From the first artificial insemination to the modern reproduction biotechnologies: traditional ways and new frontiers of animal production

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ARTIFICIAL INSEMINATION FROM THE ORIGINS UP TO TODAY

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Introduction

Artificial insemination (AI) was the first great biotechnology dealing with animal reproduction and animal breeding (1-14). The development of AI was accompanied by research, which stimulated an explosion of knowledge in the field of animal reproduction. The application of AI, particularly to dairy cattle, has been responsible for enormous economic benefits through the control of venereal and other diseases, the genetic improvement in milk production and working type traits, and the reduction of lethal genes (6, 15, 16). These demonstrated benefits to animal agriculture and consumers have paved the way for development and general acceptance by the public of many new beneficial emerging animal reproductive biotechnologies.

These emerging biotechnologies provide powerful tools for rapidly changing animal populations genetically (17-22) that previously changed slowly through natural selection and older established methods of selection by animal breeders. Application of the powerful tools available today increases the responsibility of all involved in these programs to consider with utmost care and deliberation the ethical aspects of these programs with respect to their potential impact on both animals and society. This paper will include a historical overview of AI followed by a discussion of selected major developments of AI in several species, especially in cattle. Also, we will outline briefly the role played by AI in leading toward development and acceptance of current reproductive technologies, such as sexing sperm, estrous cycle regulation, superovulation, embryo culture, embryo transfer, in vitro fertilization and cloning of animals (23-25). It will conclude with selected recent statistics on application of AI worldwide.

Changes over time have been phenomenal. Sperm studies have even reached the space age. Sperm motility patterns have been studied in space under weightless conditions (26), and interestingly, gravity does affect sperm motion.

Early history of AI

The early history of AI in several species has been chronicled by many workers (1, 2, 8, 9, 12-14, 27-30). The story is a fascinating one as it changed forever the old system of natural mating to one relying on advanced technology of sperm, egg and embryo handling, all combined with new methods of genetic selection.

The discovery of small motile cells called “animalcules” by Leeuwenhoek (31) and his pupil Hamm, using a simple microscope, opened the door to investigations of the functions of sperm cells and eventually to AI (table 1). When Leeuwenhoek sent his paper to the Royal Society of London and wrote to the king concerning its acceptance, he was afraid that it might not get published, but it was published.

Then, more than 100 years later, Spallanzani (32) inseminated a bitch with three pups being born 62 days later. This was the first documented case of artificial
insemination. Spallanzani also filtered the semen later on and found that only the residue on the filter (spermatozoa), and not the filtrate could cause fertilization.

In 1799 Hunter, in England, used artificial insemination of a woman to overcome hypospadias of her husband. Spallanzani (33) continued his investigations and observed that cooling and perhaps freezing stallion sperm did not destroy sperm, but only held them motionless until rewarmed.

Table 1. Important dates in the history of artificial insemination.

<table>
<thead>
<tr>
<th>Year</th>
<th>Description of event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1677</td>
<td>Leeuwenhoek discovered spermatozoa.</td>
</tr>
<tr>
<td>1780</td>
<td>Spallanzani successfully inseminated a bitch.</td>
</tr>
<tr>
<td>1799</td>
<td>Hunter used AI for a woman.</td>
</tr>
<tr>
<td>1803</td>
<td>Spallanzani observed that chilling sperm did not kill them.</td>
</tr>
<tr>
<td>1899</td>
<td>Ivanoff initiated organized AI research in Russia.</td>
</tr>
<tr>
<td>1902</td>
<td>Sand recommended AI in Denmark, but no program was started.</td>
</tr>
<tr>
<td>1912</td>
<td>Ishikawa organized AI research in Japan.</td>
</tr>
<tr>
<td>1914</td>
<td>Amantea devised the first artificial vagina for use in dogs.</td>
</tr>
<tr>
<td>1930s</td>
<td>Organized AI began in Denmark and the USA and quickly spread.</td>
</tr>
</tbody>
</table>

Landmarks in development of cattle AI

<table>
<thead>
<tr>
<th>Year</th>
<th>Description of event</th>
</tr>
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<tbody>
<tr>
<td>1937</td>
<td>Danish had established rectovaginal insemination, reducing sperm required.</td>
</tr>
<tr>
<td>1940</td>
<td>Phillips developed phosphate-buffered egg yolk for preserving bull sperm.</td>
</tr>
<tr>
<td>1941</td>
<td>Salisbury and others developed citrate-buffered egg yolk for preserving bull sperm.</td>
</tr>
<tr>
<td>1948</td>
<td>Almquist and Foote reported independently on the value of antibiotics in semen extenders to control microorganisms and increase fertility.</td>
</tr>
<tr>
<td>1949</td>
<td>Polge et al. discovered that glycerol protected sperm during freezing.</td>
</tr>
<tr>
<td>1950s</td>
<td>Powerful tools for progeny testing were developed by Henderson and Robertson.</td>
</tr>
<tr>
<td>1954</td>
<td>Waterloo (Canada) was the first organization to use frozen semen 100%.</td>
</tr>
<tr>
<td>1957</td>
<td>American Breeders Service developed liquid nitrogen tanks and service for frozen semen.</td>
</tr>
<tr>
<td>1963</td>
<td>Davis et al. (Cornell) developed Tris-buffered egg yolk-glycerol for fresh and frozen sperm, used later for many species.</td>
</tr>
<tr>
<td>1970</td>
<td>AI was used commercially for superovulated cows and embryo transfer and provided the initial framework for many breeding strategies.</td>
</tr>
<tr>
<td>1990s</td>
<td>Sexing bull sperm was improved with limited potential application.</td>
</tr>
</tbody>
</table>

Since about 1950 intensive studies have been conducted to develop methods of preserving sperm and use of AI in many species.

In the late 19th century (34) it was reported that AI had been used in isolated studies with dogs, and horses in several Countries. The first studies in which AI was developed on an organized basis were started in 1899 in Russia by Ivanov, or Ivanoff, as his name often is spelled. By 1909 Ivanoff had studied artificial insemination of domestic farm animals, dogs, foxes, rabbits and poultry. Incidentally, it is interesting to
note that his paper (8), received on 21 June 1922, was published in the July, 1922 issue of the "Journal of Agricultural Science". That efficiency is hard to beat, even with today's electronic technology.

The AI horse research in Russia was extensive, with good fertility, provided that fertile stallions and mares carefully selected to be in estrus were used. In 1910-1913, 77% of 201 mares (table 2) inseminated in different areas in Russia conceived (8). World War I disrupted the program, but after the war AI was again used extensively as one way of replacing the population of horses lost in the war. Ivanoff emphasized the importance of well-trained technicians, aseptic techniques and high quality fertile animals. Diluters to preserve the fertility of sperm for several days were researched extensively to increase the use of superior stallions over large geographical areas. Meanwhile isolated reports of mare insemination appeared in Western Europe.

Table 2. Conception rate in mares in Russia in 1910-1913 (from Ivanoff, 1922).

<table>
<thead>
<tr>
<th>Site of mares</th>
<th>Number of mares</th>
<th>Conception rate using AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>Overall</td>
<td>201</td>
<td>155</td>
</tr>
</tbody>
</table>

Nishikawa reported (29) that AI in domestic animals in Japan began in 1912, following Dr. Ishikawa’s return after studying with Ivanoff. The field of AI in Japan developed slowly, starting with horses, until the government supported AI programs with horses, cattle, sheep, goats, swine and chickens in the late 1930s.

In 1914 Amantea devised an artificial vagina for the dog (2). The artificial vagina was quickly modified in Russia to collect semen from bulls, stallions and rams. This seemingly simple item of equipment was of great importance for the development of AI.

An alternative to the artificial vagina, where animals have not been or cannot be trained to use it, is the electroejaculator. Gunn (35) was one of the first to experiment with electroejaculators, and they are available for many species of animals (36).

In 1936 Sørensen (37) organized the first cattle AI Cooperative in Denmark, although Sands had suggested this in 1902. By 1937 the Danish veterinarians had established the procedure of intrauterine insemination by manipulating the cervix per rectum to help pass the catheter through the cervix. Intrauterine insemination greatly reduced the number of sperm required per insemination.

The first cattle AI Cooperative was started in the United States in New Jersey in 1938 (13), patterned after the Danish system. Almost simultaneously AI organizations were established in several states in the USA, where experimental studies had been in progress for several years (13, 38). Phenomenal growth of AI in cattle followed in the
USA and Canada. This was facilitated by the establishment of research groups working with farm organizations and extension personnel to improve both genetics and reproduction.

At Cornell University, the private university provided land upon which was built a research center and an artificial breeding cooperative (The New York Artificial Breeders Cooperative Inc.) from funds provided by both New York State and the farmers’ AI Cooperative. For a detailed account see Sipher (30). The researchers and Cooperative personnel worked together, along with the extension service and veterinarians. The result was a remarkable group effort to improve AI and genetics of cattle, with a major impact worldwide.

Modern development of AI in dairy cattle

Many details of the technical development that helped to make AI of cattle so successful are described in various books (1, 9, 14, 27, 30, 39), and in review articles (12, 29, 40-42). These sources should be consulted for additional references.

