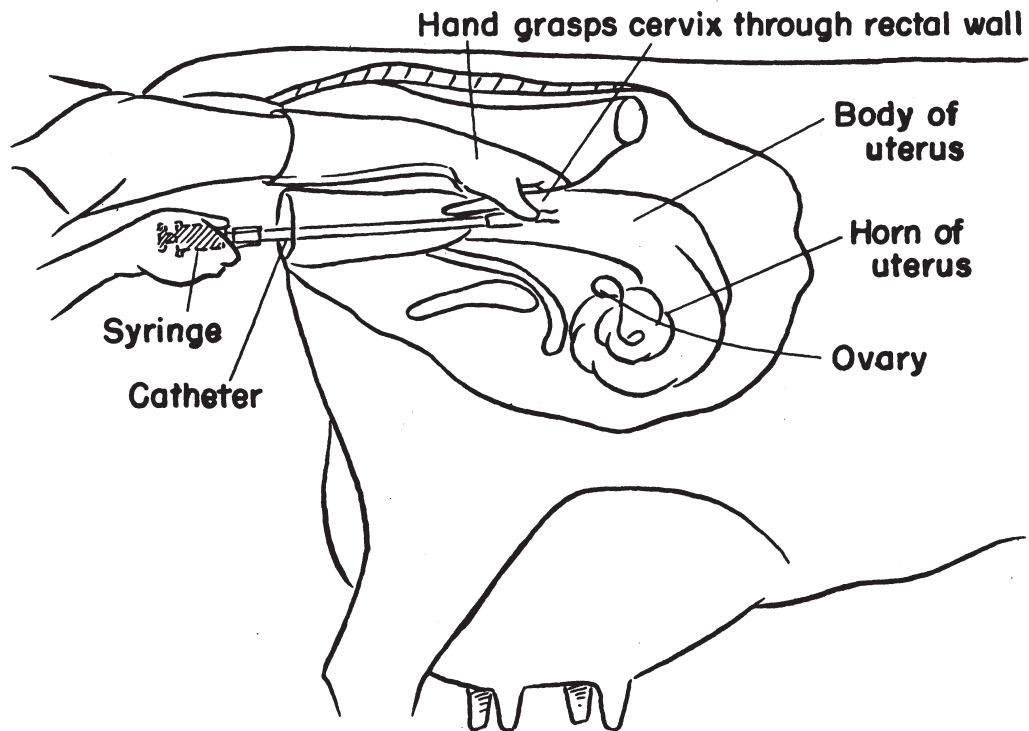
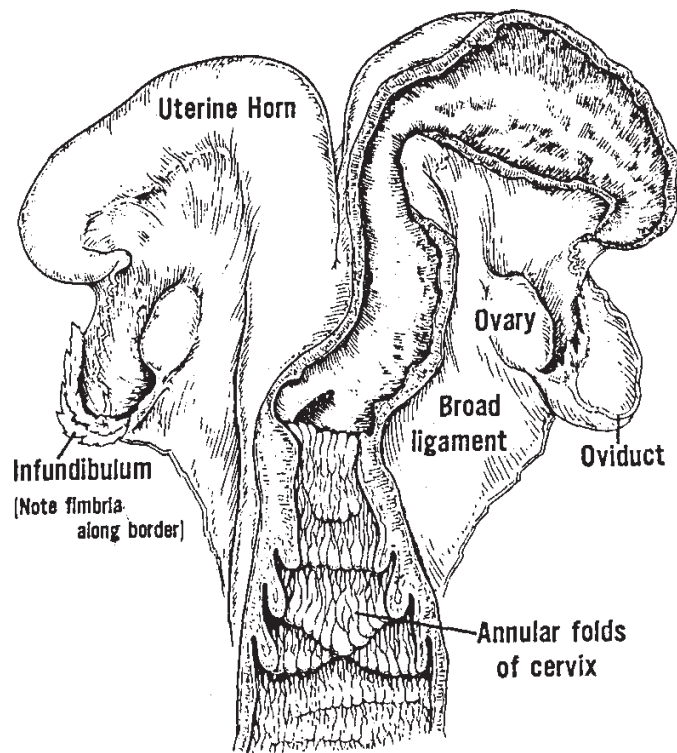


Fig. 9 Depositing semen through the cervix into the body of the uterus facilitated by using the gloved hand in the rectum to manipulate the cervix.



COW REPRODUCTIVE ORGANS

Fig. 10 The reproductive tract (less the vagina) with the cervix and right uterine horn open to show particularly the annular folds of the cervix.

The primary organs of reproduction in the female are the paired ovaries. They produce the female germ cells, the oocytes (commonly referred to as eggs), and the steroid hormones, principally estradiol and progesterone, plus small quantities of androgens and relaxin. The ovarian structure is described in many old texts summarized by Marshall (1910) and more recently by Mossman and Duke (1973) and Zuckerman and Weir (1977). The ultrastructure of the reproductive system is depicted and thoroughly described, with extensive literature citations, by Van Blerkom and Motta (1979).

Oogenesis

At the beginning of the 20th century it was known that early in fetal development the ovaries started to produce large numbers of future germ cells from oogonia dividing mitotically. This contrasts with males where gametogenesis is initiated at puberty. Opinion was divided as to whether or not new oocytes were formed in adults. Evidence obtained in the past 50 years (Kennelly and Foote, 1966; Mauleon, 1969; Baker, 1972a,b; Blandau, 1975; Foote, 1975b; Zuckerman and Weir, 1977) strongly supports the concept that neogenesis does not normally occur in adult females of most species. Recent preliminary studies have suggested that there may be ovarian stem cells which might be stimulated to form oocytes under certain conditions (Johnson et al., 2004).

As a result of multiple mitotic divisions in the fetal ovary up to several million oogonia may be produced. Simultaneously, many oogonia undergo atrophy, leaving a few hundred thousand potential germ cells stored in the paired ovaries by the time of birth. This represents the lifetime supply of oocytes in the female. An excellent summary of this process was published by Mauleon (1969).

Storage and Maturation of Oocytes

The oogonia progress to a primary oocyte in prophase I of the first of two divisions of meiosis, and then are arrested in the diplotene stage and stored in the dictyate condition. They remain divisionally dormant, often for years, until selected to resume meiosis at ovulation. So the enormous synthesis of DNA, as the paired chromosomes in each cell are duplicated, is completed in the fetal ovaries. The stored oocytes that survive grow as the female grows prepubertally. They become surrounded with layers of follicle cells as they grow from primary to secondary follicles. Some become large tertiary follicles with a fluid-filled antrum. The Graafian follicle named after de Graaf is a mature tertiary follicle 1 to 2 cm in diameter in the cow.

Fetal oogenesis is well-documented in cattle (Henricson and Rajakowski, 1959). Ericksen (1966) counted the number of surviving oocytes in the three types of follicles from birth of the calf to adult cattle 20 years old. Many oocytes degenerate, and those that survive for 20 years contain DNA that must have been stored for slightly more than 20 years. This depletion and aging of oocytes may account for some of the lower fertility in older cows, although DNA is remarkably stable. The longtime storage in women 40 years old or older appears to be related to Down's syndrome, with an extra chromosome 21. Also, the fact that no new oocytes are formed in the adult means that any ovarian accident or exposure to toxic materials that destroy the existing oocytes can result in permanent sterility.

Another contrast between spermatogenesis and oogenesis is that one primary spermatocyte produces four spermatids which potentially develop into four sperm cells. In contrast one primary oocyte produces only one ootid or potential egg. The extra chromosomes are expelled in nonfunctional compact polar bodies. The completion of meiosis I and II results in one ootid or egg containing all the cytoplasm and a single set of chromosomes. This completion of two divisions of meiosis occurs only during the process of ovulation and fertilization of the ovulated oocyte. Details of this process, as chromosomes are duplicated and exchange parts, are illustrated in many texts (Austin 1959, 1961).

The ovulated egg, with its large supply of rich cytoplasm, contains all the material necessary to start embryonic development following fertilization by a sperm cell. The first published report on bovine ova was by Hartman et al. (1931). The union of the sperm and egg, each with one complete set of chromosomes, restores the diploid paired set of chromosomes characteristic of all somatic cells. If the egg is not fertilized after ovulation, recurring estrous cycles follow in most mammals, which in primates are called menstrual cycles. A schematic diagram of the ovary representing follicle growth is shown in Fig. 11.

Estrous Cycle Dynamics

Fig. 11 shows schematically the morphologic changes that the ovary undergoes during an estrous cycle. The dominant follicle in cattle (several follicles in pigs) is selected to ovulate while several other large follicles not selected regress. A corpus luteum (CL) develops in the area where the follicle ruptured. The CL will remain throughout most of the estrous cycle. If no fertilization has occurred the CL will regress about 18 days after ovulation and a new estrous cycle will start about every 21 days. The old CL is called a corpus albicans. If the egg is fertilized it will start to develop into a young embryo as it moves through the oviduct into the uterus. About 16 days after the last ovulation with an embryo present, a signal to the CL will cause it to remain functional, critical in maintaining pregnancy. Otherwise the CL will regress, initiating a new estrous cycle on the average every 21 days in cattle.

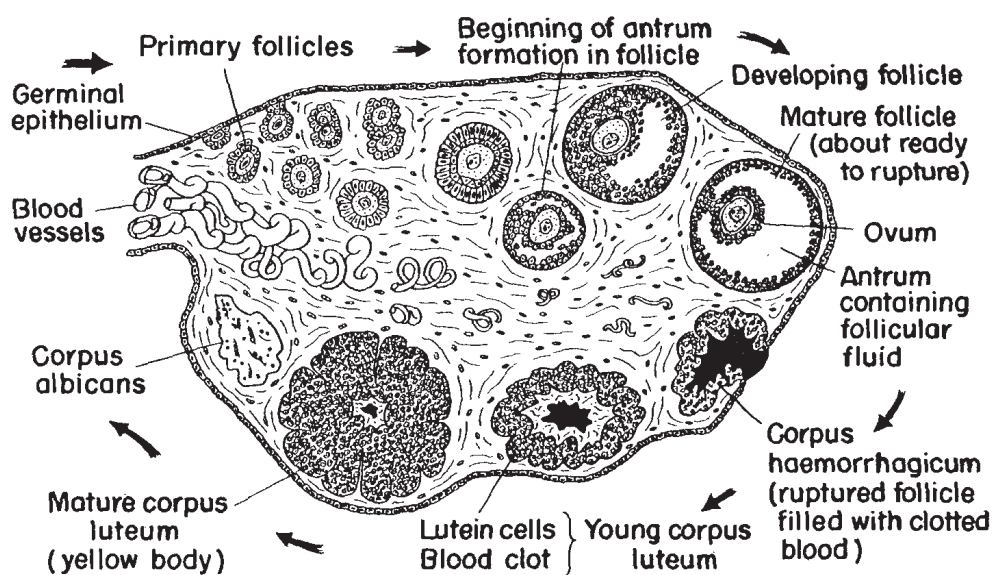


Fig. 11 Cross-section of the ovary showing various stages in the development of ovarian follicles and the development and retrogression of the corpus luteum. (Redrawn by permission from Embryology of the Pig, by Patten. Copyright 1931. The Blakiston Co., Inc.)

The ovary also is a dynamic endocrine organ, playing a pivotal role in the hypothalamic pituitary-ovarian axis. Much of the research on the estrous cycle in the first half of the century was descriptive. Many of the early studies on the relationship between the CL and large follicles were based upon observing results of ablating these structures at various stages of the estrous cycle. Studies of the hormonal relationship between the ovary and the pituitary gland were hampered by having only crude pituitary extracts of gonadotropins available at the outset. It was debated whether there were one or two gonadotropic hormones until the report by Leonard at an AAAS meeting in 1931, and published in 1931 (Fevold et al., 1931) clearly demonstrated that there were two hormones, later called follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In 2005, 100-year-old Sam Leonard (Fig. 12) clearly recounts this debate, while most of us assume that the two hormones were known forever.



Fig. 12 Professor Samuel Leonard, discoverer with Fevold et al. (1931), that there were two gonadotropic hormones, is shown here in his 80s. Photo used by permission.

Assays of progesterone (P_4) and estradiol (E_2) were developed in the 1930s, but they lacked sensitivity and E_2 in blood could not be assayed directly. Researchers extracted tons of hog ovaries and obtained 6 mg of E_2 per ton (Young, 1961). Results of studies on interactions between gonadotropin extracts administered and E_2 and P_4 often were in partial conflict because of the impure gonadotropin extracts used. However, the role of E_2 in evoking behavioral estrus and P_4 in preventing estrus, plus the powerful feedback effects on gonadotropin secretion were established by the 1940s.

