Mammalian Reproductive Biology and Advanced Mammalian Breeding Technologies: Are These Applicable to Canines?

A Report to the American Kennel Club

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Executive Summary

Background

From the very beginning of their evolution, dogs were a product of human intervention and selection. Thus, it may strike us that dog breeders should be able to take advantage of the advances in reproductive technology that now allow us to produce domestic animals—and even, by extension, humans—by artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer [ET], and even cloning. We have produced cloned monkeys, a cloned cat and cloned human embryos, but, in fact, there are no canine clones and canine clones may well be years down the pike. What new reproductive technologies could be adapted for use in canines? This report will also focus on technologies that are in use and those that have been used experimentally in canines.

Reproduction manipulation can occur in several places during the canine estrous cycle. This cycle is unique among mammals and dogs cycle into one estrus once every seven months, independent of the seasons of the year. Dogs' eggs are released at a much earlier developmental stage than those of other mammals, and they must mature while in the oviduct. These and other unique features of canine reproduction make it difficult to adapt advanced manipulated reproductive techniques used in other mammals for dogs.

Advanced Reproductive Technologies

There are many interventional reproductive technologies, ranging from those currently in use with canines, such as artificial insemination, to methods such as cloning, that are not yet possible. The most advanced assisted reproduction techniques rely on egg maturation *in vitro*

(IVM), *in vitro* fertilization (IVF), fertilization techniques that result in clones (nuclear transfer, NT), embryo manipulation, and placing the early embryo into the uterus (embryo transfer, ET). One hurdle in applying these techniques to canines is the inability to reliably regulate the estrous cycle in the dog.

Artificial insemination (AI)

Artificial insemination (AI) is the introduction of semen from a stud into a female without the act of mating. It is commonly used in dog breeding. AI may use fresh semen; chilled, preserved (extended) semen or frozen-thawed (cryopreserved and thawed) semen. Vaginal AI is the standard AI method carried out by breeders. Semen is placed directly into the bitch's vagina through a pipette attached to a syringe containing the semen. An alternative technique is intra-uterine AI, in which the semen is deposited directly into the bitch's uterus. This technique is not commonly used in dogs because it is more difficult to do and can injure the animal if done incorrectly. Deposition of semen using laparoscopy—inserting a laparoscope through a small, surgical incision in the animal's abdominal cavity--is also done and has had a good success rate, but there have been few studies carried out that consider the effectiveness of this method. A final method for intra-uterine AI is via full open surgery on a bitch that is under general anesthesia, although this is rarely done except in research facilities.

When using AI or other methods that employ preserved semen samples, it is important to determine the ability of the sperm to fertilize eggs. Standard microscopic examination of semen to detect motility (capability of motion) and normal morphological structure of sperm can be carried out in a veterinarian's office. There are more sophisticated techniques used to study sperm motility and viability and components of the sperm, such as the DNA, mitochondria, and cell membrane. These methods include computer-assisted semen analysis, and the use of more

advanced microscope optics, staining, scanning and transmission electron microscopy (SEM and TEM) and more recently, fluorescent probes (Rodriguez-Martinez, 2000). The majority of these methods have not been applied to canines to any appreciable extent. To assess fertility, binding capacity tests and oocyte penetration tests that assay the ability of sperm to bind with or penetrate the egg have been done using canine sperm (Hewitt and England, 1997; Ivanova *et al.*, 1999). There are several methods in use to sex mammalian sperm—in other words, to determine if the sperm contains an X chromosome, which will yield a female offspring, or a Y chromosome, which will yield a male offspring. Again, the literature does not indicate that these methods are in use in dogs; furthermore, intellectual property issues may limit use of such methods.

To better preserve sperm and allow them to survive longer in the female reproductive tract, researchers are studying microencapsulation methods. In microencapsulation, the sperm are enclosed in a polymeric material. This will preserve them and allow them to survive longer in the female reproductive tract, increasing the likelihood of fertilization.