Genetic selection of bulls

Tremendous progress has been made in sire selection. Originally, bulls with proofs on daughters obtained in natural service were selected, but the environmental influences were so great that the proofs were not reliable indicators of genetic superiority. The alternative established, that is now widely followed, was to make special matings of top tested cows to top AB-proven sires and progeny test these bulls (6, 15, 16). The essentials of this program, established 50 years ago were as follows:

1) obtain accurate and complete records on parents and other close relatives of the young sires to be progeny-tested, picking top AB proven bulls as sires;
2) make proper use of these records in making planned matings to produce the young bulls to be tested;
3) test several times as many bulls each year as will be required for replacements in the bull stud;
4) progeny-test each animal properly by obtaining an adequate number of progeny, giving equal opportunities in the performance testing to the progeny of the different sires, and correcting records for environmental differences.

A problem with the progeny-test system was the long generation interval between the tested sire and the tested son. One way of reducing this interval is by banking frozen semen from young bulls and using more semen from young, highly selected bulls, based on pedigree. A frozen semen bank and bull slaughter program is especially attractive economically for bulls representing breeds with small populations of females (27). Other biotechnologies, have been combined in multiple ovulation-embryo transfer (MOET) programs to produce and test sires. A great tribute to the AI industry is the tremendous improvement in dairy cattle. As a consequence of increased production without increased consumption of dairy products, cow numbers have decreased. This has placed an economic burden on many well-run AI organizations.

Unraveling and capitalizing on the power of the testis

The genetic impact of a sire depends on the ability to sire thousands of progeny, as well as being superior genetically. The ability to sire large numbers of progeny
depends primarily on testis size, as this is the chief factor determining sperm production (43, 44). Testis size can be estimated accurately by measuring scrotal circumference (27, 45, 46). Adult sperm production can be predicted by measuring the testes of young bulls. Testis size also is highly heritable, $h^2 = 0.67$ (47), so selection of sires with large testes is a trait that will be passed on to sons. Also, if their testes are firm (48), sperm produced tend to be more fertile. The contribution of large testes, as they affect sperm production, and the other components of an AI program that influence the number of progeny possible follow:

\[
\text{Number of progeny possible per sire} = \frac{\text{Number of sperm obtained per sire}}{\text{Number of sperm inseminated/cow}} \times \frac{\text{Percent of potential sperm used}}{\text{Percent of cows that calve}}
\]

Obviously, the testes form an important part of a breeding soundness examination of a bull (49). Ultrasonography (50-52) and thermography (53) can be used to produce a profile of the general internal characteristics of the testes, particularly useful when bulls with large testes are producing subnormal numbers of sperm or sperm of poor quality and low fertility. All AI organizations should measure testicular characteristics of their bulls.

**Nutrition of bulls**

The development of AI focused attention on the nutritional requirements of young, and mature bulls (54-57). Young bulls need a simple diet that will provide good growth according to nutritional standards. High energy diets will accelerate growth and allow young bulls to produce semen earlier (56, 57) and be progeny-tested sooner, but moderate feeding must follow to avoid fattening and physical problems.

Mature bulls need only a maintenance diet of good quality hay and a 12% protein concentrate. Vitamin A or E supplementation is not beneficial when a good ration is fed. High calcium diets fed to lactating cows to supply needs for calcium secreted in the milk must be avoided in mature bulls, as this will lead to excessive calcification of the bones with fusion of the vertebrae. Also, overfeeding that produces sleek fat adult bulls can lead to leg problems, lower libido and poorer semen quality (54).

**Sexual behavior and semen collection**

Studies at Cornell (58) and by Hale and Almquist (59) have led to recommendations for sexual preparation of bulls that result in harvesting the maximal number of sperm. These include a combination of attractive mounts, varied semen collection locations, active movement of the teaser bulls or steers used as mounts, and allowing a bull to mount several times before semen is actually collected. Foote et al. (60) described an unusual case of tricking a sexually uninterested young bull to lift his head to lick molasses on the mount animal. The bull then mounted for the first time and thereafter there was an unlimited display of libido.

The development of the artificial vagina in 1914 (table 1) by Amantea (2), with modifications for cattle (61), and by others later, has been critical to the success of AI. Modifications to assure no twisting of the liner to prevent injury to the penis and proper size and temperature (62) to stimulate the bull and collect most of the sperm directly into the collection tube have been studied extensively (63).
**Frequency of semen collection**

Bulls can be ejaculated more frequently than was originally believed to be desirable (27, 54, 59, 64, 65). Daily ejaculation of bulls for 8 months did not reduce semen quality or fertility (65).

A practical system is to prepare the bulls for two or three semen collections per day, each collection spaced about 30 minutes apart, and combine the ejaculates in the semen processing laboratory into one collection for optimal large batch processing. This schedule can be repeated two or three times per week.

**Semen evaluation**

The classical tests required to measure the number of sperm collected are volume of the ejaculate of semen and sperm concentration (66). For assessment of the quality of semen, sperm motility and morphology (67-74) are important. Normality of the acrosome and shape of the sperm head can be evaluated (74, 75). Induction of the acrosome reaction also is correlated with fertility (76, 77). With flow cytometry (78-80) one can detect organelle function and damage, and monitor the number of sperm per straw (81a). Semen pH and sperm metabolism have been used to monitor semen quality (9, 14, 39, 74, 81b-83), but are not used routinely. Seminal plasma also may contain components affecting fertility (84, 85), but these are not tested for routinely.

We have decreased the error of measuring sperm volume to less than 1% by weighing each ejaculate, thus avoiding errors in visual estimation due to bubbles and parallax (86). In small ejaculates, errors of visual estimate could be 10%. Sperm concentration, measured on properly taken subsamples with a cell counter or by spectrophotometry, is determined quickly and accurately.

Computer assisted sperm analysis (CASA) is effective in measuring several parameters on many sperm (87), and these multiple criteria obtained on both fresh and frozen sperm appear to be highly correlated with fertility (88-91). By using the Hoechst stain 33342 it is possible to measure sperm motion characteristics in extenders containing many particles such as whole milk (27, 91).

<table>
<thead>
<tr>
<th>Number of breedings</th>
<th>Range in fertility estimates (2 s.d.) when true % is:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>25</td>
<td>30-70(^a)</td>
</tr>
<tr>
<td>50</td>
<td>36-64</td>
</tr>
<tr>
<td>75</td>
<td>38-62</td>
</tr>
<tr>
<td>100</td>
<td>40-60</td>
</tr>
<tr>
<td>300</td>
<td>44-56</td>
</tr>
<tr>
<td>500</td>
<td>45-55</td>
</tr>
<tr>
<td>1,000</td>
<td>47-53</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation (s.d.) = \sqrt{pq/n}

where p = % pregnant; q = 100-p %

n = number of breedings.

\(^b\)The range of ± 2 standard deviations normally includes about 95% of the samples.
A hypoosmotic swelling (HOS) test has received widespread attention in human fertility clinics (92, 93). This test is based upon a large breeding experiment by Bredderman and Foote (94), who concluded that "the swelling of spermatozoa may be of greater value than their initial size as a predictor of fertility". The test has recently been used again with bull semen (88). Accessory sperm attached to eggs may provide another basis for judging semen quality (95). Freezing semen decreases the number of sperm arriving at and attaching to the egg.

Most correlation coefficients between estimates of semen quality and fertility are low, accounting for less than 20% of the covariation (27). This low relationship usually is interpreted as being due to the lack of a substantial biological relationship between the characteristic of semen quality measured and fertility. However, this may not be correct, as the true biological relationship will be underestimated considerably when there are large sampling errors in both estimating semen quality and measuring fertility. This often is true. For example, the correlation between sperm motility and fertility was increased from about 0.3 to 0.64, simply by making several independent estimates of motility, instead of individual estimates, and correlating the average with fertility of the semen used to inseminate a large number of cows (86). Thousands of inseminations are required to minimize the large nonbiological binomial variation associated with a cow being pregnant or not (0 versus 1; table 3). True fertility of a bull with an estimated 50% conception rate, based on 100 inseminations, is usually between 40 and 60%.

**Sperm evaluated by competitive fertilization and in vitro fertilization**

The number of inseminations required to achieve a prescribed precision in estimating fertility can be reduced by using competitive fertilization (96, 97). Sperm from two or more males are mixed in equal numbers and the proportion of oocytes fertilized and/or progeny produced is recorded. Markers, such as coat color, are required to identify the sire of progeny (98, 99). However, commercial trials to produce progeny with mixed sperm seldom are feasible, but *in vitro* fertilization (IVF) tests with colored sperm can be used (27).

The early IVF tests to estimate bull fertility, in comparison with semen characteristics, were performed with zona-free hamster oocytes (27). These IVF studies were greatly simplified by the work of Lu et al. (100), and Gordon and Lu (101). Literally millions of oocytes from slaughterhouse cattle have been used in sperm and embryo studies in cattle (102).