The involvement of the hypothalamus in the gonadal-anterior pituitary axis was established in the 1940s by Harris at Cambridge (1955), but details remained to be elucidated. Knowledge of endocrine function still moved slowly because there was no simple, specific, sensitive test for each hormone (Gomes and Erb, 1965). Chromatographic and fluorometric methods were tedious. Bioassays had been developed, but were neither highly sensitive nor specific. When the author obtained LH or FSH from Armour and Company in the 1950s the assessment of these semipurified products was determined by bioassay (for FSH see Steelman and Pohley, 1953). We were warned that the potency of these hormones for superovulating rabbits and cattle might vary considerably from batch to batch because the assays were not based on applications we planned to use these hormones for. They did vary, although Armour had the most advanced assays in use at that time. In the 1960s the National Institutes of Health took over the hormone program to make hormones available to researchers. Purified hormones replaced the semipurified Armour products packaged on the basis of arbitrary Armour units. These historical notes are introduced here to reflect some of the difficulties researchers faced before the 1960s compared with the

facility that similar experiments can be conducted today with purified hormones and specific assays.

Another example of a problem is the long time that elapsed between discovery by Harris and others demonstrating the involvement of the hypothalamus until tools were available to exploit this breakthrough. It was not until 1964 that Schally and Bowers (1964) first demonstrated that beef and sheep hypothalamic extracts contained a luteinizing hormone-releasing factor. They were able to demonstrate release of LH using the ovarian ascorbic acid depletion bioassay (Parlow, 1961), and acknowledged Dr. Parlow's help in setting up the assay. It was not until 1971 that the gonadotrophin-releasing hormone (GnRH) was characterized by two laboratories and synthesized (Nobel prizes shared by Drs. Schally and Guillemin). The ability to synthesize GnRH made it possible to make sufficient quantities for research with farm animals (Convey, 1973).

About the same time that GnRH was being characterized, radioimmunoassays (RIAs) were developed (Niswender et al., 1968; Midgley, 1969). With LH antiserum supplied by Dr. Niswender and GnRH available commercially, hundreds of investigations with GnRH, LH and other hormones were initiated. The voluminous literature published more recently is available electronically. Emphasis here will be limited to the basic characteristics of the estrous cycle which have laid the groundwork for developing techniques to manipulate or control the estrous cycle (Hammond and Bhattacharya, 1944; Hansel 1959, 1961; Hansel and Convey, 1983).

The gross changes in the hormones in the estrous cycle are graphed in Figure 13, and details can be found in Hansel and Convey (1983), Cupps (1991) and

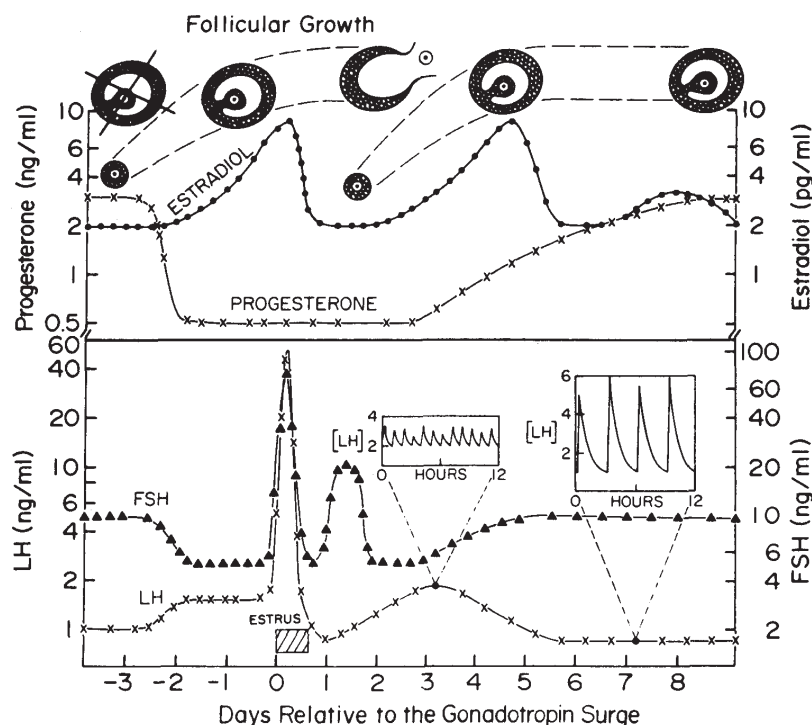


Fig. 13 Day zero is the day estrus was initiated. The top panel shows changes in E2 and P4, follicle growth and ovulation of the dominant follicle. The lower panel shows changes in FSH and LH with changes in pulsatile secretions of LH. From Hansel and Convey, *J. Anim. Sci.* 57(Suppl. 2):404-424. 1983. By permission.

Knobil and Neill (1998). Estrous cycle length averages 21 days in multiparous cows and 20 to 21 days in heifers. About 16 days after the previous ovulation, P_4 secretion decreases and LH and E_2 increase. Soon after E_2 peaks, LH secretion surges, inducing ovulation. Historically, prolactin was considered to be the luteotropic hormone, as it was found to have this function in the rat. The rat was the exception. After ovulation the CL forms in place of the ruptured follicle and secretes P_4 . As follicles develop the theca interna layer of cells secretes increasing quantities of E_2 , causing the circulating E_2 concentrations to increase. Much higher circulating concentrations of P_4 prevent estrus from occurring until the CL declines. The rectangles in Fig. 13 show changes in frequency and pulse height of LH that result from the hypothalamic GnRH pulse generator changing pulses. Knobil (1974, 1980) has reviewed the discovery of GnRH pulsatile release in his laboratory, and the extensive literature is beyond the intended scope of this overview. Knobil was a graduate student of Sam Leonard (Fig. 12).

Likewise, how one dominant follicle normally is selected to ovulate in the cow (Fortune et al., 2004; Ginther et al., 2001) is a subject of intensive investigation. As follicles sequentially develop during an estrous cycle (usually three waves) a dominant follicle emerges. It is usually the largest. It secretes relatively large amounts of E_2 . This suppresses FSH release, probably causing the smaller follicles to self-destruct. Inhibin produced by the granulosa cells in the ovary also acts directly on the anterior pituitary to inhibit FSH secretion.

Duration and Detection of Estrus

The duration of estrus and the time of ovulation vary considerably among cows, and are affected by several environmental factors. Hansel (1959) reported that average values in the literature varied from 14 to 19 hours, with a range from 6 to 34 hours. Ovulation occurred on the average about 11 hours after the end of estrus (range 2 to 22 hours, Hansel, 1959). Recently Saumande and Humblot (2005) reported that for lactating cows, the time from onset of estrus to the LH peak was 9.1 ± 2.0 hours (mean \pm S.E.), LH peak to ovulation was 29.4 ± 1.5 hours and onset of estrus to ovulation was 38.5 ± 3.0 hours. Intervals for heifers and for animals under the stress of the tropics are shorter and variable. As mentioned earlier in the section on the male, this variability was of concern in making recommendations of when to inseminate cows for optimal conception rates. Fortunately, there is a considerable window of opportunity for insemination with comparable results obtained by Dransfield et al. (1998) between 5 and 14 hours after the onset of estrus.

A big problem for the herd owner is to determine when estrus begins for each cow. The time that can be allotted each day to observe cows for signs of estrus is limited and must be balanced against the many other demands on time to complete daily tasks on a dairy farm. Researchers developed a variety of aids to supplement visual observation (Foote, 1975a). These included changes in viscosity of vaginal mucus, ferning and electrical conductivity of mucus, with electronic probes developed to quantify the conductivity of the cervical mucus. Also, heatmount detectors that changed color when compressed by animals mounting a female in standing estrus, bulls with surgically deviated penises and chin balls containing marking paint, mechanical pedometers (see references

in Foote, 1975a), and later electronic activity meters as well as radiotelemetric pressure sensing systems (Nebel et al., 2000) were developed. The presence of P_4 in milk at concentrations highly correlated with blood P_4 , and the ease of collecting milk samples, has resulted in various applications relative to estrus for more than 25 years (Foote, 1975a; Mitchell et al., 2004). However, this hormonal assay is more useful for profiling the estrous cycle and pregnancy testing than in detecting estrus precisely.

With the onset of estrus presumably timed accurately by electronic equipment continuously monitoring activity or the first standing event, it would seem that there should be a short period of time to inseminate cows that would result in maximal conception rates. However, while the conception rate peaks when cows are inseminated about 12 hours after the first standing event it is not greatly different from conception rates when the inseminations occurred 5 to 17 hours after the initiation of estrus (Nebel et al., 2000). Presumably this is due to the fertile lifespan of sperm in the female reproductive tract of at least 24 hours.

Synchronization of Ovulation and Fixed Time of Insemination

Because of the variability in duration of estrus and the time of ovulation, a predetermined fixed time of insemination is appealing to many large dairy farm operators, if the effectiveness and costs balance the more routine methods of detecting estrus and insemination. Groups of cows could be managed to be inseminated the same day and avoid insemination on weekends or during holidays and vacations. Other possible applications include grouping animals less intensively managed such as dairy heifers or beef cattle.

The first principle exploited to control the time of ovulation time in the 1950s was to extend the progestational phase of a group of animals to a simultaneous endpoint by feeding P_4 or synthetic progestagens. Steroid hormones are not destroyed by digestion. Ulberg et al. (1951), at the University of Wisconsin, reported that the time of ovulation in dairy heifers could be regulated by this treatment. While oral administration was convenient, ingestion was variable and longtime treatment followed by withdrawal resulted in both variable ovulation time and generally low fertility. Dziuk and Cook (1965) reported that steroid hormones pass through silicone rubber. Progesterone embedded in Silastic coils (PRID, Abbott) and inserted into the vagina was another method of administering P_4 . A similar device was Controlled Intravaginal Releasing Device (CIDR). These devices offered a convenient method to obtain sustained release of P_4 . Upon withdrawal of the devices simultaneously from a group of cows their estrous cycles were better synchronized.

However, various progestagens more potent than P_4 such as medroxyprogesterone (MAP, Upjon), melengestrol acetate (MGA, Upjohn) and norgestomet (Synchro-Mate B implant, Searle) were tested widely. The latter implant was administered with an estradiol valerate injection to improve synchronization. In the 1960s, hundreds of studies with different combinations of progestagens, estrogen and gonadotropins resulted in improved synchronization of ovulation, but pregnancy rates varied greatly. (See reviews by Hansel, 1959, 1961; Hansel and Beal, 1979; Hansel and Convey, 1983).

Rowson et al. (1972) established that prostaglandin F_{2 α} (PGF_{2 α}), normally secreted by the uterus, caused the regression of the corpus luteum (CL). Many reports followed showing that PGF_{2 α} regulated the life of the CL, and PGF_{2 α} injected intramuscularly or smaller doses into the uterus caused regression of the CL. However, cows near estrus without a CL were not affected by prostaglandin so either the injection had to be combined with detection of estrus, or two injections were given 10 to 12 days apart. In this way all animals were treated when an active CL was present. A potent prostaglandin analog (ICI 80,996, Cloprostenol) also was available commercially for synchronization. A combination of P₄ and PGF_{2 α} increased the precision of ovulation control (Roche, 1976). Pregnancy rates were acceptable with the best combinations of treatments, but the application was somewhat limited by the cost of labor and drugs. A combination of feeding a progestagen, MGA, plus a PGF_{2 α} and GnRH injections was one of the simplest low cost procedures for synchronizing estrus, but it is not approved for lactating dairy cows.

Since the early 1990s extensive efforts have been made to develop a synchronization program which combined control of the CL with PGF_{2 α} and GnRH to give a controlled surge in secretion of luteinizing hormone (LH) to promote ovulation. One commonly used method of synchronizing groups of cows to inseminate all at one fixed time is referred to as Ovsynch. This consists of a GnRH injection at a random stage of the estrous cycle, followed by PGF_{2 α} 7 days later, a second injection of GnRH 36 to 48 hours after PGF_{2 α} and timed AI 16 to 24 hours later. Another approach called Targeted Breeding™ (Pharmacia-Upjohn) consists of three injections of PGF_{2 α} at 14-day intervals, with insemination at any detected estrus, with no further injections of PGF_{2 α} or a timed insemination after the third PGF_{2 α} injection. Other research programs have included estrogens and bovine somatotropin (bST). The pregnancy rates following treatment with various combinations of bST, GnRH and PGF_{2 α} injections (Moreira et al., 2001) ranged from 28 to 49% for all cows, and up to 56% for cows which were cycling when treated. These results demonstrate the problem of attempting to breed cows about 2 months postpartum because of the high incidence of anestrus, and/or initiating treatment at random stages of the estrous cycle. Presynchronization with PGF_{2 α} and GnRH injections improves pregnancy rates, but involves injections five times.

A sampling of the studies from the past decade collectively involving all of the agents mentioned previously and presynchronization plus synchronization, includes Fricke et al. (1998), Pursley et al. (1997, 1998), Nebel and Jobst (1998), Stevenson and Phatak (2005), Stevenson et al. (1998), Cartmill et al., (2001) and Tallman et al., (2001). One of the problems with the programmed breeding systems is the low conception rate, averaging about 35%. The pregnancy rates with presynchronization included are somewhat improved (average about 45%), but labor and drug costs are higher. Also, this leaves 50 to 65% of the cows requiring rebreeding. What type of program is most cost-effective for these cows? Is it repeated synchronization, visual detection of estrus or a combination? There has been no consistent answer to that question, and the correct answer probably varies depending upon the facilities and management practices on individual farms. A problem still not understood is why there is embryo mortality of up to

10% in clinically normal cows after the initial pregnancy check at 30 to 35 days post insemination.