Timing Ovulation

To increase the probability of fertilization in normal matings or AI—and also to aid in harvesting eggs or embryos for *in vitro* (translated from the Latin as "in glass," as in a test tube or a glass dish) methods, it is essential to time ovulation. There are a number of hormone tests on the market that can test the female's blood for the critical hormone changes that occur immediately prior to ovulation.

Maturation of Canine Eggs In Vitro

Because canids, unlike other mammals, ovulate eggs that have not completed meiotic division, their eggs may require different strategies for *in vitro* maturation than used in other

species (Bolamba *et al*., 1998; Durrant *et al*., 1998). Among the techniques that are used in other species—but rarely in canines—for harvesting eggs is superovulation of the female. This is induction of ovulation through administering hormones that stimulate ovarian and egg development. Most researchers working on canines do not induce ovulation through extrinsic hormones because a reliable method to do this has not yet been developed.

To date, the culturing of canine eggs *in vitro* has not been very successful. In other species, such as sheep and cows, researchers have treated ovarian follicles in culture with hormones and growth factors over time. The follicles grew and matured. The eggs within the follicles also grew. The hope is that such follicular cultures could, in the future, provide large numbers of eggs for IVF and cloning.

Embryo Transfer

To receive an embryo—whether produced by IVF or produced in one dog and removed and placed into another (surrogate dam) for gestation--the female must be hormonally stimulated so that she is at the correct time in the estrous cycle to receive the embryo. Although there has been little success in embryo transfer in dogs from one female to another (Tsutsui *et al.*, 1989, 2001a, 2001b), one pup was delivered from an estrus-induced female (Kim, 1994), and Tsutsui *et al.* (2001b) produced 10 pups through embryo transfer. Although frozen embryos are used extensively in human assisted reproduction, this technique has not yet had much success in canines (Farstad, 2000a).

Cloning

Cloning is production of a genetically identical organism or organisms. There are two methods: nuclear transfer (NT) and parthenogenesis. In NT, the nucleus is removed from an egg and discarded. A somatic (nonreproductive) cell, such as a skin cell, or, in some cases, a cell

from a developing early embryo, is inserted into the enucleated egg (an egg that has had its nucleus removed) and the resulting cell is subjected to an electrical pulse in a process called electrofusion. Other methods to fuse the cells are use of viruses or chemicals. These embryos may be grown *in vitro* for several days until they have grown to blastocysts, at which stage they are implanted into a female that has been hormonally treated to regulate her estrous cycle. In parthenogenesis, the unfertilized egg is activated in some way and begins to divide, forming an embryo. This method most likely will be used to produce stem cells for treatment of diseases, and not to produce whole animals.

DNA Manipulation

Manipulation of an organism's DNA or genetic material may be attempted to correct a genetic defect (gene therapy) or to create a transgenic animal (an animal that carries genetic material from another organism). The value of transgenic animals is that they have specific genes either inserted into their genome or removed from the genome. Such animals serve as animal models for human or animal diseases in research. Transgenic dogs are most likely to be used in biomedical research.

The next major breakthrough in canine breeding will be somatic cell gene therapy—the introduction of new genetic material, probably by introducing a virus that carries the specified gene or genes (viral vector)—into the nonreproductive cells of the body to correct a hereditary disease or defect. This isn't animal reproduction at all, but genes can be inserted into embryos. This probably is the most likely of the new technologies that will have an effect on canine health and welfare. There have been a number of successful experiments in gene therapy for dogs including ameliorating the effects of hemophilia B (Mauser *et al.*, 1997) and Leber congenital

Amaurosis, a form of congenital blindness (Acland *et al.*, 2001). This method also may provide genetic-based therapies for cancer.

Germ cell gene therapy is correcting the genetic defect in the germ line—either in the egg or sperm or in the very early embryo. At this time, germ line gene therapy is unlikely to be accomplished in the canine embryo in the near future because of the difficulties involved in *in vitro* fertilization in dogs.