Fertilization depends on sperm being capacitated, a phenomenon that has been a partial mystery since first reported by Austin (103) and Chang (104). Capacitation is promoted by reagents that induce a calcium flux (102, 105) or lower the cholesterol content of sperm (106-108). *In vitro* co-culture systems have been developed (109, 110) to study sperm capacitation and other interactions within the oviduct (111, 112). It would seem possible to pretreat sperm and prepare mixtures that contain sperm conditioned to fertilize oocytes immediately following insemination, and others with a delayed reaction, thus increasing the period over which oocytes could be fertilized. Freezing sperm tends to capacitate them (113, 114), and this may influence the optimal time of insemination. Seminal plasma can delay capacitation (115).

**Development of extenders for liquid and frozen semen**

The extenders used for preserving sperm have changed little in recent years. After Phillips (116) and Phillips and Lardy (117) reported on the use of egg yolk-
phosphate, Salisbury and coworkers (118) published a report on a citrate-buffered egg yolk (50% egg yolk by volume). This greatly improved optical clarity of the medium so that sperm could be examined more critically with the aid of a microscope. This extender was widely used, along with whole milk (119-122). High fertility with liquid semen was achieved with both a Cornell University Extender (CUE; 123) and a Tris-buffered egg yolk-glycerol extender (124, 125). The CUE extender contained a self-carbonating system. Shannon and coworkers (126) modified this system with caproic acid to produce “Caprogen”, the semen extender used for the highly successful liquid semen program in New Zealand.

All of the extenders used for cattle AI contained various antibiotics (127, 128), following the publication by Almquist and coworkers (129) and Foote and Bratton (130, 131) of the dramatic effect on bacterial control in semen and an increase in fertility. A 7% boost in fertility, with antibiotics, combined with the 7% increase due to adding extender to semen before cooling (132) gave AI a major advantage over natural service, where venereal disease, particularly Vibriosis, was responsible for a high incidence of embryonic mortality.

The bacterial control exerted by antibiotics and sulfanilamide made possible the preservation of sperm at ambient temperatures without extensive bacterial growth, particularly if catalase was added and exposure to oxygen was minimal (14, 27, 133). The best motility at ambient temperature was maintained when the egg yolk content was reduced from 20% by volume to 1 to 5% (14). Shannon et al. (126) obtained good fertility with unfrozen semen by adding catalase to “Caprogen” with 5% egg yolk.

The discovery by Polge et al. (134) of the cryoprotective effect of glycerol revolutionized the processing of semen in both egg yolk-citrate (118) and milk extenders (121, 122, 135). The Tris-buffered egg yolk-glycerol (EYTG) extender was equally effective for preserving liquid and frozen semen (124, 125). Iritani (42) reported that EYTG was the most commonly used extender for cryopreservation of bull sperm worldwide. The egg yolk content of 20 to 25% by volume is widely used for cryopreservation, but this concentration can be reduced without loss of sperm motility or fertility (136).

In addition to the foregoing extenders, Nagase and Graham (137) tested many high lactose or raffinose mixtures (11-14%), with 20% egg yolk and a low concentration of glycerol (about 4.7%) that protected sperm frozen as pellets on solid carbon dioxide. Addition of detergents to reduce osmotic effects of freezing improved motility, but not fertility (138). However, detergents may be useful in some extenders (139).

Attempts to produce a completely synthetic medium for freezing bull sperm containing a balance of salts, disaccharides, lipoproteins, cryoprotectants and other agents, have failed to develop any as successful as those containing natural products such as egg yolk or milk. Addition of the agents, proline and betaine glycine, trehalose, glutathione and others, have not improved fertility (Foote, unpublished, 1980, and 27, 86, 140, 141). However, Amann et al. (142) reported preliminary promising results with a synthetic peptide. So the search continues for the perfect freezing cocktail.

Processing liquid and frozen semen

During processing, sperm should not be exposed to bright light (143). The importance of slow cooling of semen to 5°C in extender to protect against coldshock is well-known (9, 14, 27, 82). Likewise, the addition of glycerol stepwise, by the drop method or in one step, and different freezing rates have been thoroughly reviewed (14, 42, 144, 145). Optimal cooling rates vary with media and packaging but often are about
15°C per minute from +5°C to −100°C before transferring the semen to liquid nitrogen (14, 82, 146, 147). Also, a simple two-step procedure gives good protection during freezing (146). Interactions among extenders, cooling and thawing rates occur (148), so the whole processing procedure, as a unit, is important. Most semen is packaged in 0.25 ml or 0.50 ml straws (149), replacing glass ampules used originally. In 1996 the IMV Company produced 72 million 0.5 ml and 162 million 0.25 ml straws (B. Cassou, personal communication).

Sperm packaged in 0.25 ml and 0.5 ml straws give similar fertility results (150), although freezing in the 0.25 ml straws may result in less freeze-thaw damage to sperm (151). Straws should be maintained continuously at −196°C in tanks well-filled with liquid nitrogen. At this temperature the sperm remain highly fertile for many years (14, 27, 41).

Because of potential damage in routine cryopreservation due to ice crystal formation, freezing in the glassy state (vitrification), with high concentrations of cryoprotectants and extremely rapid cooling, has been reported for sperm from several species. However, bull sperm may not suffer much damage due to ice crystals during conventional freezing, as these cells are highly permeable (152), and seeding to minimize supercooling has little effect (144). No successful vitrification procedure for cryopreservation of bull sperm has been reported.

Thawing of bull sperm can be done at various rates in air, water and in the inside jacket pocket. Generally, thawing should be rapid, but the final temperature of the thawed semen should not exceed about 38°C. For semen thawed in air, the rate of air movement as well as temperature greatly affects thawing rate (Foote, unpublished).

Freeze-dried sperm
There have been many attempts to freeze-dry sperm, but early attempts were not successful. However, the possibility of applying this procedure successfully was made possible by microinjection of sperm (153).

Sperm numbers per breeding unit
One of the great advantages of AI is the ability to achieve a pregnancy with a few million sperm per cow, thus permitting the billions of sperm in a normal ejaculate of bull semen to be extended greatly (81, 83, 154-156). Often the sperm are packaged with 20 x 10^6 total sperm per straw, but reducing the number to 10 x 10^6 total sperm (about 4 to 5 million motile) only decreases fertility by 1 to 2% (157). Consequently, using 10 x 10^6 sperm nearly doubles the potential number of progeny possible per genetically superior sire. Obviously it is important to transfer most of the sperm packaged per breeding unit to the cow by careful insemination. Jondet (158) obtained 28% and 21% 60-90-day nonreturns when as few as 50,000 and 25,000 motile sperm were inseminated.

With liquid semen as few as about 2 x 10^6 total sperm are used routinely for insemination in New Zealand (126, 159). Fertility declines as sperm numbers are reduced further. The minimal number of sperm required for high fertility depends on the quality of semen, the extender used, skill of the inseminator and condition of the cow.

Insemination procedures
Major changes occurred in earlier years with the development in Denmark of the rectovaginal method of insemination, the change from glass to plastic catheters (86),
liquid to frozen semen, and to stainless steel guns for insemination with straws. Unpublished trials by Bratton and Foote, conducted with liquid semen in 1950 (86), resulted in no difference between inseminating equal numbers of sperm with special devices delivering the sperm in 1.0 ml versus 0.23 ml of extended semen. Attempts to encapsulate sperm with collagen (27) for prolonged release failed because the capsules were expelled from the uterus. More recently Nebel et al. (160) and Vishwanath et al. (161) used microencapsulated sperm for AI of cattle with promising results. Retraining of the inseminators (162), particularly as this relates to the actual site of semen deposition and sperm numbers inseminated (163) may improve fertility. However, with normal numbers of sperm per straw there appears to be no difference in fertility between semen placed in the body or horns of the uterus (164). When fewer than 1,000,000 sperm are inseminated into the uterus, decreases in fertility occur (158, 165).

Timing of insemination during estrus to optimize pregnancy rates continues to be investigated (166-168). The “A. M. to P. M. and P. M. to A. M.” rule established by Trimberger (169) has served as an excellent practical guide, with insemination 5 to 14 hours after the cow first stands giving the highest conception rate (170). Many herd managers, doing their own insemination, inseminate some problem cows twice during detected estrus. Timing with frozen semen may be more important than with fresh semen because cryopreservation may cause sperm capacitation, with a reduced sperm survival time. Aids for identifying cows in estrus (170, 171) may assist in reducing the time required for visual observation. One of the least expensive aids is chalking the rump area of cows a few days before the time they should be inseminated. Methods allowing for fixed time of insemination may be economic under selected circumstances (172).

Measurement of fertility by nonreturn rates

From the beginning of AI it was apparent that fertility estimates could not be obtained routinely from the field by pregnancy diagnosis per rectum. The nonreturn method of assessing the proportion of cows that returned for reinsemination was developed by Thompson and Salisbury (173) at Cornell University, and officially approved in 1947. This method proved to be a convenient and reliable way of monitoring fertility (174, 175), including calves born (176), but nonreturn rate is higher than the actual conception rate. Fertility associated with bulls, inseminators, extenders, laboratory and field procedures, all could be compared, providing invaluable information for researchers and AI managers.