The Postpartum Cow: Condition and Care

The previous sections have dealt with the normal physiology of the estrous cycle and attempts to modify it to facilitate the application of AI using a fixed time of insemination. Reproduction in the individual cow is much more complex, as the body intrinsically attempts to maintain homeostasis with the demands of pregnancy and the sudden major shift in demands with the onset of lactation. The attempt to maintain homeostasis is impacted by many factors such as environmental temperature, complications at calving, and metabolic and infectious diseases. The negative impact on reproduction becomes more pronounced as milk production increases due to the shift in metabolism to supply nutrients required for lactation. This phenomenon called homeorhesis has been elegantly discussed by Bauman and Currie (1980). One of the benefits of bST on lactation is through this mechanism. Thus, any use of bST to increase pregnancy rates through stimulation of early growth of the conceptus must be balanced against the lactogenic effects.

The multiple conditions affecting reproduction in dairy cattle have been documented in considerable detail by Roberts (1986), and only selected examples will be referenced here. Great advances have been made in vaccination programs and diagnostic tests. One area that needs little discussion is heredity as a cause of sterility. With the development of AI and close monitoring of bulls, congenital defects in the reproductive system have essentially been eliminated. One non-inherited congenital defect is the well-known condition referred to as a freemartin, where about 95% of the heifer calves born co-twin with a male are sterile due to fetal inhibition of the female reproductive system because of earlier development of the male gonads. A major advance in the past 50 years as a result of AI is that the bull can be eliminated as the source of a herd infertility problem where AI is used. This puts the onus on any bad semen used or used improperly, primarily on the herd manager.

Dystocia at calving can be reduced by selecting semen from bulls known to produce small calves when breeding small cows or heifers. Bull studs supply information on the size of calves produced by their bulls, as well as many characteristics of daughters. Retained placenta, associated particularly with twinning, often is associated also with metritis or endometritis. Induction of parturition by corticoids, such as dexamethasone, was not popular because of the high incidence of retained placenta (Roberts, 1986). Infectious agents may be viral, bacterial or fungal in nature. Hormonal problems can lead to or result from follicular and luteal cysts, but not from small cystic CLs. Several of these conditions may delay the involution of the uterus, delaying onset of estrous cycles even in cows with no clinical indication of infection (Foote and Riek, 1999). These conditions have been discussed thoroughly by Roberts (1986) who lamented that over many years of veterinary practice it was difficult to evaluate results of treatment as herd owners culled cows or didn't maintain regular contact allowing clinicians to evaluate treatment.

A common cause of infertility is metritis or endometritis. Intrauterine infusion with a variety of irritants such as Lugol's iodine more than 50 years

ago has continued, often with antibiotics included in more recent times. Gilbert (2000) notes that careful diagnosis is essential for rational treatment. Camann (2000) and Ginther (1998) describe the value of ultrasound in aiding in the accurate diagnosis of ovarian structures and uterine pathology, as well as in early pregnancy diagnosis. Thorough examination of the reproductive system is important in detecting a variety of abnormal conditions, as modeling and path studies reveal that infertility has multiple causes (Oltenacu et al., 1980, 1981; Erb et al., 1985). Obviously, rational treatment also depends upon correct diagnosis. Correct diagnosis is not simple with multiple possible causes, but the complicating factor of possible bull effects in a herd have been reduced where AI is used for all breedings.

Another change, as Gilbert (2000) points out, is the increasing herd size with computerized records. With a regular herd health program, the veterinarian, animal science extension specialist and the herd owner can use these records and combine resources into an effective management program. Diagnostic laboratories are available to provide valuable backups on disease and nutrition. In addition to the array of antibiotics on the market (to be used sparingly as milk must be discarded), the availability of prostaglandin $F_{2\alpha}$ and analogs, various forms of GnRH, LH, progestational agents, and cowside tests of milk P_4 increase the armament available to combat multiple problems. The extensive computerized systems and electronic identification of individual cows on modern dairy farms also facilitate the collection of detailed data for analysis, as well as for management. Such data are needed to construct more useful models and improve management programs (Oltenacu et al., 1980, 1981; Erb et al., 1985).

As the 21st century begins probably the largest single obstacle to early postpartum breeding is the substantial proportion of clinically normal cows that are in a negative energy balance and are either anestrus or otherwise are temporarily infertile. As milk production has increased, conception rates have decreased (Spalding et al., 1975; Butler, 2003). Nutritionists have done a remarkable job of formulating rations to provide an optimal balance of nutrients for individual cows depending upon their level of production and stage of lactation. Nevertheless, cows decrease in body condition score postpartum, a condition associated with temporary infertility. Cows are in a major negative

energy balance for at least a month and they do not return to a positive energy balance until 2 to 3 months postpartum.

During this period of negative energy balance, LH, pulse frequency, ovarian activity, and blood glucose, insulin, IGF-I, and leptin are reduced (Beam and Butler, 1997; Boland et al., 2001; Butler, 2003). The quality of the oocytes ovulated also may be affected (Boland et al., 2001; Moreira et al., 2001). Attempts to correct these imbalances by altering the diet (Butler, 1998) have not been successful in restoring homeostasis for factors optimal for reproduction. It is likely

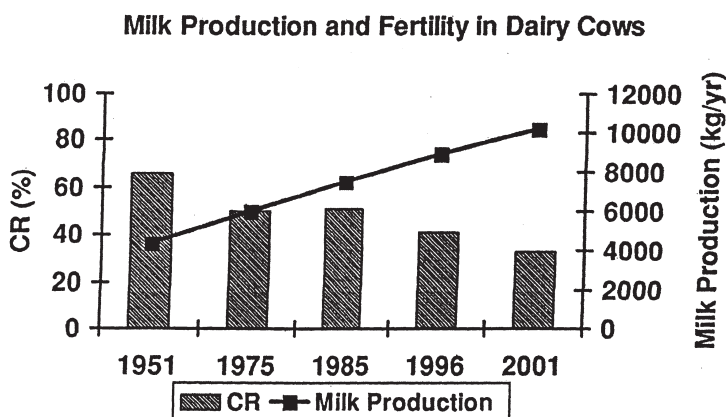


Fig. 14 Reprinted from Butler, W.R. *Livestock Prod. Sci.* 83:211-218. 2003. With permission from Elsevier.

that selection pressure during evolution favored mechanisms that enabled mothers to nurse their offspring before reproduction was renewed. Now selection for production has magnified that condition, with conception rate decreasing (Fig. 14) as milk production has increased (Butler, 2003).

Calving Interval and Milk Production

It is remarkable that genetic selection and improved feeding and management of dairy cows has resulted in more than tripling milk production per cow per lactation, or per year. With this high milk production, it is difficult to achieve the old goal of an average herd calving interval of 12 months. In most high producing herds the calving interval is between 13 and 14 months. This extended calving interval puts less stress on the cow and may be economically profitable (Arbel et al., 2001). It is not the objective of this overview to establish either physiologic or economic optimums. What is important, as our knowledge increases, is to consider carefully how to apply this knowledge for the benefit of both the cow and the cow manager, the producers in this system, as well as the relationship of the industry to society. That gaps exist in our knowledge is obvious.

Biotechnology Applied to the Cow

The contribution of the male to genetic improvement was greatly multiplied by AI. Although the female contribution was somewhat increased by selection of dams to produce sons for use in AI, other methods were sought to increase the contribution of superior cows to the pool of herd replacements. These developments occurred primarily in the last 50 years of the 20th century (Brackett et al., 1981; Foote, 1996, 2001).

Superovulation

Science and practice has come a long way since Fevold, Hisaw and Leonard reported in 1931 that there were two gonadotropins, and Rowson (1951) published methods of inducing superovulation in cattle. Umbaugh (1949) had previously worked for several years on superovulation and embryo transfer of cattle, but without any calves resulting. After the successful bovine embryo transfer by Willett and associates (1951), interest in producing and transferring embryos took a big leap, but progress was slow at first (Foote and Onuma, 1970; Elsdon et al., 1978; Seidel, 1981; Hasler, 1992; Betteridge, 2003). Studies in laboratory animal models were conducted (Kennelly and Foote, 1965; Maurer and Foote, 1971). The development of nonsurgical methods for cattle embryo transfer (ET) in the 1970s decreased the cost and increased the market for embryos. Inseminating enough sperm into superovulated cows (Hawk et al., 1988) and better methods of handling the embryos increased the pregnancy rates, also important in increasing market size.

Embryo Evaluation

The first cattle ETs were performed surgically. The embryos were collected in their cleavage stage (morula) of development. It was difficult to evaluate their quality. When nonsurgical procedures became routine, embryos were collected 6 to 8 days after cows were inseminated. The embryos usually were in the blastocyst

stage. Evaluation was based primarily upon morphologic appearance when embryos were viewed microscopically. This evaluation included an appropriate stage of development for embryos of a particular age, normality of blastomeres with sharply defined membranes, degree of fragmentation, and other signs of degeneration (Lindner and Wright, 1983; Hasler et al., 1987). This system could be applied quickly without manipulating the embryos. The classification of each embryo was compared with pregnancy results following ET. More complex procedures tested in research labs added little to the reliability of evaluation and were not used commercially.

Embryo Culture

Much of the pioneering work on embryo culture was done in the 1950s and 1960s with embryos from mice and rabbits (reviewed by Biggers, 1987; Bavister, 1995). These media were modified from tissue culture media such as TC199 or Ham's F10. To simulate more natural conditions for bovine embryo development, they were co-cultured in these media with oviductal epithelial cells (Eyestone and First, 1989; Ellington et al., 1990; Bavister, 1995). In vitro culture with epithelial cells resulted in most of the culture media being utilized by the massive number of epithelial cells compared to a few embryos. Also, the unknown production of waste products by these cells could have been good or bad for the embryos. Consequently, these systems were unsatisfactory from a scientific and knowledge standpoint because, with this mixture of unknowns, one did not know why certain treatments succeeded or failed. Eventually a satisfactory completely defined medium for fertilization and culture was developed (Liu and Foote, 1995). The medium was modified from a KSOM medium developed for mouse embryos in Biggers laboratory (Lawitts and Biggers, 1991).

It is worth noting here that in the author's lab some of the completely defined media first formulated did not produce good results. The "whip had to be cracked" occasionally to convince the researchers that we were pursuing new knowledge and that was more important than being pleased that the old mixes still produced moderately successful results. Good research is hard work. It takes time.

Embryo Transfer

The development of a nonsurgical technique for embryo transfer (ET) was published in 1965 by Sugie and associates in Japan (see Foote and Onuma, 1970). Subsequently Rowson and Moor (1966) obtained three pregnancies after many



Fig. 15 The Holstein cow was superovulated. Embryos recovered were transferred to 10 recipients (at the left side) producing 10 calves related to the Holstein embryo donor. With permission from Colorado State University. Photo by J. Messineo. Seidel, S.E., Jr. *Science* 211:351-358. 1981.

years of obtaining negative results. Techniques of transfer improved rapidly thereafter (Betteridge, 2003), greatly reducing the difficulty and cost of ET when surgery and general anesthesia were no longer required (Fig. 15). Commercial application expanded (Seidel, 1981).

Another cost associated with the transfer of fresh embryos was the need to have many recipient cows in the same stage of the estrous cycle or that were synchronized using protocols similar to those described for fixed time insemination, except embryos were usually collected and transferred 7 days after estrus. One never knew in advance how many good embryos would be collected and therefore how many recipients were needed.

Embryo Freezing

The successful freezing of mouse embryos (Whittingham, 1971), using glycerol as a cryoprotectant as had been used for bull sperm cryopreservation, was adapted for freezing bovine embryos (Maurer, 1978; Betteridge, 2003). This greatly extended the usefulness of ET. Embryos could be collected from superior females previously inseminated with sperm from genetically superior sires and embryos stored. They could be shipped to introduce desired genetic material into any place in the world by using liquid nitrogen storage tanks, synchronization of recipients, and transfer of embryos, patterned after the technology and infrastructure developed first for AI.

Cells and tissues to preserve banks of somatic material can be similarly frozen. Diploid cells from superior bulls could be frozen for possible cloning. Banks of superior male and female diploid germ plasm would insure genetic diversity being preserved without maintaining as many lines of live animals.

Embryo Splitting

One embryo can be split into two parts and produce identical twins, but repeated splits are not successful (Betteridge, 2003). A splitting technique developed by Willadsen (1979) for sheep was adopted on a limited basis to increase the number of progeny obtained from the embryos collected from each superovulated cow (Williams et al., 1984). However, because pregnancy rate is reduced about 10% by splitting embryos into halves, it has not been applied widely.