Summary and Conclusions

Although there are near-weekly reports of a new species being cloned or a new method for cloning, assisted reproductive technologies are, in general, much tougher for canines than for other species. The reasons are twofold: the difficulty in maturing canine eggs *in vitro* and the difficulty in regulating the bitch's estrous cycle to allow harvesting of eggs, and implantation of embryos produced by IVF or cloning. Furthermore, each new technique has its advantages and disadvantages, many of them health related, and many, such as cloning, present serious ethical problems to the canine breeding community.

Researchers recommend that significant additional funds be made available for long-term research into canine reproductive physiology, especially the reproductive physiology of the bitch. Additionally, funds are needed to devise methods to extend the viability of canine sperm for AI or IVF. Studies on IVM of eggs are also required. The next likely area for research breakthroughs in canine genetic technologies is the use of molecular screens for genetically mediated diseases and abnormalities, followed with somatic cell gene therapy to correct the problem. These advances are a direct result of the research done in the Canine Genome Project, an offshoot of the Human Genome Project. These are all areas for which the AKC may wish to

consider naming a panel of advisors—recognized experts in the field of canine theriogenology—to advise on research needs in the field.

Introduction

Historical evidence shows that wolves, the ancestors of the domestic dog, lived with humans some 400,000 years ago (Clutton-Brock, 1995). The first bones that appear to be those of a domestic dog are about 14,000 years old (Clutton-Brock, 1995). Thus, domestication of the wolf into the dog took thousands of years of evolution. During that time, dogs became clearly distinct from wolves; although they remain so genetically close they are able to interbreed. Dogs developed a smaller skull; smaller cranium and brain; fluffy, tightly curled tail; unwolf-life coat colors; floppy ears, along with a decrease in auditory ability; a heavy coat; hair that falls over the eyes (decreasing visual acuity) and manageable behavior (Clutton-Brock, 1995; Coppinger & Schneider, 1995). Humans selected animals with puppy-like features and submissive behaviors that persisted into adulthood for breeding (Copping& Schneider, 1995). About 3,000-4,000 years ago, what we first recognize as distinct canine breeds—greyhound-like dogs, mastiff-type dogs, and dogs with short legs--were specifically bred by humans for hunting and protection (Clutton-Brock, 1995).

From the very beginning of their evolution, dogs were a product of human intervention and selection. Thus, it may strike us that dog breeders should be able to take advantage of the advances in reproductive technology that now allow us to produce domestic animals—and even, by extension, humans—by artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer [ET], and even cloning. Headlines about Dolly the sheep, cloned cows, cloned mice, cloned monkeys, and now a cloned housecat may have convinced us that cloning canines is right around the corner. In early 2000, newspaper articles touted the seemingly imminent production of the first canine clone. But, in fact, there are no canine clones as yet. And canine clones may well be years down the pike, as this report will explain. But the question remains: What new

reproductive technologies could be adapted for use in canines? This report will attempt to answer this. It will also focus on technologies that are in current use or have been used experimentally.

Background

An understanding of advanced reproductive technologies requires an understanding of normal canine reproductive anatomy and physiology so that it is clear at what stage of reproduction manipulation can occur.

The canine female reproductive tract consists of a pair of ovaries, a pair of Fallopian tubes, the uterus with left and right uterine horns, the cervix, the vagina, and the external genitalia (Fig. 1). The uterus is cornuated, which means that it has two horns or cornu. The left and right uterine horns emanate from the body of the uterus and end in Fallopian tubes, each leading to an ovary. The cervix or entryway into the uterus is deep within a vagina that opens to the outside, below the anal opening. The external genitalia of the bitch are called the vulva, which has external lips (labia) that are folds of skin, and the clitoris, erectile tissue that is structurally similar—and, in fact, derived from the same embryonic tissue—to the male's penis (Feldman and Nelson, 1995b).