Fertility studies must be carefully designed (177, 178) so that large bull differences, insemination of cows versus heifers, cows in DHI herds and other factors affecting fertility are accounted for (90, 176). For example, most heifers are inseminated with semen from bulls that produce small calves and the nonreturn rate of heifers is about 9% higher than for cows. Thus, bulls producing small calves tend to have high fertility. Procedures for correcting nonreturn rates for several extraneous effects have been published (179-181). Fertility has been measured by IVF as described previously. Other laboratory tests of fertility become increasingly important as less feedback by inseminators occurs with direct sales of semen.

The technology for semen collection and processing for beef cattle is the same as for dairy cattle, but management of cows and selection of sires is much different. Special facilities and programs to detect cows in estrus or to synchronize those to be inseminated, along with associated confinement chutes, are necessary. For additional information on dairy and beef cattle see appendix table I.
Modern development of AI in other domestic species

Much of the research with bull sperm has found application in other species and the principles of sperm handling are similar. Procedures for different species have been reviewed (1, 9, 13, 14, 182, 183), and discussed at international conferences on AI (12, 40, 42, 184). Only a few highlights for each species will be included here.

Sheep and goats

The simplest method of collecting semen is to train males to ejaculate into an artificial vagina (183), although rams and bucks that do not respond to mounts or have not been trained to use the artificial vagina, can be electroejaculated (35, 36, 183). Major problems with AI of small ruminants are the cost per animal, and the difficulty of intrauterine insemination.

Semen evaluation is done essentially as described for bulls (5, 183). Also, the swirling of undiluted semen, due to the high concentration of sperm (up to $6 \times 10^9$ sperm/ml), is easily seen when highly motile sperm are viewed microscopically.

With the high concentration of sperm in semen only a small volume is needed to provide about $300 \times 10^6$ sperm for vaginal insemination, or $10 \times 10^6$ sperm in fresh semen or frozen-thawed sperm with intrauterine insemination. Various simple diluents, or an egg yolk-Tris-fructose extender, developed originally at Cornell for bull semen (124, 125), and heated milk or egg yolk-sugar extenders (137), are commonly used for freezing ram sperm in straws or pellets (183, 185).

Frozen ram sperm remain fertile after storage for many years (186). Frozen semen potentially has a great advantage in distributing the genetics of superior rams in the field, but vaginal or intracervical insemination of frozen semen, even with large doses of sperm, results in poor fertility. Apparently, transport through the cervix is limited and slow, and sperm survival with possible shorter capacitation times following freezing (187), could alter the optimal timing of insemination.

Obviously, a method of extending, freezing and thawing sperm that would yield high fertility with deep intra-cervical insemination is desirable. Researchers have modified the egg yolk-Tris extender (188), added various antioxidants (189), proline and betaine glycine (152), and have packaged and frozen and thawed ram sperm differently (185, 190). None of the procedures have completely nullified the damage due to freezing.

At the present time, the most practical method of inseminating frozen ram semen is intrauterine placement, aided by laparoscopy. More than 500,000 ewes in Australia are inseminated this way (187). Large groups of ewes with estrus synchronized with fluorogesterone acetate (FGA), or medroxy-progesterone acetate (MAP) can be inseminated efficiently (187).

Researchers continue to find several causes of membrane damage to sperm during freezing (152, 185-187). These include partitioning of mitochondrial and plasma membrane damage, using such stains as Rhodamine 123 for mitochondria (191), and SYBR-14 (80), and propidium iodide (PI) for plasma membrane integrity tests (187). Further research is needed, as theories of optimal methods of freezing sperm do not always apply in practice (192).

Under field conditions, it is usually not possible to obtain fertility information on individual rams. Sperm penetration following heterospermic insemination of zona-free hamster eggs has been reported to correlate highly ($R^2 = 0.69$) with in vivo fertility (193). Additional information on AI is given in appendix table II.
Other aspects of ram sperm used in conjunction with sexing, superovulation, embryo culture and transfer have been summarized elsewhere (102, 194). Efforts to improve the superovulatory response of ewes or to simplify the hormonal injection schedule continue (195).

Cloning has become a technique with powerful agricultural and medical potentials utilizing sheep and other farm animals (24). This was particularly stimulated by the report of the cloning of Dolly from an adult somatic mammary gland cell (196). The earlier successes of developing AI, leading to embryo production, embryo culture and transfer greatly facilitated the experiments with cloning.

Goat artificial insemination

Goat artificial insemination techniques are similar to sheep, but goat sperm survive freezing better than ram sperm (182, 183). However, there is an enzyme in the seminal plasma that can coagulate the semen preserved in 20% egg yolk (29, 42, 183), but it is not a problem if the egg yolk concentration is reduced to about 2.5% (vol/vol). Others have used up to 20% egg yolk with the egg yolk-Tris extender (197), and did not report any complications, possibly due to breed differences. The compound in seminal plasma responsible for coagulation may be the same lipase reported by Pellicer-Rubio et al. (198) to cause poor goat sperm survival in milk extenders.

Various researchers have studied procedures to optimize survival of frozen-thawed buck sperm (183). Deka and Rao (199) recommended 6.4% glycerol in the egg yolk-Tris extender and a 5-hour equilibration time before freezing. Tuli and Holtz (197) found the standard egg yolk-Tris to be superior to the use of other zwitterion-buffered egg yolk extenders. Singh et al. (200) found that lactose added to the egg yolk-Tris extender decreased enzyme loss during semen processing. Both yolk-based and milk-based extenders can be used to fertilize goat oocytes in vitro (201).

Insemination of does should be done by skillfully placing a small volume of concentrated sperm through the cervix into the uterus. This is facilitated by elevating the hind quarters and using a lighted speculum to view the cervix. Otherwise fertility is lower with frozen sperm and a greater number of fresh sperm are required with vaginal deposition.

One of the problems with goat AI is the lack of fertility information in the field to relate to semen quality, and evaluate buck fertility. As a laboratory test Berger et al. (202) found that fusion of buck sperm with zona-free hamster oocytes was highly correlated with fertility in vivo ($R^2 = 0.78$), whereas CASA and acrosomal morphology were not highly correlated with fertility. We have found (Foote, unpublished, 1980) that high progesterone in goat milk samples taken 20 to 21 days after insemination is a reliable indicator of pregnancy and could be used in field studies. Additional information is given in appendix table II on AI.

Batt et al. (203), Gordon (102) and Amoah and Gelaye (204) have summarized other reproductive technologies with goats. They include IVF, embryo transfer and production of transgenic goats. As good milk producers, transgenic goats are useful producers of pharmaceutical products in their milk (205, 206).

Swine

Boars have large testes and a relatively short spermatogenic cycle. Therefore, they are the largest sperm producers among farm animals. Collection of the ejaculate of
boar semen in fractions, by the gloved or bare hand or artificial vagina, has been described in numerous texts. Although some semen can be obtained by electroejaculation (36) it causes considerable stress on boars (Foote, unpublished) and the training of boars to mount a dummy is simple and effective.

Semen evaluation assessed by sperm concentration, sperm motility, acrosomal integrity, live-dead staining and the use of fluorescent markers to evaluate membrane damage and integrity of the sperm DNA have been described (207-209). Juonala et al. (210) conducted an extensive evaluation of boar semen and found that total motility measured by CASA after 7 days of storage at 16-20°C was the most highly correlated variable with litter size, $r = 0.26$.

An early objective in preserving boar sperm, was to provide long time preservation through cryopreservation, as was accomplished with bull sperm (42). Many extenders were developed (207, 211). These were mainly of two types. One type was composed of buffered egg yolk, similar to the egg yolk-Tris described for bull and ram sperm, but with EDTA added, or the Beltsville BF5 extender. The other type of frozen semen extender contained no buffer, and was composed of egg yolk with a sugar such as lactose for sperm frozen in pellets. Glycerol was the most effective cryoprotectant, with 2-3% usually considered to be optimum.

Numerous attempts have been made to overcome damage during freezing and thawing and to evaluate freezing effects with a variety of criteria (152, 212-214). These include studies of the interaction of thawing rate with freezing rate and glycerol concentration (215), studies of osmotic properties of boar sperm (216), addition of detergents and antioxidants (207, 217), and surgical insemination into the uterus (209). Low concentrations of glycerol are used because high concentrations are toxic to boar sperm. Perhaps post-thaw dialysis to remove glycerol would be beneficial to fertility and reduce polyspermy. Post-thaw motility of sperm is satisfactory. However, none of the procedures result in fertility as high as is obtained with unfrozen semen, and litter size is smaller. Consequently, frozen boar semen is primarily limited to its use for special matings and when distribution is worldwide.

Swine AI has grown rapidly with the acceptance of AI by breed organizations and the successful use of unfrozen semen extended and maintained at about 18°C, although it can be stored at temperatures as low as 12°C (218). In 1957 we inseminated 81 purebred gilts and sows (219) and the herd owner planned to have the “first sale in the USA of hogs produced by AI”, but the breed organization would not allow it. Both mental attitudes and technical progress have improved.