Sexing of Embryos

A single blastomere removed from a multicellular young embryo (blastocyst) provides sufficient material to detect the sex by amplification of the DNA with the polymerase chain reaction (Anderson, 1987; Bondioli et al., 1989; Kobayashi et al., 1998). Sexing of embryos approaches 100% in accuracy. Application has been limited in cattle because once a valuable embryo has been obtained it is uneconomic to discard it. Bull studs often use this procedure to select male embryos for transfer, with the female embryos being transferred to cows in herds desiring heifer calves. An alternative for bull studs is to obtain sperm sorted to produce sexed calves, and use these sperm to inseminate a few selected cows.

Embryos Made in the Lab by IVF of Oocytes from Slaughtered Cattle

The development of in vitro fertilization (IVF) procedures for oocytes from slaughtered cattle (Lu et al., 1987; Goto et al., 1988; Gordon and Lu, 1990; Brackett,

in Wolf and Zelinski-Wooten, 2001) made it possible to produce large numbers of embryos to study and improve culture conditions as well as to produce embryos for ET. Rowson (1971) predicted that this would happen. The genetics of oocytes obtained from a slaughterhouse for IVF are unknown. However, if one selects ovaries from older cows slaughtered in an area where superior dairy farms exist, and sperm from superior sires are used, the average genetic merit of the embryos would be higher than found in cattle many places in the world. Also, since only thousands of sperm are needed for IVF, a small portion of an ejaculate of semen sexed for females could be used to produce valuable embryos, as nearly all would be genetic females. Thus, multiple biotechnologies can be combined to produce a new valuable product.

The IVF of Oocytes from Live Calves and Cows

A technique was developed by Pieterse et al. (1991) and others to aspirate oocytes repeatedly directly from follicles in cows in any stage of the estrous cycle and during pregnancy. These oocytes can be fertilized in vitro, cultured into blastocysts and transferred to produce calves (Hasler, 1992). Thus, cows that do not respond to superovulation could also be included in this type of biotech program.

Calves can also be used as donors of oocytes. Seidel et al. (1971) demonstrated that calf oocytes are totipotent. Oocytes from calves approaching puberty have been aspirated (Armstrong et al., 1992; Tervit, 1996) to increase the number of progeny from females so as to increase the accuracy of genetic analysis and to decrease the generation interval involved in proving sires for use in AI (Lohuis,

1995; Nicholas, 1996). Various genetic markers are being tested for possible use in early genetic selection.

Genetic Progress by Combining Reproductive Biotechnologies

Van Vleck (1981) discussed the potential rate of genetic progress if the various technologies (Brackett et al., 1981) discussed here were ever developed. Since their development, many studies have been conducted to predict the expected genetic impact and cost of adopting various biotechnologies (Dekkers and Shook, 1990; Lohuis, 1995; Nicholas and Smith, 1983; Nicholas, 1996). Overall, the major contributor to genetic improvement is through the male because of the extensive testing and intensive selection of sires used to produce thousands of progeny per sire through AI.

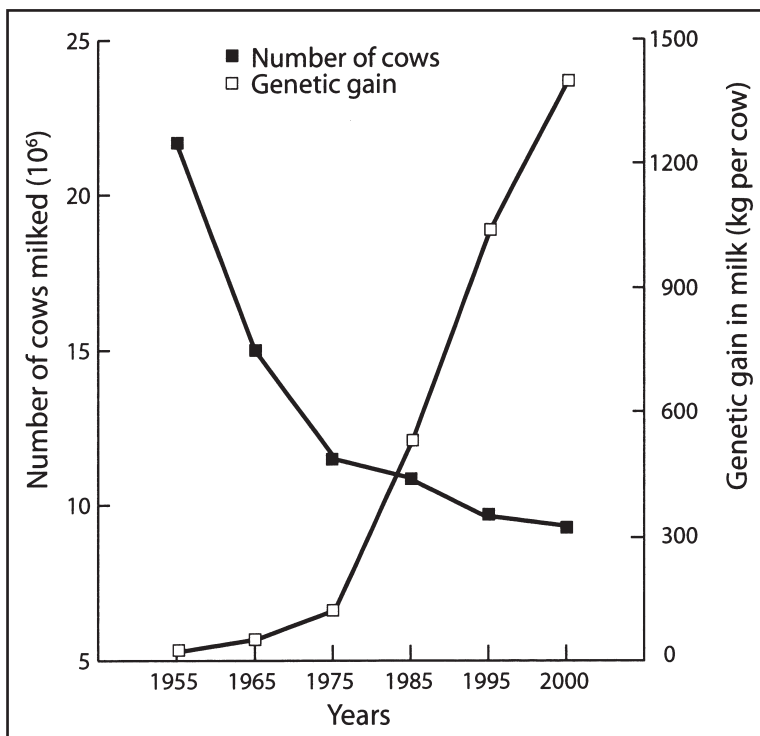


Fig. 16 The number of milk cows decreased by 57% between 1955 and 2000. Milk production per cow nearly tripled during this time, with nearly 1400 kg of this increase due to genetic gain. In 2000 the 9.2 million dairy cows in the U.S. produced about 30% more milk than 21.6 million cows produced in 1955.

The combined genetic ability of cows to produce milk and the improvement in feeding and management practices has greatly increased the efficiency of milk production (Fig. 16).

Cloning and Transgenic Animals

The development of the many reproductive biotechnologies and advances in genomic analysis and molecular biology has led to the production of transgenic and cloned animals (Hammer et al., 1985; Prather et al., 1987; Foote and Yang, 1992; Wall et al., 1997, 2005; Wolf and Zelinski-Wooten, 2001; Cibelli et al., 2002, 2003). A major stimulus in this field was the successful production of Dolly, a lamb derived from the somatic cells of a 6-year-old ewe (Wilmut et al., 1997). Occasional cloning of the best sires in AI could provide insurance against accidental loss of these sires. Animals genetically engineered to produce hornless offspring or sexed offspring, or improved food products from animals of medical importance would be worth cloning. Females engineered to produce human blood proteins in milk are being cloned, as they are of enormous potential value. Widespread production of clones in commercial agriculture is unlikely because of the high cost and inefficiency.

Stem cell research, cloning of millions of cells, and controlling their differentiation and replication has mushroomed after Dolly. These studies have enormous potential benefits for society, far beyond their agricultural applications.

Concluding Statement

The use of AI in many species, but especially in dairy cattle, has become routine, with its advantages widely accepted. Earlier generations worried that AI would produce abnormalities. In the 1950s cartoons showed calves born with icicles in their mouth following the use of frozen semen. Instead, dairy animals have been bred into miraculous sperm producing males and milk producing cows. However, research on the many biotechnologies described here, also should include a consideration of the efficiency, safety, and costs, along with predicted impacts of the biotechnology on animals and on the environment.

Current research, based on the discoveries of the past 100 years or more, could hardly have been imagined 100 years ago. In the future all cows will be permanently identified and tracked to monitor disease. The potential applications of stem cells and understanding how genes are turned on and off in development, health and disease will be as exciting in the 21st century as landing on the moon was in the 20th century. Furthermore, the discoveries of all aspects of regulation of cellular and mammalian reproduction are far more important to the future of *Homo sapiens* than are the discoveries on outer space. These advances increase the importance of our cultural and ethical development to appreciate and respect each other and our environment. In Cibelli et al. (2002), Foote wrote “These powerful techniques also put powerful emphasis on us to discern how these technologies might best be applied, especially to our own species. We should all ask and ponder the question, just because we can do it, should we? Those who blaze trails should also assume some responsibility for the consequences.”

Think about these things!

References

- Albert, A. The mammalian testes. In: Sex and Internal Secretions, Vol. 1. W.C. Young (ed.) Williams & Wilkins, Baltimore. pp. 305-365. 1961.
- Almquist, J.O. Effects of sexual preparation on sperm output, semen characteristics and sexual activity of beef bulls with a comparison to dairy bulls. *J. Anim. Sci.* 36:331-336. 1973.
- Almquist, J.O. Effect of long term ejaculation at high frequency on output of sperm, sexual behavior, and fertility of Holstein bulls; relation of reproductive capacity to high nutrient allowance. *J. Dairy Sci.* 65:814-823. 1982.
- Almquist, J.O. and E.W. Wickersham. Diluents for bovine semen. XII. Fertility and motility of spermatozoa in skim milk with various levels of glycerol and methods of glycerolization. *J. Dairy Sci.* 45:782-787. 1962.
- Almquist, J.O., P.J. Glantz, and H.E. Shaffer. The effect of a combination of penicillin and streptomycin upon the livability and bacterial content of bovine semen. *J. Dairy Sci.* 32:183-190. 1949.
- Amann, R.P. Reproductive capacity of dairy bulls. IV. Spermatogenesis and testicular germ cell degeneration. *Am. J. Anat.* 110:69-78. 1962.
- Amann, R.P. Sperm production rates. In: The Testis, Vol. 1. A.D. Johnson, W.R. Gomes and N.L. VanDemark (eds.). Academic Press, New York. pp. 443-482. 1970.
- Amann, R.P. A critical review of methods for evaluation of spermatogenesis from seminal characteristics. *J. Androl.* 2:39-58. 1981.
- Amann, R.P. Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *J. Dairy Sci.* 66:2606-2622. 1983.
- Amann, R.P. Can the fertility potential of a seminal sample be predicted accurately? *J. Androl.* 10:89-98. 1989.
- Amann, R.P. and D.F. Katz. Reflections on CASA after 25 years. *J. Androl.* 25:317-325. 2004.
- Amann, R.P. and B.D. Schanbacher. Physiology of male reproduction. *J. Anim. Sci.* 57 (Suppl 2):380-403. 1983.
- Amann, R.P. and G.E. Seidel, Jr. (eds.) Prospects for Sexing Mammalian Sperm. Colorado Associated Univ. Press, Boulder. 288pp. 1982.
- Amann, R.P., G.E. Seidel, Jr. and Z.A. Brink. Exposure of thawed frozen bull sperm to a synthetic peptide before artificial insemination increases fertility. *J. Androl.* 20:42-46. 1999.
- Anderson, G.B. Identification of embryonic sex by detection of H-Y antigen. *Theriogenology* 27:81-89. 1987.

- Arbel, R., Y. Bigan, E. Ezra, H. Sturman and D. Hojman. The effect of extended calving intervals in high lactating cows on milk production and profitability. *J. Dairy Sci.* 84:600-608. 2000.
- Armstrong, D.T., P. Holm., B. Irvine, B.A. Petersen, R.B. Stubbings, D. McLean, G. Stevens and R.F. Seamark. Pregnancies and live birth from in vitro fertilization of calf oocytes collected by laparoscopic follicular aspiration. *Theriogenology* 38:667-678. 1992.
- Asdell, S.A., *Patterns of Mammalian Reproduction*, 2nd ed. Cornell University Press, Ithaca, N.Y. 1964.
- Austin, C.R. Fertilization and development of the egg. In: *Reproduction in Domestic Animals*, Vol. I, 1st ed. H.H. Cole and P.T. Cupps (eds.). Academic Press, New York. pp. 399-432. 1959.
- Austin, C.R. *The Mammalian Egg*. C.C. Thomas, Springfield, IL. 1961.
- Baker, T.G. Gametogenesis. *Acta Endocrin. (Suppl. 166)* 71:18-41. 1972a.
- Baker, T.G. Oogenesis and ovarian development. In: *Reproductive Biology*. H. Balin and S. Glasser (eds.). Excerpta Medica, Amsterdam. pp. 398-437. 1972b.
- Barth, A.D. and R.J. Oko. *Abnormal Morphology of Bovine Spermatozoa*. Iowa State University Press, Iowa City, IA. 279 pp. 1989.
- Bauman, D.E. and W.B. Currie. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. *J. Dairy Sci.* 63:1514-1529. 1980.
- Bavister, B.D. Culture of preimplantation embryos: Facts and artifacts. *Hum. Reprod. Update* 1:91-148. 1995.
- Beam, S.W. and W.R. Butler. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biol. Reprod.* 56:133-142. 1997.
- Beatty, R.A. The genetics of the mammalian gamete. *Biol. Revs.* 45:73-119. 1970.
- Berndtson, W.E. and C. Desjardins. The cycle of the seminiferous epithelium and spermatogenesis in the bovine testis. *Am. J. Anat.* 140:167-180. 1974.
- Betteridge, K.J. A history of farm animal embryo transfer and some associated techniques. *Anim. Reprod. Sci.* 79:203-244. 2003.
- Biggers, J.D. Pioneering mammalian embryo culture. In: *The Mammalian Preimplantation Embryo*. B.D. Bavister (ed.). Plenum, New York, NY. pp. 1-22. 1987.
- Bishop, D.W. Sperm motility. *Physiol. Rev.* 42:1-59. 1962.
- Blandau, R.J. (ed.) *Aging Gametes*. S. Karger, New York, NY. 1975.
- Blom, E. A one-minute live-dead sperm stain by means of nigrosin-eosin. *Fertil. Steril.* 1:176-177. 1950a.
- Blom, E. Interpretation of spermatic cytology in bulls. *Fertil. Steril.* 1:223-238. 1950b.