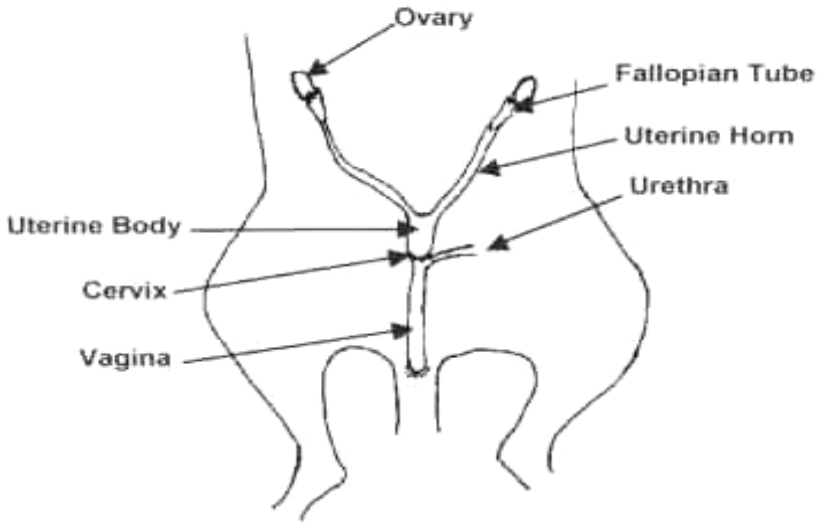

Fig. 1 Schematic of the canine female reproductive tract.

The female is receptive to the male and mating only during a hormonally stimulated period called estrus (heat) (Fig. 2). Within estrus, there is only a small window of opportunity during which fertilization of the egg or ovum (ova, plural) can occur. In the bitch, the first estrous cycle may occur anytime between the ages of five to 24 months of age (Aiello and Mays, 1998; Feldman and Nelson, 1995b). Unlike other mammals that may go into estrus several times a year, dogs—and canids in general—at most, will go into estrus twice in a year, perhaps seven months apart (Feldman and Nelson, 1995b). Some dogs may go into estrus only once a year. Estrus is determined by hormonal cycles of two areas of the brain, the hypothalamus and the pituitary gland, and the ovaries. The estrous cycle can be divided into four distinct periods: anestrus, when the bitch is not receptive to mating; proestrus, when there may be a bloody vaginal discharge, which is actually blood from the uterus; estrus, the period when the female will accept mating by the male; and diestrus, the end of the period of receptivity. Each part of