It is estimated that 27% of the world’s 71 x 10^6 sows are inseminated by AI. The development of rapid transportation has made boar sperm available for use within 24 hours of collection in most places of high pig density. Semen is usually extended to about 3 x 10^9 up to 6 x 10^9 total sperm per insemination dose in a variety of semen extenders that maintain viable sperm for at least one week and good fertility for several days (appendix table III). The extenders used include those with registered trademarks as “Sperm-Aid”, “Modena”, “MR-A”, “Androhep”, “X-CELL” and “BTS”. The BTS is used for short time storage, and “Androhep” and several others as X-CELL can be used with semen held for more than 3 days at 18°C. By obtaining freshly collected semen at least twice per week from boars on swine farms and/or from AI stations most weekly semen needs can be met. Most of the large swine operators do their own inseminating. The farrowing rate exceeds 80% with 10 to 12 piglets per litter.

Optimal times to inseminate relative to ovulation have been studied by monitoring ovarian function ultrasonically. Nissen et al. (220) recommended
inseminating sows with stored liquid semen from 28 hours before up to 4 hours after ovulation. Waberski et al. (221) recommended insemination of gilts 12 hours before up to ovulation time with fresh sperm, and from 4 hours before up to ovulation time with frozen-thawed sperm. Freezing probably reduces capacitation time. More information on boar semen and AI is in table III.

Boar sperm also can be sexed, but the rate of sexing sperm is too slow for commercial application. Development of several reproductive technologies in swine have received little attention due to the natural synchronization at the post-weaning estrus of sows and high fecundity at this time.

Horses

The application of AI for controlling genital diseases and increasing the availability of semen from selected stud horses has been limited by breed organizations opposed to AI and by the lower fertility of frozen semen, but AI of mares has increased in recent years as more breed associations approve the use of AI. Techniques of semen collection and evaluation have been described (9, 222) and reviewed recently by Malmgren (223).

Fresh stallion semen is evaluated similar to procedures used for other species (62, 224, 225). Jasko et al. (226) reported a correlation of 0.63 between CASA of the percentage of motile sperm and fertility. Love and Kenney (227) evaluated sperm DNA denaturation and fertility. Vidament et al. (228) conducted many tests, including CASA before and after freezing stallion sperm, but fertility was not reported. Coculture with oviductal epithelium (229) is being utilized to mimic sperm behavior in vivo, as is done for several other species.

The semen should be centrifuged to remove seminal plasma and concentrate the sperm. Unfrozen semen can be preserved in a variety of extenders (230). One of the more common ones is the Kenney extender (231) consisting of 2.4 g of dried skim milk, 4.9 g of glucose, 92 ml of sterile distilled water, 2 ml of 7.5% sodium bicarbonate and 2 ml of gentamycin sulfate. Padilla et al. (232) modified this extender to include more potassium which prolonged sperm preservation and maintained high egg penetrating ability in vitro for up to 72 hours.

Semen should be cooled slowly to about 5°C for storage and shipment for AI (233). Consequently, insulated containers should be used to maintain temperature, and semen stored at 5°C can be used for 2 days with good fertility (230).

For frozen semen one of the widely used extenders proposed by Martin (see 230) has been modified with various ratios of egg yolk, milk and lactose. Vidament et al. (234) added 2% egg yolk and 2.5% glycerol to INRA-82, which contains UHT sterilized skim milk, glucose, raffinose, lactose, sodium and potassium citrate and antibiotics (235). Braun et al. (236) examined several combinations of egg yolk, skim milk, and BSA added to the Martin extender. Skim milk improved the percentage of motile sperm and sperm with intact plasma membranes after freezing and thawing. Liposomes composed of phosphatidylserine and cholesterol had minor effects on survival of cryopreserved stallion sperm (237).

It is clear from the foregoing that stallion sperm are exposed to stress by centrifuging to reduce seminal plasma and concentrate the sperm, by cooling and by freezing and thawing. Blach et al. (238) reported that the percentages of motile sperm in fresh, centrifuged, and frozen-thawed stallion semen were 67, 62 and 37%, respectively.
Insemination doses of sperm and the optimal time of insemination vary with different systems (239). For single inseminations the best fertility was obtained using up to \(400 \times 10^6\) motile sperm 0-24 hours before ovulation (230, 240, 241). When mares were inseminated with \(150 \times 10^6\) or \(300 \times 10^6\) sperm, two inseminations per estrus resulted in 34% foaling versus 26% following one insemination (234), but Shore et al. (242) found no difference in pregnancy rate between one and two inseminations on consecutive days.

Samper and Morris (243) surveyed stallion semen cryopreservation in 14 Countries. A majority of respondents froze stallion sperm after centrifugation in Martin’s extender or modifications of it, with at least \(700 \times 10^6\) sperm per insemination dose, and performed 1 or 2 inseminations within 12 hours before ovulation. Most respondents expected at least a 35% first cycle pregnancy rate. See appendix table IV for additional information.

Stallion sperm can be sexed. A foal with sexed sperm has been reported (Seidel, personal communication, 1999). Stallion sperm also are used with other reproduction technologies, such as embryo transfer.

**Dogs**

Although the earliest studies on artificial insemination were initiated in the dog by Spallanzani (32), most research on preservation of canine semen for use in AI has occurred in the past 40 years. Detailed studies by Boucher et al. (244) indicated that the simplest and highly effective method of collecting semen from dogs was to apply pulsating pressure to the base of the penis of a dog, with fractionation of the ejaculate into a prewarmed funnel attached to a collection tube. Semen can be collected at 1- to 2-day intervals. Dog sperm in fresh semen can then be evaluated as for sperm from males of other species, including the use of flow cytometry (245) and CASA. Hay et al. (246) tested the oocyte penetration method as a possible way of evaluating fertility of sperm in a species where fertility testing by AI was impractical.

Sperm can be preserved at 5°C by extending with heated whole milk, an egg yolk-citrate-glycine-glucose extender or with egg yolk-Tris-citric acid and glucose or fructose (27, 222, 247). Good fertility is maintained for at least 2 days. The first pups registered by the American Kennel Club resulting from AI are believed to have been produced at Cornell University (Foote and Kirk, unpublished, 1958).

For freezing dog sperm, glycerol is the most commonly used cryoprotectant (248-251), added to the egg yolk-Tris-citric acid-fructose or glucose extender (27, 124, 125), or in an unidentified extender (252). Egg yolk with lactose also has been used for freezing sperm in pellets, but Olar et al. (253) obtained better results with the Tris extender.

Most reported inseminations are from clinics in Sweden, Finland and Norway, using \(150 \times 10^6\) to \(700 \times 10^6\) sperm, with the higher doses used with frozen semen. Prolonged elevation of the hind quarters of the bitch often practiced with intravaginal insemination apparently is not necessary (254). With intrauterine insemination a 74% whelping rate (79/107) and a litter size of 5.5 pups have been achieved on a commercial basis (249). Commercial AI is limited, and fertility results using fresh and frozen canine semen have been summarized recently (252, 255).

Researchers continue to investigate methods of improving canine sperm cryopreservation (256-260). The addition of 0.5% Equex STM (active detergent is sodium dodecylsulfate) to the conventional Tris extender increased the proportion of sperm with an intact plasmalemma, increased the percentage of motile sperm following freezing (257, 261), and supported high fertility following intrauterine insemination.
Peña et al. (262) reported that proline added to the conventional Tris extender improved motility, longevity, and acrosomal integrity of cryopreserved canine sperm.

**Rabbits**

Artificial insemination can be done easily (27), producing a large number of embryos and young (263, 264). Males can be ejaculated daily with 1,000 $x 10^6$ sperm collected weekly. An insemination dose of 1 $x 10^6$ sperm in fresh semen is more than adequate, if it is placed in the anterior vagina close to the cervices. Thus, there is the potential for inseminating 50,000 does per year with unfrozen semen from genetically superior males. This could result in at least 300,000 progeny. In France, one of the few Countries practicing extensive AI of rabbits, 20 $x 10^6$ total sperm are used with a pregnancy rate of 85% on 5,000,000 inseminations.

Sperm from some rabbits do not freeze well. With frozen rabbit semen more sperm are required for high fertility, but 100,000 progeny per buck per year are possible with frozen semen. In Countries where rabbit meat consumption is high, AI of rabbits could play a major role in multiplying the genes of males with high growth rate potential and high fertility.

Semen quality can be estimated as for sperm from other species, including the use of CASA (27). However, special techniques are required for CASA to distinguish dead sperm from the dense granules in rabbit semen (27, 89).

The Tris-yolk extender (124, 125) is commonly used to preserve liquid and frozen rabbit semen (265-267), with DMSO and glycerol added for cryopreservation. Maurer et al. (265) stored frozen semen for one year at −196°C with no loss in fertility when 10 $x 10^6$ motile sperm were used per insemination. Chen et al. (113) obtained higher sperm survival following freezing with the acetamide cryoprotectant (99, 140, 268) than with the Weitze (267) extender. Dalimata and Graham (269) found nonpermeating trehalose and methyl cellulose to be beneficial, as rabbit sperm have a low water permeability. Little research has been done on thawing rates (270).

Insemination time may be more critical with frozen semen as sperm may be partly capacitated, with reduced survival time (99, 266, 271). This difference can be compensated for easily in the rabbit by administering GnRH or LH 5 hours after insemination (5 to 7 hours before ovulation).