- Blom, E. and N.O. Christensen. Studies on pathological conditions in the testis, epididymis and accessory sex glands in the bull. *Skand. Vet. Tidskr.* 37:1-49. 1947.
- Boland, M.P., P. Lonergan and D. O'Callaghan. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. *Theriogenology* 55:1323-1340. 2001.
- Bondioli, K.R., S.B. Ellis, J.H. Prior, M.W. Williams and M.M. Harpold. The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. *Theriogenology* 31:95-104. 1989.
- Brackett, B.G., G.E. Seidel, Jr., and S.M. Seidel (eds.). *New Technologies in Animal Breeding*. Academic Press. New York. 1981.
- Branton, C., R.W. Bratton, and G.W. Salisbury. Total digestible nutrients and protein levels for dairy bulls used in artificial breeding. *J. Dairy Sci.* 30:1003-1013. 1947.
- Branton, C., R.W. Bratton and G.W. Salisbury. Semen production and fertility of dairy bulls fed rations containing proteins of plant and animal origin. *J. Dairy Sci.* 32:292-300. 1949.
- Bratton, R.W. and R.H. Foote. Semen production and fertility of dairy bulls ejaculated either once or twice at intervals of either four or eight days. *J. Dairy Sci.* 37:1439-1443. 1954.
- Bratton, R.W., R.H. Foote, C.R. Henderson, S.D. Musgrave, R.S. Dunbar, Jr., H.O. Dunn, and J.P. Beardsley. The relative usefulness of combinations of laboratory tests for predicting the fertility of bovine semen. *J. Dairy Sci.* 39:1542-1549. 1956.
- Bratton, R.W., S.D. Musgrave, H.O. Dunn and R.H. Foote. 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. *Cornell Univ. Expt. Sta. Bul.* 964:1-24. 1961.
- Bratton, R.W., G.W. Salisbury, T. Tanabe, C. Branton, E. Mercier and J.K. Loosli. Breeding behavior, spermatogenesis and semen production of mature dairy bulls fed rations low in carotene. *J. Dairy Sci.* 31:779-791. 1948.
- Braundmeier, A.G., J.M. Demers, R.D. Shanks, R.G. Saacke and D.J. Miller. Examination of the binding ability of bovine spermatozoa to the zona pellucida as an indicator of fertility. *J. Androl.* 23:645-651. 2002.
- Bredderman, P.J. and R.H. Foote. Volume of stressed bull spermatozoa and protoplasmic droplets, and the relationship of cell size to motility and fertility. *J. Anim. Sci.* 28:496-501. 1969.
- Bredderman, P.J. and R.H. Foote. Factors stabilizing bull sperm cell volume and prolonging motility at high dilution. *Exp. Cell Res.* 66:458-464. 1971.
- Brockett, C.C., G.A. Presicce, R.H. Foote, M.T. Kaproth and H.E. Rycroft. Semen quality and behavior of Holstein bulls exposed to estradiol-treated bulls for mounts. *J. Dairy Sci.* 77:124-131. 1994.

- Butler, W.R. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *J. Dairy Sci.* 81:2533-2539. 1998.
- Butler, W.R. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livestock Prod. Sci.* 83:211-218. 2003.
- Camann, C. Incorporating ultrasound into your reproduction programs. Mimeo. Cornell Conference on Optimizing Reproductive Performance in Dairy Herds. 9pp. 2000.
- Cartmill, J.A., S.Z. El-Zarkoung, B.A. Hensley, G.C. Lamb and J.S. Stevenson. Stage of cycle, incidence, and timing of ovulation, and pregnancy rates in dairy cattle after three breeding protocols. *J. Dairy Sci.* 84:1051-1059. 2001.
- Cassou, R. La méthode des pailletes en plastique adaptée à la généralisation de la congélation. In: *Proc. 5th Int. Congr. Anim. Reprod.* Trento, Italy. 4:540-546. 1964.
- Cates, W.F. Examination of the bull for breeding soundness. *Proc. Soc. Theriogenology*. pp. 1-18. 1975.
- Celebi, C., T. Guillaudeau, P. Auvray, V. Vallet-Erdtmann and B. Jégou. The making of "transgenic spermatozoa". Minireview. *Biol. Reprod.* 68:1477-1483. 2003.
- Chandler, J.E., A.M. Canal, J.B. Paul and E.B. Moser. Collection frequency affects percent Y chromosome-bearing sperm head area and quality of bovine ejaculates. *Theriogenology* 57:1327-1346. 2002.
- Cibelli, J., R.P. Lanza, H.S.K. Campbell and M.D. West (eds.). *Principles of Cloning*. Academic Press, Amsterdam. 2002.
- Clay, J.S., B.T. McDaniel and C.H. Brown. Variances and correlations among progeny tests for reproductive traits of cows sired by AI bulls. *J. Dairy Sci.* 87:2307-2317. 2004.
- Clermont, Y. Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Revs.* 52:198-236. 1972.
- Clermont, Y. and C.P. Leblond. Renewal of spermatogonia in the rat. *Am. J. Anat.* 93:475-502. 1953.
- Cole, H.H. and P.T. Cupps (eds.). *Reproduction in Domestic Animals*. Vol. I, II. Academic Press, New York. 1959.
- Collins, W.J., R.W. Bratton and C.R. Henderson. The relationship of semen production to sexual excitement of dairy bulls. *J. Dairy Sci.* 34:224-227. 1951.
- Comstock, R.E. and W.W. Green. Methods for semen evaluation. I. Density, respiration and glycolysis of semen. *Am. Soc. Anim. Prod. Proc.* 32:213-216. 1939.
- Convey, E.M. Neuroendocrine relationships in farm animals: A review. *J. Anim. Sci.* 37:745-757. 1973.
- Cordoba, M.C., R. Sartori and P.M. Fricke. Assessment of a commercially available early conception factor (ECF) test for determining pregnancy status of dairy cattle. *J. Dairy Sci.* 84:1884-1889. 2001.

- Cormier, N., M.A. Sirard and J.L. Bailey. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *J. Androl* 18:461-468. 1997.
- Coulter, G.H. and D.R.C. Bailey. Effects of ultrasonography on bovine testis and semen quality. *Theriogenology* 30:743-749. 1988.
- Coulter, G.H. and R.H. Foote. Bovine testicular measurements as indicators of reproductive performance and their relationship to productive traits in cattle: A review. *Theriogenology* 11:297-311. 1979.
- Coulter, G.H., T.R. Rounsaville and R.H. Foote. Heritability of testicular size and consistency in Holstein bulls. *J. Anim. Sci.* 43:9-12. 1976.
- Cran, D.G., L.A. Johnson, N.G.A. Miller, D. Cochrane and C. Polge. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilization. *Vet. Rec.* 132:40-41. 1993.
- Cupps, P.T. (ed.). *Reproduction in Domestic Animals*. 4th ed. Academic Press, Inc. San Diego. 1991.
- Davis, A.P. and R.H. Foote. Relationship of sire fertility to acrosome-reacted motile spermatozoa after treatment with liposomes. *J. Dairy Sci.* 70:850-857. 1987.
- Davis, I.S., R.W. Bratton and R.H. Foote. Livability of bovine spermatozoa at 5, -25, and -85°C in tris-buffered and citrate-buffered yolk-glycerol extenders. *J. Dairy Sci.* 46:333-336. 1963.
- Dekkers, J.C.M. and G.E. Shook. Genetic and economic evaluation of nucleus breeding schemes for commercial artificial insemination firms. *J. Dairy Sci.* 73:1920-1937. 1990.
- Dransfield, M.B.G., R.L. Nebel, R.E. Pearson and L.D. Warnick. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. *J. Dairy Sci.* 81:1874-1882. 1998.
- Dunn, H.O. Estimation and use of variance components in A.I. research and development. *Proc. 4th Int. Congr. Anim. Reprod.* The Hague. 4:746-761. 1961.
- Dvoskin, S. Local maintenance of spermatogenesis by intratesticular implanted pellets of testosterone in hypophysectomized rats. *Am. J. Anat.* 75:289-320. 1944.
- Dykeman, D.A., L.S. Katz and R.H. Foote. Behavioral characteristics of beef steers administered estradiol, testosterone and dihydrotestosterone. *J. Anim. Sci.* 55:1303-1309. 1982.
- Dziuk, P.J. Review. Factors that influence the proportion of offspring sired by a male following heterospermic insemination. *Anim. Reprod. Sci.* 43:65-88. 1996.
- Dziuk, P.J. and B. Cook. Passage of steroids through silicone rubber. *Endocrinology* 78:208-211. 1966.
- Dziuk, P.J., E.F. Graham, J.D. Donker, G.B. Marion and W.E. Petersen. Some observations in collection of semen from bulls, goats, boars and rams by electrical stimulation. *Vet. Med.* 49:455-458. 1954.

- Ehrenwald, E., J.E. Parks and R.H. Foote. Cholesterol efflux from bovine sperm: II. Effect of reducing sperm cholesterol on penetration of zona-free hamster and in vitro matured bovine ova. *Gamete Res.* 20:413-420. 1988.
- Ellington, J.E., E.W. Carney, P.B. Farrell, M.E. Simkin and R.H. Foote. Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell co-culture systems. *Biol. Reprod.* 43:97-104. 1990.
- Elsden, R.P., L.D. Nelson and G.E. Seidel, Jr. Superovulating cows with follicle stimulating hormone and pregnant mare's serum gonadotropin. *Theriogenology* 9:17-26. 1978.
- Erb, H.N., R.D. Smith, P.A. Oltenacu, C.L. Guard, R.B. Hillman, P.A. Powers, M.C. Smith and M.E. White. Path model of reproductive disorders and performance, milk fever, mastitis, milk yield, and culling in Holstein cows. *J. Dairy Sci.* 68:3337-3349. 1985.
- Erickson, B.H. Development and senescence of the postnatal bovine ovary. *J. Anim. Sci.* 25:800-805. 1966.
- Evenson, D.P., Z. Darzynkiewicz and M.R. Melamed. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 210:1131-1133. 1980.
- Evenson, D.P., K.L. Larson and L.K. Jost. Sperm chromatin structure assay, its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* 23:25-43. 2002.
- Everett, R.W. and B. Bean. Semen fertility: an evaluation system for artificial insemination sires, technicians, herds and systematic fixed effects. *J. Dairy Sci.* 69:1630-1641. 1986.
- Eyestone, W.H. and N.L. First. Coculture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *J. Reprod. Fertil.* 85:715-720. 1989.
- Farrell, P.B., R.H. Foote, M.M. McArdle, V.L. Trouern Trend and A.L. Tardif. Media and dilution procedures tested to minimize handling effects on human, rabbit, and bull sperm for computer-assisted sperm analysis (CASA). *J. Androl.* 17:293-300. 1996a.
- Farrell, P.B., R.H. Foote and M.J. Zinaman. Motility and other characteristics of human sperm can be measured by computer-assisted sperm analysis of samples stained with Hoechst 33342. *Fertil. Steril.* 66:446-453. 1996b.
- Farrell, P.B., G.A. Presicce, C.C. Brackett and R.H. Foote. Quantification of bull sperm characteristics measured by CASA and the relationship to fertility. *Theriogenology* 49:871-879. 1998.
- Fawcett, D.W. and J.M. Bedford (eds.). *The Spermatozoon*. Urban and Schwarzenberg, Baltimore-Munich. 441 pp. 1979.
- Fevold, H.L., F.L. Hisaw and S.L. Leonard. The gonad stimulating and the luteinizing hormones of the anterior lobe of the hypophysis. *Am. J. Physiol.* 97:291-301. 1931.

- Foote, R.H. Research techniques to study reproductive physiology in the male. *Techniques and Procedures in Animal Science Research*. ASAP, Albany, NY. pp. 81-110. 1969.
- Foote, R.H. Fertility of bull semen at high extension rates in Tris-buffered extenders. *J. Dairy Sci.* 53:1475-1477. 1970a.
- Foote, R.H. Inheritance of fertility in dairy cattle – facts, opinions and speculations. *J. Dairy Sci.* 53:936-944. 1970b.
- Foote, R.H. Aging of spermatozoa during storage in liquid nitrogen. *Proc. IVth Tech. Conf., Anim. Reprod. and A.I., NAAB.* pp. 28-30. Feb. 1972a.
- Foote, R.H. How to measure sperm cell concentration by turbidity optimal density. *Proc. IVth Tech. Conf., Anim. Reprod. and A.I., NAAB.* pp. 56-61. Feb. 1972b.
- Foote, R.H. Estrus detection and estrus detection aids. *J. Dairy Sci.* 58:248-256. 1975a.
- Foote, R.H. The gametogenic function of the aging ovary in the mammal. In: *Aging Gametes*, R.J. Blandau (ed.). S. Karger, New York. pp. 179-200. 1975b.
- Foote, R.H. Reproductive performance and problems in New York dairy herds. *Search* 8(2): 18 pp. 1978.
- Foote, R.H. Time of insemination and fertility in dairy cattle. *J. Dairy Sci.* 62:355-358. 1979.
- Foote, R.H. The artificial insemination industry. In: *New Technologies in Animal Breeding*. B.C. Brackett, G.E. Seidel, Jr. and S.M. Seidel (eds). Academic Press, New York pp. 13-39. 1981.
- Foote, R.H. General evaluation of male reproductive capacity. 10th Int. Congr. on Anim. Reprod. and A.I., Urbana, IL. IV (Sec. X):1-8. 1984.
- Foote, R.H. Review: Dairy cattle reproductive physiology research and management – past progress and future prospects. *J. Dairy Sci.* 79:980-990. 1996.
- Foote, R.H. Artificial Insemination to Cloning. *Tracing 50 Years of Research*. Published by the Author, Ithaca, NY. 231 pp. 1998.
- Foote, R.H. Artificial insemination from its origins up to today. *Proc. of the Spallanzani International Symposium*. V. Russo, S. Dall'Olio and L. Fontanesi (eds.). Reggio Emilia, Italy. pp. 23-67. 1999.
- Foote, R.H. Developments in animal reproductive biotechnology. In: *Assisted Fertilization and Nuclear Transfer in Mammals*. D.P. Wolf and M. Zelinski-Wooten (eds.). Humana Press, Inc. Totowa, NY. pp. 3-20. 2001.
- Foote, R.H. Effect of processing and measuring procedures of estimated sizes of bull sperm heads. *Theriogenology* 59:1765-1773. 2003a.
- Foote, R.H. Fertility estimation: a review of past experience and future prospects. *Anim. Reprod. Sci.* 75:119-139. 2003b.
- Foote, R.H. and W.E. Berndtson. The germinal cells. In: *Reversibility in Testicular Toxicity Assessment*. A.R. Scialli and E.D. Clegg (eds.). CRC Press, Boca Raton, FL. pp. 1-55. 1992.