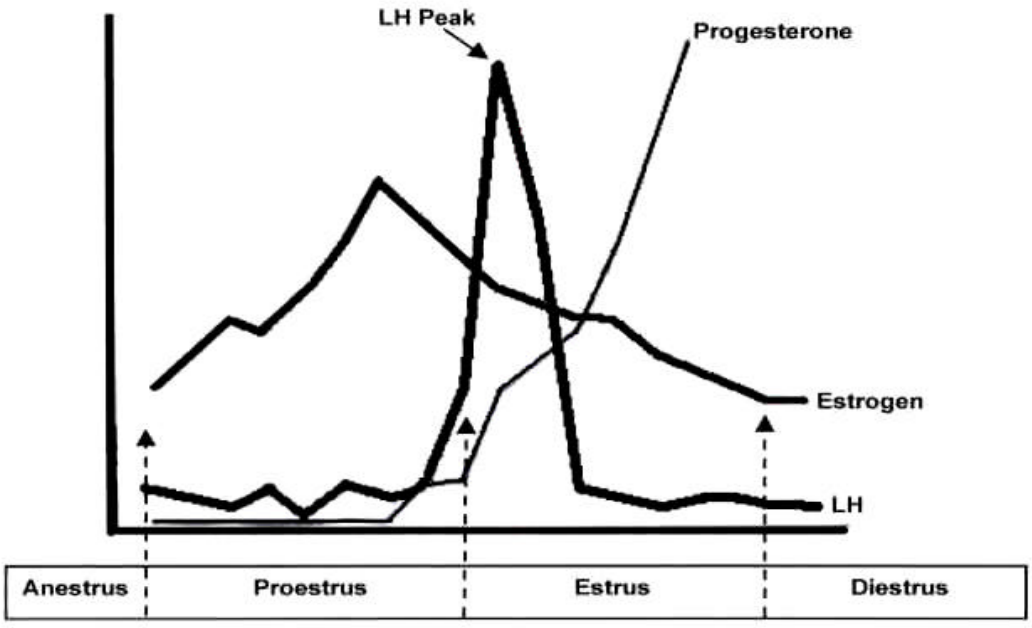

Fig. 2 Hormones of the canine estrous

the cycle is distinguished by hormonal changes that can be monitored with blood tests, and changes in the cells lining the vagina, which can be determined by cytological (cell structure) examination and gross examination of the vagina using an endoscope (Feldman and Nelson, 1995a, 1995b, 1995c).

In proestrus, the pituitary will produce increasing quantities of two hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH). In response to these two hormones eggs develop in the ovaries (Fig. 3). These eggs develop in bubble-like enclosures called follicles. In the presence of these hormones, the developing follicles will begin to produce the hormone estrogen. It is the increase in estrogen that results in the behavioral changes that we think of as estrus, such as increased nervousness, changes in appetite, increased frequency of

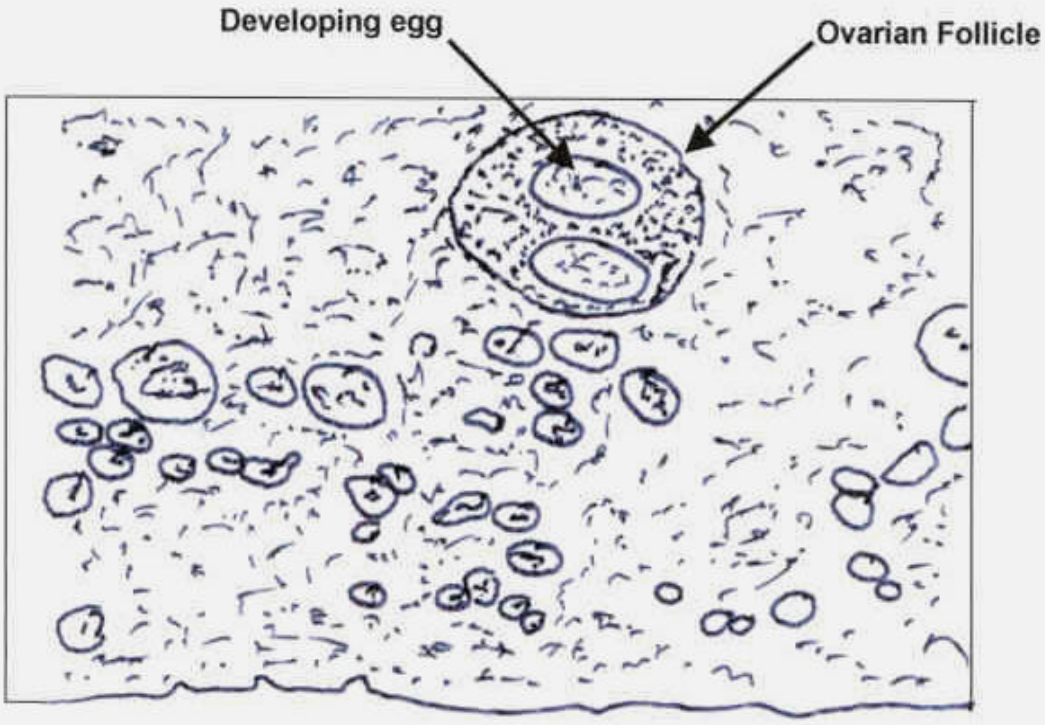

Fig. 3 The developing egg in the canine ovary.

urination, and, eventually, acceptance of the male for mating. Estrogen also stimulates the tissue lining the vagina to thicken, preparing it for mating. Thus, the change in cell types within the

lining can aid the dog owner in accurately predicting where a bitch is in her estrous cycle. Proestrus can last for as long as 22 days, although the average length is about six days. As estrogen levels reach their peak, then rapidly decline, progesterone levels increase, and the pituitary releases a surge of LH. This LH surge is followed by a rapid increase in progesterone. Estrus begins at about the time the LH levels begin to increase. It is at this time that the female indicates a willingness to accept the male. This period is called "standing heat."