Rabbits serve as a model for semen evaluation, capacitation, insemination, superovulation, embryo development, sexing of sperm and cloning (27). They would be a good model to test using sperm mixtures of capacitated, control and encapsulated sperm. Rabbits were the first species in which successful embryo transfer was reported (272).

**Poultry**

Artificial insemination has been employed successfully in propagation of a variety of avian species (247, 273, 274). The following brief discussion is limited to the commercially important chickens and turkeys. For more detailed information and references consult reviews (273-276). The first paper on cryopreservation of poultry sperm was by Shaffner et al. (277).

Many tests of semen quality are the same as those employed to evaluate sperm of other species, including sperm motility, morphology hypo-osmotic swelling and
density gradient penetration (274, 278, 279). Kirby et al. (280) used the competitive fertilization method to efficiently rank fertility of males. An egg binding assay (281) was reported to be highly correlated with fertility of roosters, \( r = 0.83, n = 40 \).

Fresh semen on large poultry farms is used within a few hours of semen collection. The dual purpose of extending the semen is to increase the volume slightly for accurate deposition of the planned number of sperm, and to assist in maintaining high sperm survival for a few hours. Fertility approaches 100% when used within a few hours, if held at 5°C. Many extenders have been tested to provide the proper physiological environment.

Turkeys are produced 100% by artificial insemination with fresh semen, because of the difficulty of natural mating. Toms can be collected three times per week, producing enough sperm per ejaculate to inseminate 20 hens, with best results when hens are inseminated weekly.

Turkey sperm require high oxygen for best survival, while chicken sperm survive either aerobically or anaerobically. Nevertheless, antioxidants in extenders improve fertility of rooster sperm (282), and systems to control the oxygen concentration have been developed (274).

Cryopreservation of poultry semen, particularly for maintaining breeding stocks where records are kept for genetic selection (growth in broilers and turkeys and egg production in chickens) can be useful. However cryopreservation of poultry sperm has given variable results, generally with a considerable reduction in fertility (274-276). Cryoprotectants, such as glycerol are toxic, with reduced sperm survival (283). Dialysis before insemination may improve fertility (284). Dialysis of glycerol also was beneficial in freezing peregrine falcon sperm (285). The nonpermeating methyl cellulose offers some protection (286) during freezing.

Recent developments with sperm cryopreservation are encouraging. Specially designed containers to freeze-thaw and deglycerolate the semen before insemination without cumbersome handling have been reported (287, 288). Hens inseminated three times at 4-day intervals produced 64% fertile eggs over a 10-day period (288). Addition of a synthetic peptide enhances fertility (289), making cryopreserved sperm useful for the broiler industry, where natural mating is becoming more difficult.

AI in non-domestic animals

Mice

Valuable genetic stocks of mice have been preserved inexpensively and protected against disease outbreaks by freezing embryos (290). Cryopreservation of sperm offers many advantages in preserving genetic material, but successful freezing of mouse sperm has been difficult (291, 292). However, sufficient progress has been made with cryoprotectant cocktails and freezing, seeding and vitrification procedures to fertilize oocytes and reproduce valuable genetic strains of mice.

Foxes

Artificial insemination with frozen fox sperm is widely practiced in Northern Europe. Collection and processing of fox semen is similar to dog semen, with frozen semen used almost exclusively for AI. The procedures have been summarized by Farstad (249). When 37.5 to 150 x 10^6 cryopreserved sperm were deposited by transcervical insemination
into the uterus twice, 24 hours apart, 81% of 617 blue fox females whelped (293). When 608 vixens were inseminated once with $150 \times 10^6$ sperm, 75% whelped (249).

**Zoo and other animals**

Information on artificial insemination of many species, ranging from marsupials to primates, has been summarized (247). Novel methods of collecting semen have been used for alpacas (294).

One species of commercial interest is deer. They are farmed, primarily for meat in several Countries. Various assisted reproductive technologies are used, such as electroejaculation of semen, freezing of semen (modified Tris extender), synchronization of estrus and intrauterine insemination with the aid of a laparoscope (295). Approximately 50% of the hinds produce inseminated produced calves (295, 296).

**Endangered species**

Various reproductive technologies for the propagation of many endangered species have been reviewed (297-299). Domestic cat AI (299) has been used as a model for the larger wild members of the cat family. Fertile sperm from animals that have recently died may be removed from the epididymis (300), as we did from a prize bull in 1951 and used the semen successfully in AI to produce progeny (Bratton and Foote, unpublished). In addition to cryopreservation of sperm, sperm have been sexed and used for *in vitro* fertilization, and intracytoplasmic sperm injection (ICSI) followed by cryopreservation of embryos produced.

In small animals, such as the ferret, successful artificial insemination requires depositing the semen past the cervix into the uterine horn. This usually requires anesthesia and laparoscopy (301), with the accompanying hazards of overdose of anesthesia and postoperative adhesions. Recently, Kidder et al. (302) developed successful nonsurgical procedures for intrauterine insemination as well as for embryo collection and transfer (303).

**Humans**

Many children have been born in the past 50 years as a result of using husbands or donor semen to overcome various problems of infertility (304). With the onslaught of AIDS the use of frozen human sperm became the only kind of sperm recommended for AI. Frozen sperm are held frozen for 6 months before use while the donor is checked again for AIDS to be sure that the donor was free from the AIDS virus when semen was collected.

Another use of human sperm is for *in vitro* fertilization (IVF). Sperm for IVF were initially obtained primarily by masturbation. However, with blockage of efferent ducts from the testes, sperm have been aspirated from the epididymides (305) or testis. Only one sperm is needed for ICSI. Such sperm have resulted in pregnancies at a low rate, and a concern is that these problems of infertility may be passed on to the next generation. Sexed sperm have been used, particularly when parents carried sex-linked diseases, or had many children, all of one sex.

**Combining artificial insemination with newer reproductive biotechnologies**

Successful AI provided the base for many of the remarkable reproductive biotechnologies which have followed (102). These are listed in table 4, and all utilize
sperm in some way. They have been combined in special animal breeding programs (21, 22, 306). They are described briefly here to provide an integrated picture of the role of AI as a starting point for many biotechnologies.

**Sexing sperm**

Sexing of sperm so as to produce the desired sex has been the subject of conversations, theories and failed experiments for centuries (41, 307). A successful sperm sexing procedure would produce embryos and young of the desired sex, and thus be more valuable than sexing embryos. A flow cytometer designed specially to produce laminar flow at Livermore Laboratories (308) was reproduced for use at the US Department of Agriculture (194, 309), to separate sperm from many species, based upon the difference in DNA content of the X and the Y chromosomes. While technically a marvelous achievement, the rate of sperm separation has been too slow to make sperm separation useful on a wide scale commercially. However, it may have application under selected circumstances when relatively few sperm are inseminated (165).

The system is useful for *in vitro* fertilization (IVF) where one sperm per oocyte is enough to cause fertilization with sperm microinjection. Further modification to speed the sorting and increase the efficiency, coupled with fewer sperm per insemination, as is currently being tested (165), may make this system commercially feasible for selected use in cattle, horses and some other species, including endangered species. In the latter group production of more females is important.

**Table 4. Ways sperm can be used to increase the impact of superior males or assist reproduction.**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine artificial insemination (AI with fresh or frozen sperm)</td>
<td>Cattle, sheep, swine, horses, goats, dogs, poultry, rabbits and endangered species</td>
</tr>
<tr>
<td>AI combined with estrous cycle regulation</td>
<td>Cattle, sheep, goats, and horses</td>
</tr>
<tr>
<td>Special insemination techniques</td>
<td>Endangered species and small lab animals</td>
</tr>
<tr>
<td>AI combined with superovulation and production of fresh or frozen embryos&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cattle, sheep, goats, swine and rabbits</td>
</tr>
<tr>
<td>AI with sexed sperm</td>
<td>Mostly cattle, but other species experimentally</td>
</tr>
<tr>
<td><em>In vitro</em> fertilization&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mostly cattle, but possible in many species and a major use in humans</td>
</tr>
<tr>
<td>Intracytoplasmic sperm injection&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Possible in many species</td>
</tr>
</tbody>
</table>

<sup>a</sup>Horses cannot be effectively superovulated, but this system has potential, where useful, in most species. Also, embryos can be sexed in different species.

<sup>b</sup>*In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have had widespread application in human infertility clinics, including some use of sexed sperm where genetic problems exist.
Estrous cycle regulation

An essential component of a successful AI program obviously is to inseminate the female at the appropriate time of the estrous cycle to obtain optimal fertility. Many aids to assist farm managers in detecting estrus and arranging for insemination at the optimal time have been developed. Simultaneously, methods to regulate estrus (310, 311) in cattle and other species have been widely investigated. The early research was based primarily on the use of progestagens and estrogen.

The identification of important natural compounds, such as prostaglandin F$_2\alpha$ and GnRH, by physiologists and biochemists, followed by large scale production by pharmaceutical companies, has revolutionized many aspects of reproductive management and treatment of reproductive problems by veterinarians and farm managers. These compounds have been helpful in developing current regimens to control ovulation (312).