- Foote, R.H. and R.W. Bratton. The fertility of bovine semen cooled with and without the addition of citrate-sulfanilamide-yolk extender. *J. Dairy Sci.* 32:856-861. 1949.
- Foote, R.H. and R. W. Bratton. The fertility of bovine semen in extenders containing sulfanilamide, penicillin, streptomycin, and polymyxin. *J. Dairy Sci.* 33:544-547. 1950.
- Foote, R.H. and A. Heath. Effect of sperm losses in semen collection equipment on estimated sperm output by bulls. *J. Dairy Sci.* 46:242-244. 1963.
- Foote, R.H. and H. Onuma. Superovulation, ovum collection, culture and transfer. A review. *J. Dairy Sci.* 53:1681-1692. 1970.
- Foote, R.H. and P.M. Riek. Gonadotropin-releasing hormone improves reproductive performance of dairy cows with slow involution of the reproductive tract. *J. Anim. Sci.* 77:12-16. 1999.
- Foote, R.H. and G.W. Salisbury. The effect of pyridium, penicillin, furacin, and phenoxethol upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. *J. Dairy Sci.* 31:763-768. 1948.
- Foote, R.H. and X. Yang. Cloning of bovine embryos. *Reprod. Dom. Anim.* 27:13-21. 1992.
- Foote, R.H., N. Munkenbeck and W.A. Green. Testosterone and libido in Holstein bulls of various ages. *J. Dairy Sci.* 59:2011-2013. 1976.
- Fortune, J.E., G.M. Rivera and M.Y. Yang. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Anim. Reprod. Sci.* 82-83:109-126. 2004.
- Fricke, P.M. J.N. Guenther and M.C. Wiltbank. Efficacy of decreasing the dose of GnRH used in a protocol for synchronization of ovulation and timed AI in lactating dairy cows. *Theriogenology* 50:1275-1284. 1998.
- Garner, D.L., C.A. Thomas and C.H. Allen. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. *J. Androl.* 18:324-331. 1997a.
- Garner, D.L., C.A. Thomas, H.W. Joerg, J.M. DeJarnette and E. Marshall. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol. Reprod.* 57:1401-1406. 1997b.
- Gilbert, R.O. Hormones, antibiotics and fresh cow programs. Mimeo. Cornell Conference on Optimizing Reproductive Performance in Dairy Herds. 8 pp. 2000.
- Ginther, O.J. *Ultrasound Imaging and Animal Reproduction: Cattle (Book 3)*. Equiservices Pub., Cross Plains, WI. 1998.
- Ginter, O.J., M.A. Beg, D.R. Bergfelt, F.X. Donadeu and K. Kot. Follicle selection in monovular species. *Biol. Reprod.* 65:638-647. 2001.
- Gledhill, B.L. Studies on the DNA content, dry mass and optical area of morphologically normal and abnormal bull spermatozoal heads. *Acta. Vet. Scand.* 7:1-20. 1966.

- Gledhill, B.L. Engima of spermatozoal deoxyribonucleic acid and male infertility. A review. *Am. J. Vet. Res.* 31:539-545. 1970.
- Gledhill, B.L. Cytometry of mammalian sperm. *Gamete Res.* 12:423-438. 1985.
- Gomes, W.R. and R.E. Erb. Progesterone in bovine reproduction. A review. *J. Dairy Sci.* 48:314-330. 1965.
- Gordon, I. Laboratory Production of Cattle Embryos. CAB International, Wallingford, Oxon, UK. 1994.
- Gordon, I. and L.H. Lu. Production of embryos in vitro and its impact on livestock production. *Theriogenology* 33:77-87. 1990.
- Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa. Pregnancies after co-culture of cumulus cells with bovine embryos derived from in vitro fertilization of in-vitro matured follicular oocytes. *J. Reprod. Fertil.* 83:753-758. 1988.
- Goto, K., A. Kinshita, Y. Takuma and K. Ogawa. Fertilization of bovine oocytes by the injection of immobilized, killed spermatozoa. *Vet. Rec.* 127:517-520. 1990.
- Graham, J.K., E. Kunze and R.H. Hammerstedt. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. *Biol Reprod.* 43:55-64. 1990.
- Grossman, M., W.J. Koops and J.H.G. Den Daas. Multiphasic analysis of reproductive efficiency of dairy bulls. *J. Dairy Sci.* 78:2871-2876. 1996.
- Hafs, H.D. and M.S. McCarthy. Endocrine control of testicular function. In: *Animal Reproduction*. H.W. Hawk, C.A. Kiddy and H.C. Cecil (eds.). John Wiley & Sons, New York. pp. 345-364. 1979.
- Hafs, H.D., R.W. Bratton, C.R. Henderson and R.H. Foote. Estimation of some variance components of bovine semen criteria and their use in the design of experiments. *J. Dairy Sci.* 41:96-104. 1958.
- Hafs, H.D., R.S. Hoyt and R.W. Bratton. Libido, sperm characteristics, sperm output and fertility of mature dairy bulls ejaculated daily or weekly for thirty-two weeks. *J. Dairy Sci.* 42:626-636. 1959.
- Hafs, H.D., R.C. Knisely and C. Desjardins. Sperm output of dairy bulls with varying degrees of sexual preparation. *J. Dairy Sci.* 45:788-793. 1962.
- Hahn, J., R.H. Foote and E.T. Cranch. Tonometer for measuring testicular consistency of bulls to predict semen quality. *J. Anim. Sci.* 29:483-489. 1969.
- Hale, E.B. and J.O. Almquist. Relation of sexual behavior to germ cell output in farm animals. *J. Dairy Sci. Suppl.* 43:149-169. 1960.
- Hammer, R.E., V. Pursel, C.E. Rexroad, Jr., R.J. Wall, D.J. Boldt, K.M. Ebert, R.D. Palmiter and R.L. Brinster. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* 315:680-683. 1985.
- Hammond, J., Jr. and P. Bhattacharya. Control of ovulation in the cow. *J. Agric Sci.* 34:1-15. 1944.

- Hancock, J.L. The morphology of bull spermatozoa. *J. Exp. Biol.* 29:445-453. 1952.
- Hansel, W. The estrous cycle of the cow. In: *Reproduction in Domestic Animals*. Vol. I. H.H. Cole and P.T. Cupps (eds.). Academic Press, New York. pp. 223-265. 1959.
- Hansel, W. Estrous cycle and ovulation control in cattle. *J. Dairy Sci.* 44:2307-2329. 1961.
- Hansel, W. and S.A. Asdell. The effects of estrogen and progesterone on the arterial system of the uterus of the cow. *J. Dairy Sci.* 34:37-44. 1951.
- Hansel, W. and W.E. Beal. Ovulation control in cattle. In: *Animal Reproduction*, H.W. Hawk, C.A. Kiddy and H.C. Cecil (eds.). John Wiley & Sons, New York. pp. 91-110. 1979.
- Hansel, W. and E.M. Convey. Physiology of the estrous cycle. *J. Anim. Sci.* 57(Suppl. 2):404-424. 1983.
- Harris, G.W. *Neural Control of the Pituitary Gland*. Williams and Wilkins, Baltimore, Maryland. 1955.
- Hartman, C.G., W.H. Lewis, F.W. Miller and W.W. Swett. First findings of tubal ova in the cow together with notes on estrus. *Anat. Rec.* 48:267-275. 1931.
- Hasler, J.F. Current status and potential of embryo transfer and reproductive technology in dairy cattle. *J. Dairy Sci.* 75:2857-2879. 1992.
- Hasler, J.F., A.D. McCauley, W.F. Lathrop and R.H. Foote. Effect of donor-embryo-recipient interactions on pregnancy rate in a large-scale bovine embryo transfer program. *Theriogenology* 27:139-168. 1987.
- Hawk, H.W., H.H. Conley, R.J. Wall and R.O. Whitaker. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. *Theriogenology* 29:1131-1142. 1988.
- Heape, W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Proc. Roy. Soc. B* 48:457-458. 1891.
- Henderson, C.R. Selecting and sampling young sires. *Proc. 7th Ann. Conf., Natl. Assoc. Artificial Breeders*. pp. 93-103. 1954.
- Hendrikse, J. and F.C. van der Kaay (Translated title). Fertility results with different methods of artificial insemination. *Tijdschr. Diergeneesk.* 75:983-999. 1950.
- Henricson, B. and E. Rajakowski. Studies of oocyto-genesis in cattle. *Cornell Vet.* 49:494-503. 1959.
- Herman, H.A. *Improving Cattle by the Millions. NAAB and the Development and Worldwide Application of Artificial Insemination*. University of Missouri Press, Columbia, MO. 377 pp. 1981.
- Hough, S.R., M.T. Kaproth and R.H. Foote. Induction of the acrosome reaction and zona-free hamster oocyte penetration by a bull with complete teratospermia versus a half brother with normal sperm. *J. Androl.* 23:98-106. 2002.

- Ivanoff, E.I. On the use of artificial insemination for zootechnical purposes in Russia. *J. Agr. Sci.* 12:244-256. 1922.
- Jeyendran, R.S., H.H. Van der Ven, M. Perez-Pelaez, B.G. Crabo and L.J. Zaneveld. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil* 70:219-228. 1984.
- Johnson, A.D., W.R. Gomes and N.L. Vandemark (eds.). *The Testis. Vol I.* Academic Press, New York. 1970.
- Johnson, J., J. Canning, T. Kaneko, J.K. Pru and J.L. Tilly. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 428:145-150. 2004.
- Johnson, L.A. Gender preselection in domestic animals using flow cytometrically sorted sperm. *J. Anim. Sci.* 70:(Suppl. 2):8-18. 1992.
- Kane, M.T. and R.H. Foote. Factors affecting blastocyst expansion of rabbit zygotes and young embryos in defined media. *Biol. Reprod.* 4:41-47. 1971.
- Kaproth, M.T., J.E. Parks, G.C. Grambo, H.E. Rycroft, J.A. Hertl and Y.T. Gröhn. Effect of preparing and loading multiple insemination guns on conception rate in two large commercial dairy herds. *Theriogenology* 57:909-921. 2002.
- Kastelic, J.P., R.B. Cook and G.H. Coulter. Contribution of the scrotum, testes, and testicular artery to scrotal/testicular thermoregulation in bulls at two ambient temperatures. *Anim. Reprod. Sci.* 45:255-261. 1997.
- Kennelly, J.J. and R.H. Foote. Superovulatory response of pre- and post-pubertal rabbits to commercially available gonadotrophins. *J. Reprod. Fertil.* 9:177-188. 1965.
- Kennelly, J.J. and R.H. Foote. Oocytogenesis in rabbits: The role of neogenesis in the formation of the definitive ova and the stability of oocyte DNA measured with tritiated thymidine. *Amer. J. Anat.* 118:573-589. 1966.
- Killian, G.J. Fertility factors in seminal plasma. 14th Tech. Conf. Artif. Insem. Reprod. Natl. Assoc. Anim. Breeders, Columbia, MO. pp. 33-38. 1992.
- Knobil, E. On the control of gonadotropin secretion in the rhesus monkey. *Recent Prog. Horm. Res.* 30:1-36. 1974.
- Knobil, E. The neuroendocrine control of the menstrual cycle. *Recent Prog. Horm. Res.* 36:53-88. 1980.
- Knobil, E. and J.D. Neill (eds.). *Encyclopedia of Reproduction. Vol. I-IV.* Academic Press, New York. 1998.
- Kobayashi, J., A. Sekimoto, H. Uchida, T. Wada, K. Sasaki, H. Sasada, M. Umezu and E. Sato. Rapid detection of male-specific DNA sequence in bovine embryos using fluorescence in situ hybridization. *Mol. Reprod. Dev.* 51:390-394. 1998.
- Krook, L. L. Lutwak and K. McEntee. Dietary calcium, ultimobranchial tumors and osteopetrosis in the bull. *Am. J. Clin. Nutr.* 22:115-118. 1969.
- Lagerlof, N. Changes in the spermatozoa and in the testes of bulls with impaired or abolished fertility. *Acta Path. Microbiol. Scand. Suppl.* 19:1-254. 1934.