Two days after the LH surge, the bitch ovulates, releasing eggs from the ovarian follicles (Concannon, 2000). These eggs are not yet mature or ready to be fertilized by sperm. In fact, canine eggs are released from the ovary (ovulated) early in the first meiotic division. It takes two meiotic divisions to reduce the chromosomes in an egg from a pair of each chromosome to one of each chromosome. In the midst of the second division, the eggs are ready for fertilization (Farstad, 2000b; Luvoni, 2000). Three days after ovulation, the eggs mature.

The sperm may penetrate the egg even before the egg matures. Because sperm can survive and remain viable within the female for as long as nine days, or perhaps longer, the bitch can be successfully mated beginning about three days before the LH surge through eight days after the surge, although these later matings are not often successful (Concannon, 2000).

In mating, the male deposits sperm directly to the opening of the cervix. Within minutes, some of those sperm will have traveled to the oviducts (Fallopian tubes), where fertilization occurs. If the eggs are fertilized, the early embryos will remain in the oviducts, not moving into the uterus until at least 12 days after the LH surge (Concannon, 2000). The embryo itself does not attach to the uterine wall until about 22 days after the LH surge. The entire length of gestation is about 63 to 67 days after the LH surge. In looking at advanced reproductive technologies currently employed or undergoing research in other mammals, there are a number

of times in the cycle when interventional methods can augment normal conception and gestation. The first is at the introduction of sperm and egg. The most basic method is artificial insemination (AI), which has been standard practice in canine breeding for years. But new methods of preserving semen, sexing sperm, testing sperm for fertility, and introducing it into the female's reproductive tract can render AI more efficient.

Moving the fertilization out of the animal and into the laboratory is another step. This is called *in vitro* fertilization (IVF). A bitch (or extracted ovaries) would serve as an egg donor, and the egg would be united with sperm in the laboratory in a dish. The fertilized eggs would be put directly into the egg donor's uterus, or they could be placed in a surrogate dam. These eggs could be cultured into early embryos in the laboratory and then placed into the egg donor or a surrogate. Cloning is a unique means of IVF. It unites an egg without a nucleus with the nucleus from a cell from an adult animal, producing a "clone" of the adult. We can also intervene during gestation by removing early embryos from a pregnant bitch's uterus and then placing them into another bitch.

Any intervention that requires manipulation of eggs or embryos, however, makes it almost imperative that the female's estrous cycle be under control of administered hormones, because timing is of the utmost important. And that has been a problem.

Advanced Reproductive Technologies

Artificial insemination (AI)

Artificial insemination (AI) is the introduction of semen from a stud into a female without the act of mating. The animals never have to be in the same room—and, in fact, the stud can be long dead. There are numerous reasons to use AI. Among these are temperamental incompatibility between the bitch and the stud, desired mating with an animal that is at another

location, producing pups from a dead champion whose semen has been banked, physical problems that may prevent natural mating (such as arthritis in the stud or a vaginal stricture in the bitch), or even inexperience in the stud (Carricato, 1992; Feldman & Nelson, 1995a). A single AI requires a semen sample containing 150-200 million motile (capable of movement) sperm that are morphologically (structurally) normal; for best results, there should be two inseminations of the bitch (Linde-Forsberg, 2001b).

AI may use fresh semen; chilled, preserved (extended) semen or frozen-thawed (cryopreserved and thawed) semen. Usually, the breeder or a person skilled in the technique (often a veterinarian) deposits the semen directly into the bitch's vagina. Linde-Forsberg (2001a) noted that there is a lack of data comparing results using semen that is fresh versus that preserved by various techniques, as well as a lack of data comparing different insemination techniques. In a recent comparison, Linde-Forsberg (2001a) noted greatest whelping rates and litter sizes using fresh or chilled semen versus frozen semen and a newer technique, intra-uterine AI as opposed to vaginal AI. In intra-uterine AI, semen is deposited directly into the bitch's uterine cavity, as occurs in natural mating.