Superovulation

Methods to obtain a large number of fertilizable oocytes (313-318) have been investigated intensively. The most single striking characteristic is the variability in response among animals to gonadotropin treatment. In addition, more sperm per insemination of superovulated cows are required for high fertility than for untreated cows (75, 319). However, millions of progeny have been born following superovulation, insemination and embryo transfer in cattle.

Calves can be superovulated (320), as one approach to improve progeny testing programs in AI. The first calf born following transfer of an embryo collected from a live calf at 4 months of age and transferred to a mature recipient was by Seidel et al. in 1971. Insemination of calves and collection of embryos from the infantile reproductive tract of calves is difficult, and the procedure has been superseded by oocyte collection (321) and IVF.

Embryo transfer

The development of nonsurgical embryo transfer in cattle (23, 326, 317, 320, 322-324) to increase the genetic impact of superior females and to facilitate introduction of complete genotypes into various areas of the world benefitted from AI and the regulation of estrus. The latter permitted the donors and the desired number of recipients of embryos to have their estrous cycles synchronized, so important for a successful embryo transfer program. This reduced the size of the recipient herd needed. Also, if a group of donor cows within a herd was synchronized, they could all be efficiently inseminated at one time and embryos collected later at one time. Synchronization is especially useful in beef cattle to minimize labor costs of handling cattle under range conditions.

Embryo freezing

The successful freezing of bovine embryos has greatly extended the usefulness of the embryo transfer technology (316, 325, 326). Embryos can be collected at one time and place and used as needed with the exact number of recipients required at another time and place worldwide. Glycerol, the same cryoprotectant used for cryopreservation of bull sperm (134) is effective for freezing embryos. Sires produced by this procedure and eventually selected for AI have a huge multiplier effect on dissemination of genes from both parents.
Embryo splitting

This is a relatively simple technique (327) that can increase the number of potential embryos for transfer (19), with only a minor reduction in fertility. Pregnancy rate is reduced about 10% by splitting into two halves.

Sexing of embryos

Sexing (328-330) is carried out commercially using Y chromosome specific probes. Only one blastomere is required to obtain DNA, and then amplify it with the polymerase chain reaction. The accuracy of sexing cattle embryos approaches 100%. The combination of superovulation, sexing and freezing embryos with or without splitting is an excellent way for AI organizations to produce a planned number of young bulls to progeny test through the program called MOET.

Embryos made in the lab by IVF from slaughtered cattle

The IVF of oocytes obtained from ovaries of slaughtered cattle has resulted in making essentially millions of embryos from oocytes available inexpensively (102, 323). The oocytes are fertilized by sperm capacitated with calcium ionophore A23187 (331) or with heparin (105). This system permits testing fertilizing ability of sperm, oocyte maturation, and the study of early sperm-oocyte interactions. Microtechniques permit injection of individual sperm (including sexed sperm) into an oocyte (332, 333). The IntraCytoplasmic Sperm Injection (ICSI) produces a zygote by artificial fertilization as distinguished from artificial insemination. Sperm from males that otherwise are infertile can fertilize an oocyte by ICSI (334), so this technique should be used with caution in performing IVF, especially with human patients.

The IVF of oocytes from live calves and cows

After Lu et al. (100) demonstrated that viable embryos could be produced from ovarian oocytes collected from slaughtered cattle, a procedure predicted by Rowson (20), a technique was developed by Pieterse et al. (335) and others to aspirate oocytes repeatedly from mature cows at any stage of the estrous cycle and pregnancy. These oocytes can be matured, fertilized in vitro and cultured into blastocysts, often with coculture (336), and result in pregnancies (316, 337). Thus, cows that do not respond to superovulation can be included in this type of artificial reproduction program.

Calves also can be used as oocyte donors to obtain fertilizable “eggs” that will develop (321, 338), as demonstrated years ago in vivo (339). This program of reproduction to produce sires for AI can reduce calving intervals, increase the number of progeny, and increase the rate of genetic progress over that obtained with the conventional progeny testing programs (17, 22, 306).

Culturing of embryos

Culture of embryos is a vital step to transform them from a zygote or a one-cell product of nuclear transfer into a blastocyst for embryo transfer (102). Great advances have been made in culturing embryos (340-342). The ability to culture laboratory-made embryos, such as cloned embryos produced by culturing enucleated oocytes, each
injected with a single blastomere, (343-345), or from embryonic stem cells, fetal cells or adult somatic cells (25, 196), is essential for transgenic animal production.

Transgenic animals and cloning

Transgenic animals were originally produced mostly by microinjection of gene constructs into the zygote (205). Sperm may also be used as a vector (346-348). However, with the cloning of somatic cells (349), such as fibroblasts (350), including those transfected with various genes, the initial reproductive process of sperm cell fusing with an oocyte is bypassed. The cloning field offers dramatic potential for improving animal agriculture and especially human health (351).

Summary of achievements

Artificial breeding has been the most powerful tool for widespread improvement of livestock. Techniques have been developed to harvest the maximal number of sperm, and to evaluate, process, and preserve the sperm so as to inseminate the minimal number of sperm consistent with high fertility. Venereal diseases have been eliminated where AI is used. The development of genetic evaluation programs, and the combination of AI with various other reproductive biotechnologies has been researched and monitored with great care. They have been responsible for bringing about the greatest advance in animal propagation and improvement in the history of mankind. Yes, the demonstrated low cost, highly effective and safe AI has paved the way in the laboratory and in the field for acceptance by the public of other reproductive biotechnologies that followed.

Current statistics

Following are appendix tables I-IV on the current status of AI in the world. Extensive detailed reports have appeared previously (4, 12, 29, 40, 42, 184, 352).

Acknowledgments


References


freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology, 43: 141-152.


## Appendix

**Table I. Summary of cattle AI statistics with frozen semen from different Countries.**

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of inseminations(^a)</th>
<th>Frozen (%)(^b)</th>
<th>Number of sperm inseminated</th>
<th>Fertility (%)</th>
<th>Non-return (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>915,490</td>
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<td>25 x 10^6</td>
<td>72.3</td>
<td>59</td>
</tr>
<tr>
<td>Australia</td>
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<td>60</td>
<td>---</td>
</tr>
<tr>
<td>Belgium</td>
<td>581,000</td>
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<td>12-15 x 10^6</td>
<td>71</td>
<td>56</td>
</tr>
<tr>
<td>Brazil</td>
<td>2,861,852</td>
<td>100</td>
<td>40 x 10^6</td>
<td>60-70</td>
<td>---</td>
</tr>
<tr>
<td>Canada</td>
<td>1,500,000</td>
<td>100</td>
<td>15 x 10^6</td>
<td>68</td>
<td>60-90</td>
</tr>
<tr>
<td>China</td>
<td>10,000,000</td>
<td>100</td>
<td>---</td>
<td>70</td>
<td>---</td>
</tr>
<tr>
<td>Denmark</td>
<td>787,848</td>
<td>100</td>
<td>15 x 10^6</td>
<td>67.8</td>
<td>56</td>
</tr>
<tr>
<td>Finland</td>
<td>462,852</td>
<td>100</td>
<td>20 x 10^6</td>
<td>63.8</td>
<td>60</td>
</tr>
<tr>
<td>France</td>
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<td>20 x 10^6</td>
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<td>48</td>
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<tr>
<td>Germany</td>
<td>5,577,981</td>
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<td>10-20 x 10^6</td>
<td>64.6</td>
<td>60-90</td>
</tr>
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<td>Hungary</td>
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<td>100</td>
<td>20-25 x 10^6</td>
<td>50</td>
<td>Pregnant</td>
</tr>
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<td>100</td>
<td>20 x 10^6</td>
<td>55</td>
<td>59</td>
</tr>
<tr>
<td>Italy</td>
<td>2,450,000</td>
<td>100</td>
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<td>63.8</td>
<td>59</td>
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<td>58</td>
<td>60-90</td>
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<td>Korea</td>
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<td>35 x 10^6</td>
<td>40</td>
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<td>Netherlands</td>
<td>1,659,496</td>
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<td>Varies by bull</td>
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<tr>
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<td>69</td>
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<td>Norway</td>
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<td>18 x 10^6</td>
<td>70.9</td>
<td>60-90</td>
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<td>20 x 10^6</td>
<td>70</td>
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</tr>
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<td>30 x 10^6</td>
<td>65-70</td>
<td>59</td>
</tr>
<tr>
<td>Sweden</td>
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<td>100</td>
<td>15 x 10^6</td>
<td>68</td>
<td>56</td>
</tr>
<tr>
<td>Switzerland</td>
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<td>100</td>
<td>20 x 10^6</td>
<td>68.8</td>
<td>75</td>
</tr>
<tr>
<td>USA</td>
<td>10,466,000</td>
<td>100</td>
<td>10-30 x 10^6</td>
<td>68</td>
<td>59</td>
</tr>
</tbody>
</table>

\(^a\)Data are based primarily on dairy cattle. In Italy 1,500 buffaloes are inseminated with fresh semen in EYT.

\(^b\)Most semen was frozen in egg yolk-Tris, with yolk-citrate milk, Biociphos, and Trilady also used. Frozen-thawed sperm were reported to be 45-60% motile.