- Larsson, B. and H. Rodriguez-Martinez. Can we use in vitro fertilization tests to predict semen fertility? *Anim. Reprod. Sci.* 60-61:327-336. 2000.
- Lawitts, J.A. and J.D. Biggers. Optimization of mouse embryo culture media using simplex methods. *J. Reprod. Fertil.* 91:513-556. 1991.
- Leblond, C.P. and Y. Clermont. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N.Y. Acad. Sci.* 55:548-573. 1952.
- Leeuwenhoek, A. De natis è semine genitali animalculis. *Roy. Soc. London. Philos. Trans.* 12:1040-1043. 1678.
- Lefebvre, R. and S.S. Suarez. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biol. Reprod.* 54:557-582. 1996.
- Lein, D., I. Erickson, A.J. Winter and K. McEntee. Diagnosis, treatment and control of vibriosis in an artificial insemination center. *J. Am. Vet. Med. Assoc.* 153:1574-1578. 1968.
- Lessard, C., S. Parent, P. Leclerc, J.L. Bailey and R. Sullivan. Cryopreservation alters the level of the bull surface protein P25b. *J. Androl.* 21:700-707. 2000.
- Leuchtenberger, C. The relation of the deoxyribonucleic acid (DNA) to sperm cell fertility. *J. Dairy Sci.* 43(Suppl. 1):31-35. 1960.
- Leuchtenberger, C., I. Murmanis, L. Murmanis, S. Ito and D.R. Weir. Interferometric dry mass and micro-spectrophotometric arginine determinations on bull sperm nuclei with normal and abnormal DNA content. *Chromosoma.* 8:73-86. 1956.
- Lindahl, P.E. and L.O. Drevius. Observations on bull spermatozoa in a hypotonic medium related to sperm motility mechanisms. *Exp. Cell Res.* 36:632-637. 1964.
- Lindner, G.M. and R.W. Wright, Jr. Bovine embryo morphology and evaluation. *Theriogenology* 20:407-416. 1983.
- Liu, Z. and R.H. Foote. Effects of amino acids on the development of in vitro matured/in vitro fertilization bovine embryos in a simple protein-free medium. *Hum. Reprod.* 10:2985-2991. 1995.
- Liu, Z. and R.H. Foote. Bull sperm motility and membrane integrity in media varying in osmolality. *J. Dairy Sci.* 81:1868-1873. 1998a.
- Liu, Z. and R.H. Foote. Osmotic effects on volume and motility of bull sperm exposed to membrane permeable and nonpermeable agents. *Cryobiology* 37:207-218. 1998b.
- Lohuis, M.M. Potential benefits of bovine embryo-manipulation technologies to genetic improvement programmes. *Theriogenology* 43:51-60. 1995.
- Lu, K.H., I. Gordon, M. Gallaher, and H. McGovern. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. *Vet. Rec.* 121:259-260. 1987.
- MacMillan, K.L., H.D. Hafs, C. Desjardins and K.T. Kirton. Some semen characteristics in dairy bulls ejaculated with artificial vaginas at varying temperatures. *J. Dairy Sci.* 49:1132-1134. 1966.

- Malmgren, L. Assessing the quality of raw semen: a review. *Theriogenology* 48:523-530. 1997.
- Mann, T. *Biochemistry of Semen and of the Male Reproductive Tract*. John Wiley and Sons, New York. 1964.
- Marshall, F.H.A. *The Physiology of Reproduction*. Longmans, Green and Co., London. 1910.
- Maule, J.P. *The Semen of Animals and Artificial Insemination*. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England. 1962.
- Mauleon, P. Oogenesis and folliculogenesis. In: *Reproduction in Domestic Animals*, 2nd ed. H.H. Cole and P.T. Cupps (eds.). Academic Press, New York. pp. 187-215. 1969.
- Maurer, R.R. Freezing mammalian embryos. A review of the techniques. *Theriogenology* 9:45-68. 1978.
- Maurer, R.R. and R.H. Foote. Maternal ageing and embryonic mortality in the rabbit. I. Repeated superovulation, embryo culture and transfer. *J. Reprod. Fertil.* 25:329-341. 1971.
- McDonald, T.J., R.H. Foote, M. Drost, L. Lu, M. Patrascu and C.E. Hall. Preparation of teaser bulls and steroid-implanted steers and their effectiveness in detecting estrus. *Theriogenology* 6:51-60. 1976.
- McEntee, K. H.L. Gilman, D.E. Hughes, W.A. Wagner and H.O. Dunn. Insemination of heifers with penicillin- and dihydrostreptomycin-treated semen from *Vibrio fetus* carrier bulls. *Cornell Vet.* 49:175-183. 1959.
- McKenna, T., R.W. Lenz, S.E. Fenton and R.L. Ax. Nonreturn rates of dairy cattle following uterine body or cornual insemination. *J. Dairy Sci.* 73:1779-1783. 1990.
- Mercier, E. and G.W. Salisbury. Effect of techniques of preparing semen smears for staining on the morphology of bull spermatozoa. *J. Anim. Sci.* 6:60-66. 1947.
- Meryman, H.T. Drying of living mammalian cells. *Ann. NY Acad. Sci.* 85:729-739. 1960.
- Midgley, A.R. Principles for assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). In: *Karolinska Symposia on Research Methods in Reproductive Endocrinology: Immunoassay of Gonadotropins*. E. Diczfalusy (ed.). Stockholm, Sweden. pp. 163-184. 1969.
- Mitchell, J.S., Y. Wu, C.J. Cook and L. Main. Protein conjugate-based immunoassay of whole milk progesterone. *J. Dairy Sci.* 87:2864-2867. 2004.
- Moreira, F., C. Orlandi, C.A. Risco, R. Mattos, F. Lopes and W.W. Thatcher. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *J. Dairy Sci.* 84:1649-1659. 2001.
- Morrell, J.M. and D.W. Dresser. Offspring from inseminations with mammalian sperm stained with Hoechst 33342, either with or without flow cytometry. *Mutation Res.* 224:177-183. 1989.

- Mossman, H.W. and K.L. Duke. Comparative Morphology of the Mammalian Ovary. University of Wisconsin Press, Madison, WI. 1973.
- Nebel, R.L. and S.M. Jobst. Evaluation of systematic breeding programs for lactating dairy cows. A review. *J. Dairy Sci.* 81:1169-1174. 1998.
- Nebel, R.L., J.H. Bame, R.G. Saacke and F. Lim. Microencapsulation of bovine spermatozoa. *J. Anim. Sci.* 60:1631-1639. 1985.
- Nebel, R.L., M.G. Dransfield, S.M. Jobst and J.H. Bame. Automated electronic systems for the detection of oestrus and timing of AI in cattle. *Anim. Reprod. Sci.* 60-61:713-723. 2000.
- Nebel, R.L., W.L. Walker, M.L. McGilliard, C.H. Allen, and G.S. Heckman. Timing of artificial insemination of dairy cows: Fixed time once daily versus morning and afternoon. *J. Dairy Sci.* 77:3185-3191. 1994.
- Nicholas, F.N. Genetic improvement through reproductive technology. *Anim. Reprod. Sci.* 42:205-214. 1996.
- Nicholas, F.N. and C. Smith. Increased rates of genetic change in dairy cattle by embryo transfer and splitting. *Anim. Prod.* 36:341-353. 1983.
- Niswender, G.D., A.R. Midgley and L.E. Reichert, Jr. Radioimmunologic studies with murine, bovine, ovine and porcine luteinizing hormone. In: *Gonadotropins*. E. Rosenberg (ed.). Gerox-X, Inc. Los Altos. pp. 299-306. 1968.
- O'Dell, W.T. and J.O. Almquist. Freezing bovine semen. I. Techniques for freezing bovine spermatozoa in milk diluents. *J. Dairy Sci.* 40:1534-1541. 1957.
- Oltenacu, P.A., R.A. Milligan, T.R. Rounsaville and R.H. Foote. Modelling reproduction in a herd of dairy cattle. *Agri. Systems* 5:193-205. 1980.
- Oltenacu, P.A., T.R. Rounsaville, R.A. Milligan and R.H. Foote. Systems analysis for designing reproductive management programs to increase production and profit in dairy herds. *J. Dairy Sci.* 64:2096-2104. 1981.
- Ortavant, R. Contribution à l'étude de la durée du processus spermatogénétique du bélier à l'aide du P32. *Compt. Rend. Soc. Biol.* 148:804-806. 1954.
- Parks, J.E. Applications of flow cytometry in semen processing and handling. 14th Tech. Conf. Artif. Insem. Reprod. Natl. Assoc. Anim. Breeders, Columbia, MO. pp. 12-17. 1992.
- Parks, J.E., E. Ehrenwald and R.H. Foote. Counting mammalian spermatozoa in biological fluids containing particulate matter. *J. Dairy Sci.* 68:2329-2336. 1985.
- Parlow, A.F. Bio-assay of pituitary luteinizing hormone by depletion of ovarian ascorbic acid. In: *Human Pituitary Gonadotropins*. A. Albert (ed.). Thomas, Springfield, IL. pp. 300-310. 1961.
- Paufler, S.K. and R.H. Foote. Influence of light on nuclear size and deoxyribonucleic acid content of stored bovine spermatozoa. *J. Dairy Sci.* 50:1475-1480. 1967.
- Perry, E.J. *the Artificial Insemination of Farm Animals*, 4th ed. Rutgers University Press, New Brunswick, NJ. 473 pp. 1968.

- Peters, J.L. and P.L. Senger. Radiographic method for evaluation of bovine inseminating techniques. *J. Dairy Sci.* 66:1760-1764. 1983.
- Phillips, P.H. and H.A. Lardy. A yolk-buffer pabulum for the preservation of bull semen. *J. Dairy Sci.* 23:399-404. 1940.
- Pickett, B.W. and W.E. Berndtson. Preservation of bovine spermatozoa by freezing in straws. A review. *J. Dairy Sci.* 57:1287-1301. 1974.
- Pieterse, M.C., P.L.A.M. Vos, T.A.M. Kruip, Y.A. Wurt, T.H. van Beneden, A.H. Willemse and M.A.M. Taverne. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology* 35:857-862. 1991.
- Polge, C. Frozen semen and the A.I. programme in Great Britain. In: *Proc. 2nd Tech. Conf. Artif. Insem. Reprod.* NAAB, Columbia, MO. pp. 46-51. 1968.
- Polge, C., A.U. Smith and A.S. Parkes. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164:666. 1949.
- Prather, R.S., F.L. Barnes, M.M. Sims, J.M. Robl and N.L. First. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol Reprod.* 37:859-866. 1987.
- Purdy, P.H. and J.K. Graham. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Biol. Reprod.* 71:522-527. 2004.
- Pursley, J.R., M.R. Kosorok and M.C. Wiltbank. Reproductive management of lactating dairy cows using synchronization of estrus. *J. Dairy Sci.* 80:301-306. 1997.
- Pursley, J.R., R.W. Silcox and M.C. Wiltbank. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss and gender ratio after synchronization of ovulation in lactating dairy cows. *J. Dairy Sci.* 81:2139-2144. 1998.
- Rikmenspoel, R., G. van Herpen and P. Eijkhout. Cinematographic observations of the movements of bull sperm cells. *Phys. Med. Biol.* 5:167-182. 1960.
- Roberts, S.J. *Veterinary Obstetrics and Genital Diseases (Theriogenology)*, 3rd ed. Published by the author. Printed by Edwards Brothers, Inc., Ann Arbor, MI. 1986.
- Roche, J.F. Fertility in cows after treatment with a prostaglandin analogue with or without progesterone. *J. Reprod. Fertil.* 46:341-345. 1976.
- Rodriguez-Martinez, H. Optimization of sperm quality in AI bulls. *Reprod. Dom. Anim.* 33:233-237. 1998.
- Roosen-Runge, E.C. and L.O. Giesel. Quantitative studies on spermatogenesis in the albino rat. *Am. J. Anat.* 87:1-30, 1950.
- Rothschild, L. A new method of measuring the activity of spermatozoa. *J. Exp. Biol.* 30:178-199. 1953.
- Rowson, L.E.A. Methods of inducing multiple ovulations in cattle. *J. Endocrinol.* 7:260-270. 1951.