SEMEN

Fresh semen—Fresh semen is the method of choice for many dog breeders.

Advantages--It survives in the bitch's reproductive tract for seven to 11 days (Carricato, 1992, p.89), rendering timing of AI less critical than using chilled or frozen semen (see below).

Disadvantages--The downside is that the stud must be in the same location as the bitch or very close by, so that the semen may be kept warm as it is brought to the bitch.

Thus, AI with fresh semen often requires that either the stud or the bitch must be transported.

Chilled, preserved (extended) semen—Chilled, preserved semen may be from a sperm bank company or may have been freshly collected, treated and shipped.

Advantages--In Linde-Forsberg's study (2001a) of 2,041 AIs, such semen produced only slightly fewer pregnancies than fresh semen in vaginal AI and slightly more pregnancies than fresh semen in intra-uterine AI. It produced approximately the same number of pups per litter in both vaginal and intra-uterine AI.

Disadvantages—According to Carricato (1992, p.89), extended, chilled semen survives for only two to three days, thus, timing of the bitch's cycle so that the AI corresponds with ovulation is imperative. Linde-Forsberg (2001b) states that extended, chilled semen remains motile "for at least four days," but it is not known for how long it can retain its ability to fertilize eggs. Carricato notes that this method (including shipping) can be expensive.

Problems noted by Linde-Forsberg (2001a) and Cornell University's Vicki Meyers-Wallen (personal communication) are that extended and cryopreserved semen are often products of commercial operations. Their methods to preserve semen, including the materials used as extenders to extend the viability of sperm, are not published in the literature and remain unknown to the scientific community. Little is known about the physiology of canine sperm, so this lack of openness can impede scientific research progress.

Frozen semen—Frozen semen is semen that has been frozen, often in a cryogenic fluid. It is thawed prior to use (Linde-Forsberg, 2001b). Researchers are studying effects of

freezing on sperm from other species. This includes studies of membrane stability (which affects sperm viability and ability to penetrate the egg) and flagellar movement (which affects motility). Decreases in either or both could decrease the sperm's ability to fertilize an egg. Treatment of sperm samples with glycerol decreases their ability to fertilize eggs (Hay *et al*., 1997).

Advantages--Frozen semen can be stored indefinitely. New regimens for freezing have increased the pregnancy rate when frozen semen is used (Farstad, 2000a, 2000b; Thomas *et al*., 1992). Researchers are trying to derive new media in which the sperm can be placed before AI or *in vitro* fertilization that will stimulate the acrosome reaction and increase the fertility of the sperm (Kawakami *et al.*, 2000; Sirivaidyapong *et al.*, 2000).

Disadvantages--According to Linde-Forsberg's study (2001a), frozen semen produced fewer pregnancies and smaller litter sizes than either fresh or chilled semen. Frozen sperm appear to have decreased motility in comparison to sperm that were not frozen (Carricato, 1992). This may affect their ability to fertilize an egg. Frozen sperm are damaged by the addition of glycerol and in the thawing process, which is required before AI (Hay *et al.*, 1997).

Ethical Issues--Carricato (1992) notes that there is concern that use of frozen semen may decrease the gene pool for any given breed, as breeders may wish to inseminate their bitches with semen from a small number of champions.

General Caveat--No matter what format the semen is in, semen shipped across national borders may require permits and/or health certificates. This is reviewed by Linde-Forsberg (2001b).

ARTIFICIAL INSEMINATION METHODS

Vaginal AI—In vaginal AI, which is the standard AI method carried out by breeders, semen is placed directly into the bitch's vagina through a pipette attached to a syringe containing the semen.

Advantages--This method is relatively simple and, although it should be performed by someone experienced with the procedure, it need not be done by a veterinarian except in countries that require it.