\(^c\)New Zealand uses 1 - to 2 x 10^6 total sperm for liquid semen in Caprogen® during their restricted breeding season.
Table II. Summary of sheep and goat AI statistics from different Countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of inseminations</th>
<th>Fresh semen (%)</th>
<th>Semen extenders</th>
<th>Number of sperm inseminated</th>
<th>Motile (%)</th>
<th>Fertility (%)</th>
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<tbody>
<tr>
<td><strong>Sheep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>200,000</td>
<td>80</td>
<td>Egg yolk-Tris</td>
<td>30 x 10^6</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>France</td>
<td>832,000</td>
<td>89</td>
<td>Skimmilk, Lactose-EY</td>
<td>300 x 10^6</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Hungary</td>
<td>10,000</td>
<td>95</td>
<td>Skimmilk, biosyn</td>
<td>80 x 10^6</td>
<td>60-70</td>
<td>72</td>
</tr>
<tr>
<td>Italy</td>
<td>105,000</td>
<td>97</td>
<td>Modified Tris</td>
<td>62 x 10^6</td>
<td>---</td>
<td>50</td>
</tr>
<tr>
<td>Israel</td>
<td>50,000</td>
<td>---</td>
<td>Skimmilk</td>
<td>400 x 10^6</td>
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<td>---</td>
</tr>
<tr>
<td>Norway</td>
<td>9,175</td>
<td>79</td>
<td>Skimmilk; SM-EY-Gly</td>
<td>150 x 10^6</td>
<td>70</td>
<td>66</td>
</tr>
<tr>
<td>Poland</td>
<td>456</td>
<td>66</td>
<td>---</td>
<td>100 x 10^6</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Spain</td>
<td>45,000</td>
<td>67</td>
<td>INIA, test, skimmilk</td>
<td>250 x 10^6</td>
<td>50</td>
<td>50-65</td>
</tr>
<tr>
<td>Sweden</td>
<td>1,000</td>
<td>75</td>
<td>EYC, milk based</td>
<td>150 x 10^6</td>
<td>&gt;50</td>
<td>65</td>
</tr>
<tr>
<td><strong>Goats</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>1,000</td>
<td>20</td>
<td>Egg yolk-Tris</td>
<td>30 x 10^6</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>France</td>
<td>580,000</td>
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<td>Skimmilk</td>
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<td>30</td>
<td>65</td>
</tr>
<tr>
<td>Italy</td>
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<td>0</td>
<td>Modified skimmilk</td>
<td>100</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Norway</td>
<td>940</td>
<td>0</td>
<td>Modified skimmilk</td>
<td>100 x 10^6</td>
<td>&gt;50</td>
<td>64</td>
</tr>
<tr>
<td>Poland</td>
<td>51</td>
<td>0</td>
<td>Modified milk</td>
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<td>50</td>
<td>75</td>
</tr>
<tr>
<td>Spain</td>
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<td>INIA, skimmilk</td>
<td>200 x 10^6</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>Switzerland</td>
<td>1,635</td>
<td>0</td>
<td>Egg yolk-Tris</td>
<td>180 x 10^6</td>
<td>50</td>
<td>55-65</td>
</tr>
</tbody>
</table>

*The majority of sheep inseminations are with fresh semen, whereas frozen semen is commonly used for goats in some Countries.

*Fertility is determined in various ways, but the fertility with unfrozen sperm was higher than with frozen sperm.
Table III. Summary of Swine AI statistics from different Countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of inseminations</th>
<th>Fresh semen (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Semen extenders</th>
<th>Number of sperm inseminated</th>
<th>Motile (%)</th>
<th>Fertility (%)</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>316,518</td>
<td>100</td>
<td>Minitub MIII</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>70</td>
<td>80</td>
<td>10.5</td>
</tr>
<tr>
<td>Belgium</td>
<td>1,000,000</td>
<td>100</td>
<td>Merk III</td>
<td>3-4 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>China</td>
<td>9,000,000</td>
<td>100</td>
<td>---</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>---</td>
<td>85</td>
<td>11.0</td>
</tr>
<tr>
<td>Finland</td>
<td>181,906</td>
<td>100</td>
<td>MR-A</td>
<td>2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>70</td>
<td>84</td>
<td>12.0</td>
</tr>
<tr>
<td>France</td>
<td>1,300,000</td>
<td>100</td>
<td>BTS o derivati</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>65</td>
<td>88</td>
<td>12.0</td>
</tr>
<tr>
<td>Germany</td>
<td>1,300,000</td>
<td>100</td>
<td>Androhep, BTS</td>
<td>2.5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>50</td>
<td>85</td>
<td>---</td>
</tr>
<tr>
<td>Hungary</td>
<td>180,000</td>
<td>100</td>
<td>Modena, Pigboy</td>
<td>2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>70</td>
<td>70</td>
<td>9.5</td>
</tr>
<tr>
<td>Italy</td>
<td>450,000</td>
<td>100</td>
<td>Merk, BTS, Modena, Androhep</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>70</td>
<td>79</td>
<td>9.8</td>
</tr>
<tr>
<td>Japan</td>
<td>106,401</td>
<td>98</td>
<td>Modena</td>
<td>5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>80</td>
<td>87-90</td>
<td>&gt;10</td>
</tr>
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<td>193,706</td>
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<td>SK-L. SUS-L. BTS, Modena</td>
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<td>85</td>
<td>10.5</td>
</tr>
<tr>
<td>Netherlands</td>
<td>1,100,000</td>
<td>100</td>
<td>BTS</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>---</td>
<td>85</td>
<td>---</td>
</tr>
<tr>
<td>Norway</td>
<td>80,000</td>
<td>100</td>
<td>BTS</td>
<td>2.5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>80</td>
<td>88</td>
<td>12.3</td>
</tr>
<tr>
<td>Poland</td>
<td>600,241</td>
<td>100</td>
<td>BTS, Merck, Dilsem</td>
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<td>60</td>
<td>79</td>
<td>---</td>
</tr>
<tr>
<td>Spain</td>
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<td>100</td>
<td>MRA, Acromax, BTS, SCK, Androhep</td>
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<td>75</td>
<td>85</td>
<td>10.2</td>
</tr>
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<td>11.0</td>
</tr>
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<td>Modena</td>
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<td>86</td>
<td>11.1</td>
</tr>
<tr>
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<td>100</td>
<td>BTS, Androhep</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>80</td>
<td>85</td>
<td>11.0</td>
</tr>
<tr>
<td>USA</td>
<td>3,800,000</td>
<td>&gt;95</td>
<td>Androhep, BTS</td>
<td>3-5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>80</td>
<td>85-90</td>
<td>11-12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Frozen semen is used on a small scale for special breeding programs and international shipment with lower fertility and smaller litter size. Swine AI with liquid semen is growing rapidly.
### Table IV. Summary of horse AI statistics from different Countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of inseminations</th>
<th>Fresh semen (%)</th>
<th>Frozen (%)</th>
<th>Semen extenders</th>
<th>Number of sperm inseminated</th>
<th>Motile (%)</th>
<th>Fertility (%)</th>
</tr>
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<tbody>
<tr>
<td>Australia</td>
<td>8,000</td>
<td>98</td>
<td>2</td>
<td>Kenny, milk-egg yolk</td>
<td>500 x 10^6</td>
<td>75</td>
<td>---</td>
</tr>
<tr>
<td>Belgium</td>
<td>7,858</td>
<td>92</td>
<td>8</td>
<td>Skimmilk-egg yolk</td>
<td>300 x 10^6</td>
<td>67-70</td>
<td>70</td>
</tr>
<tr>
<td>Finland</td>
<td>35,790</td>
<td>96</td>
<td>4</td>
<td>Kenney, French, German</td>
<td>1,000 x 10^6</td>
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<td>70</td>
</tr>
<tr>
<td>France</td>
<td>20,000</td>
<td>50</td>
<td>50</td>
<td>Kenney or INRA 82</td>
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<td>40</td>
</tr>
<tr>
<td>Germany</td>
<td>30,924</td>
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<td>14</td>
<td>---</td>
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<td>---</td>
<td>71</td>
</tr>
<tr>
<td>Hungary</td>
<td>3,000</td>
<td>92</td>
<td>8</td>
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<td>50</td>
<td>70</td>
</tr>
<tr>
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<td>5,000</td>
<td>76</td>
<td>24</td>
<td>---</td>
<td>52 x 10^6</td>
<td>---</td>
<td>75</td>
</tr>
<tr>
<td>Japan</td>
<td>327</td>
<td>100</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Norway</td>
<td>1,350</td>
<td>84</td>
<td>16</td>
<td>EZ mixin; EY-lactose for frozen</td>
<td>500 x 10^6</td>
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<td>---</td>
</tr>
<tr>
<td>Poland</td>
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<td>52</td>
<td>Palmer, CAMY</td>
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<td>40</td>
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</tr>
<tr>
<td>USA</td>
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<td>3</td>
<td>Skimmilk, egg yolk-lactose</td>
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<td>40</td>
<td>30-40</td>
</tr>
</tbody>
</table>

*Many Countries reported that they had no reliable statistics collected for horse AI.

*Fertility is measured many ways. The low percentages of fertility (30-40) are per cycle and other percentages are per season.*