- Rowson, L.E.A. The role of reproductive research in animal production. *J. Reprod. Fertil.* 26:113-126. 1971.
- Rowson, L.E.A. and R.M. Moor. Non-surgical transfer of cow eggs. *J. Reprod. Fertil.* 11:311-312. 1966.
- Rowson, L.E.A., R. Tervit and A. Brand. Synchronization of estrus by means of prostaglandin F₂. *Proc. 7th Int. Congr. Anim. Reprod. Artificial Insem.*, Munich, Germany. II:865-869. 1972.
- Saacke, R.G. and C.E. Marshall. Observations on the acrosomal cap of fixed and unfixed bovine spermatozoa. *J. Reprod. Fertil.* 16:511-514. 1968.
- Saacke, R.G., C.E. Marshall, W.E. Vinson, M.L. O'Connor, J.E. Chandler, J. Mullins, R.P. Amann, R.A. Wallace, W.N. Vincel and H.C. Kellgren. Semen quality and heterospermic insemination in cattle. *9th Int. Congr. Anim. Reprod. A.I.*, Madrid, Spain. 5:75-78. 1980.
- Saacke, R.G., R.L. Nebel, D. Pennington and R. Miller. Artificial vagina design: preparation and sanitation. *Proc. 9th Tech. Conf. on Artif. Insem. Reprod.*, Milwaukee, WI. pp. 63-67. 1982.
- Salisbury, G.W. A controlled experiment in feeding wheat germ oil as a supplement to the normal ration of bulls used for artificial insemination. *J. Dairy Sci.* 27:557-562. 1944.
- Salisbury, G.W. and E.L. Willett. An artificial vagina for controlled temperature studies of bull semen. *Cornell Vet.* 30:25-29. 1940.
- Salisbury, G.W., H.K. Fuller and E.L. Willett. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. *J. Dairy Sci.* 24:905-910. 1941.
- Salisbury, G.W., G.H. Beck, P.T. Cupps and I. Elliott. The effect of dilution rate on the livability and the fertility of bull spermatozoa used for artificial insemination. *J. Dairy Sci.* 26:1057-1069. 1943a.
- Salisbury, G.W., G.H. Beck, I. Elliott. and E.L. Willett. Rapid methods for estimating the number of spermatozoa in bull semen. *J. Dairy Sci.* 26:69-78. 1943b.
- Salisbury, G.W., N.L. VanDemark and J.R. Lodge. *Physiology of Reproduction and Artificial Insemination of Cattle*, 2nd ed. W.H. Freeman Co., San Francisco. 1978.
- Saumande, J. and P. Humblot. The variability in the interval between estrus and ovulation in cattle and its determinants. *Anim. Reprod. Sci.* 85:171-182. 2005.
- Sawyer, G.J. and W.J. Fulkerson. The effectiveness of steers and heifers treated with oestrogen or testosterone to detect oestrus in cattle. *Anim. Reprod. Sci.* 3:259-269. 1981.
- Schally, A.V. and C.Y. Bowers. In vitro and in vivo stimulation of the release of luteinizing hormone. *Endocrinology* 75:312-320. 1964.
- Schanbacher, B.D. Importance of the episodic nature of luteinizing hormone secretion for normal development of the bovine testis during puberty: interference with oestradiol-17. *J. Endocrinol.* 88:393-400. 1981.

- Seidel, G.E., Jr. Superovulation and embryo transfer in cattle. *Science* 211:351-358. 1981.
- Seidel, G.E., Jr. and R.H. Foote. Influence of semen collection interval and tactile stimuli on semen quality and sperm output in bulls. *J. Dairy Sci.* 52:1074-1079. 1969a.
- Seidel, G.E., Jr. and R.H. Foote. Motion picture analysis of ejaculation in the bull. *J. Reprod. Fertil.* 20:313-317. 1969b.
- Seidel, G.E., Jr. and R.H. Foote. Variance components of semen criteria from bulls ejaculated frequently and their use in experimental design. *J. Dairy Sci.* 56:399-405. 1973.
- Seidel, G.E., Jr., C.H. Allen, L.A. Johnson, M.D. Holland, Z. Brink., G.R. Welch, J.K. Graham and M.B. Cattell. Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. *Theriogenology* 48:1255-1264. 1997.
- Seidel, G.E., Jr., L.L. Larson, C.H. Spilman, J. Hahn and R.H. Foote. Culture and transfer of calf ova. *J. Dairy Sci.* 54:923-926. 1971.
- Setchell, B.P. *Male Reproduction*. Van Nostrand Reinhold Co., Inc., New York. 1984.
- Shannon, P. and R. Vishwanath. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. *Anim. Reprod. Sci.* 39:1-10. 1995.
- Shannon, P., B. Curson and A.P. Rhodes. Relationship between total spermatozoa per insemination and fertility of bovine semen stored in Caprogen at ambient temperature. *N. Z. J. Agri. Res.* 27:35-41. 1984.
- Sipher, E. *The Gene Revolution. The History of Cooperative Artificial Breeding in New York and New England, 1938-1940*. Eastern AI Cooperative, Inc., Ithaca, NY. 1991.
- Smikle, C. and P.J. Turek. Hypo-osmotic swelling can accurately assess the viability of nonmotile sperm. *Mol. Reprod. Dev.* 47:200-237. 1997.
- Sørensen, E. *Kunstig saedeverforing hos huspattedyrene*. Kongelig Veterinaer-og Landbohojskole Aarsskrift, Copenhagen. 1938.
- Spalding, R.W., R.W. Everett and R.H. Foote. Fertility in New York artificially inseminated Holstein herds in Dairy Herd Improvement. *J. Dairy Sci.* 58:718-723. 1975.
- Spallanzani, L. *Dissertations Relative to the Natural History of Animals and Vegetables*. Trans. by T. Beddoes. J. Murray, London. 2:195-199. 1784.
- Sprott, L.R., M.D. Harris, D.W. Forrest, J. Young, H.M. Zhang, J.N. Oyarzo, M.E. Bellin and R.L. Ax. Artificial insemination outcomes in beef females using bovine sperm with a detectable fertility-associated antigen. *J. Anim. Sci.* 78:795-798. 2000.

- Steelman, S.L. and F.M. Pohley. Assay of the follicle-stimulating hormone based on the augmentation with human chorionic gonadotropin. *Endocrinology* 53:604-616. 1953.
- Stevenson, J.S. and A.P. Phatak. Insemination at estrus induced by presynchronization before application of a synchronized estrus and ovulation. *J. Dairy Sci.* 88:399-405. 2005.
- Stevenson, J.S., G.C. Lamb, Y. Kobayashi and D.P. Hoffman. Luteolysis during two stages of the estrous cycle: Subsequent endocrine profiles associated with radio-telemetrically detected estrus in heifers. *J. Dairy Sci.* 81:2897-2903. 1998.
- Suarez, S.S. Hyperactivated motility of sperm. Minireview. *J. Androl* 17:331-335. 1996.
- Suarez, S.S., K. Brockman and R. Lefebvre. Distribution of mucus and sperm in bovine oviducts after artificial insemination: The physical environment of the oviductal sperm reservoir. *Biol. Reprod.* 56:447-453. 1997.
- Swierstra, E.E. and R.H. Foote. Cytology and kinetics of spermatogenesis in the rabbit. *J. Reprod. Fertil.* 5:309-322. 1963.
- Swierstra, E.E. and R.H. Foote. Duration of spermatogenesis and spermatozoan transport in the rabbit based on cytological changes, DNA synthesis and labeling with tritiated thymidine. *Am. J. Anat.* 116:401-412. 1965.
- Tallam, S.K., T.L. Kerbler, K.E. Leslie, K. Bateman, W.H. Johnson and J.S. Walton. Reproductive performance of postpartum dairy cows under a highly intervenient breeding program involving timed insemination and combinations of GnRH, prostaglandin F_{2α} and human chorionic gonadotropin. *Theriogenology* 56:91-104. 2001.
- Tardif, A.L., P.B. Farrell, V. Trouern-Trend, M.E. Simkin and R.H. Foote. Use of Hoechst 33342 stain to evaluate live, fresh and frozen bull sperm by computer assisted analysis. *J. Androl.* 19:201-206. 1998.
- Tervit, H.R. Laparoscopy/laparotomy oocyte recovery and juvenile breeding. *Anim. Reprod. Sci.* 42:227-238. 1996.
- Thomas, C.A., D.L. Garner, J.M. DeJarnette and C.E. Marshall. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. *Biol. Reprod.* 50:991-998. 1997.
- Thompson, A.W. and G.W. Salisbury. A suggested procedure for the establishment of standard and comparable breeding efficiency reports in artificial breeding. Mineo. Pub. 1, Cornell University, Ithaca, NY. 16 pp. 1947.
- Trimberger, G.W. Breeding efficiency in dairy cattle from artificial insemination at various intervals before and after ovulation. *Nebraska Agric. Expt. Sta. Bul.* 153: 26 pp. 1948.
- Tubman, L.M., Z. Brink, T.K. Suh and G.E. Seidel, Jr. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. *J. Anim. Sci.* 82:1029-1036. 2004.
- Ulberg, L.C., R.E. Christian and L.E. Casida. Ovarian response in heifers to progesterone injections. *J. Anim. Sci.* 10:752-759. 1951.

- Umbaugh, R.E. Superovulation and ovum transfer in cattle. *Am. J. Vet. Res.* 10:295-305. 1949.
- Van Berkomp, J. and P. Molta. *The Cellular Basis of Mammalian Reproduction.* Urban and Schwarzenberg, Baltimore. 1979.
- VanDemark, N.L. Time and site of insemination in cattle. *Cornell Vet.* 42:215-222. 1952.
- VanDemark, N.L., G.L. Fritz and R.E. Mauger. Effect of energy intake on reproductive performance of dairy bulls. II. Semen production and replenishment. *J. Dairy Sci.* 47:898-904. 1964.
- van Duijn, C., Jr. Mensuration of the heads of bull spermatozoa. *Microscopie.* 14:265-276. 1960.
- Van Raden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn and G.R. Wiggans. Development of a national genetic evaluation for cow fertility. *J. Dairy Sci.* 87:2285-2292. 2004.
- van Tienhoven, A. *Reproductive Physiology of the Vertebrates*, 2nd ed. Cornell University Press, Ithaca, NY. 1983.
- Van Vleck, L.D. Potential genetic impact of artificial insemination, sex selection, embryo transfer, cloning and selfing in dairy cattle. In: *New Technologies in Animal Breeding.* B.C. Brackett, G.E. Seidel, Jr., and S.M. Seidel (eds.). Academic Press, New York. pp. 221-242. 1981.
- Vishwanath, R., R.L. Nebel, W.H. McMillan, C.J. Pitt and K.L. Macmillan. Selected times of insemination with microencapsulated bovine spermatozoa affect pregnancy rate of synchronized heifers. *Theriogenology* 48:369-376. 1997.
- Wakayama, T. and R. Yanagimachi. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotech.* 16:639-641. 1998.
- Wall, R.J., D.E. Kerr and K.R. Bondioli. Transgenic dairy cattle: Genetic engineering on a large scale. *J. Dairy Sci.* 80:2213-2224. 1997.
- Wall, R.J., A.M. Powell, M.J. Paape, D.E. Kerr, D.D. Bannerman, V.G. Pursel, K.D. Wells, N. Talbot and H.W. Hawk. Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nature Biotech.* 23:445-451. 2005.
- Way, A.L. and G.J. Killian. Capacitation and induction of the acrosome reaction in bull spermatozoa with norepinephrine. *J. Androl* 23:352-357. 2002.
- Wells, L.J. Anatomy of female reproductive organs. In: *Reproduction in Domestic Animals*, Vol. I., 1st ed. H.H. Cole and P.T. Cupps (eds.). Academic Press, NY. pp. 1-27. 1959.
- Whittingham, D.G. Survival of mouse embryos after freezing and thawing. *Nature* 220:592-593. 1971.
- Willett, E.L., W.G. Black, L.E. Casida, W.H. Stone and P.J. Buckner. Successful transplantation of a fertilized bovine ovum. *Science* 113:247. 1951.
- Williams, T.J., R.P. Elsdon and G.E. Seidel, Jr. Pregnancy rates with bisected bovine embryos. *Theriogenology* 22:521-531. 1984.

- Wilmut, I., A.E. Schnieke, J. McWhir, A.J. Kind and H.S.K. Campbell. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810-813. 1997.
- Wilson, R.D., K.A. Weigel, P.M. Fricke, J.J. Rutledge, M.L. Leibfried-Rutledge, D.L. Matthews and V.R. Schutzkus. In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows. *J. Dairy Sci.* 88:776-782. 2005.
- Wolf, D.P. and M. Zelinski-Wooten (eds.). *Assisted Fertilization and Nuclear Transfer in Mammals*. Humana Press, Totowa, NJ. 2001.
- Young, W.C. (ed.). *Sex and Internal Secretions*, 3rd ed. The Williams and Wilkins Co., Baltimore. 1961.
- Zhang, B.R., B. Larson, N. Lundeheim and H. Roderiquez-Martinez. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int. J. Androl.* 21:207-216. 1998.
- Zuckerman, S. and B.J. Weir. *The Ovary* Vol. 1, 2nd ed. Academic Press, New York. 1977.