Disadvantages--AI often is "done at a non-optimal time during estrus" (Linde-Forsberg, 2001a), contributing to relatively low success rates. Deposition of semen in the vagina and not in the uterus is not as successful as natural breeding. The latter results in deposition of the sperm through the cervix and into the uterus, and uterine contractions aid the sperms' passage into the oviducts, where fertilization occurs (Carricato, 1992; Feldman & Nelson, 1995b; Linde-Forsberg, 2001a). Sperm rapidly lose their tails within the vagina and thus lose their ability to fertilize ova (Linde-Forsberg, 2001a), so larger quantities of sperm are necessary than in intra-uterine techniques.

Intra-uterine AI (also called transcervical AI)—In intra-uterine AI, the semen is deposited directly into the bitch's uterus. It is noted in the literature that this technique is not commonly used in canines. It is more complicated than standard AI. According to one theriogenologist (veterinary reproduction specialist), the method is not always necessary or recommended. Intra-uterine AI may be accomplished in several ways. One is via catheter. Several are surgical (see below under "More Invasive Methods").

Advantages--Although this method is more complex than vaginal AI and requires greater expertise to perform, as the cervix must be palpated (located manually), or

visualized with an endoscope (Linde-Forsberg, 2001a; Wilson, 2001), it has a better success rate than vaginal AI and usually is not particularly stressful to the bitch. It is usually considered to be safe.

Disadvantages--It must be done by a veterinarian. In very rare cases, if handled incorrectly, the catheter can perforate the vagina, bladder, or other structures.

More Invasive Methods— Deposition of semen using laparoscopy—inserting a laparoscope through a small, surgical incision in the animal's abdominal cavity--is also done and has had a good success rate, but there have been few studies on this method.

A final method for intra-uterine AI is via full open surgery on a bitch that is under general anesthesia. The uterus is exposed through an abdominal incision and the semen is injected directly into the uterine body.

Advantages--There are no advantages to using this in general canine breeding, but it may be appropriate as a research technique.

Disadvantages--These procedures are expensive and time-consuming.

Ethical Issues--Linde-Forsberg (2001a) indicates that there are serious ethical issues in subjecting a bitch to the risk of surgery solely for the purpose of establishing a pregnancy. Farstad (2000a) noted that such surgical techniques are considered to be unethical in Europe, but apparently are accepted in the U.S. Brittain *et al.* (1995) recommend surgical intra-uterine AI for managing canine infertility in colonies of research dogs.

General Issues with Artificial Insemination

Advantages-- Standard AI does not risk injuries to the bitch during mating, as sometimes occur in natural mating from fighting, size differences between mates, etc. In

some cases, semen can be prechecked for signs of fertility (number of sperm, sperm motility, and appearance of normality). AI can allow sperm to be sorted by sex, resulting in all-male or all-female litters.

Disadvantages-- In using fresh semen, it is still necessary that the male and female be brought to the same location or nearby, thus, resulting in transportation of animals and limiting the dogs with which a bitch is bred. This also requires careful timing, as the bitch must be in estrus. If mistimed, even by a day or so, the entire process is valueless.

TECHNOLOGIES FOR USE IN CONJUNCTION WITH ARTIFICIAL INSEMINATION

Examination of sperm--Standard microscopic examination of semen samples to detect motility and normal morphological structure of sperm can be carried out in a veterinarian's office. More sophisticated techniques include computer-assisted semen analysis, which yields information on sperm motility patterns. This method, which is used to assess the fertilizing ability of bull semen (Rodriguez-Martinez, 2000), has been applied to canine semen (Surman *et al.*, 1992). In cattle, the use of more advanced microscope optics, staining, scanning and transmission electron microscopy (SEM and TEM), and more recently, fluorescent probes to study components of the sperm, such as the DNA, mitochondria, and cell membrane, may aid in determining sperm viability (Rodriguez-Martinez, 2000). There is no indication in the published literature that these methods are being used for canine sperm.

Fertility Assays--Fertilization entails penetration of the ovum by the sperm. This is a multistep process in which the sperm first binds to the zona pellucida, the transparent layer that surrounds the ovum. This binding is mediated by specific carbohydrate

molecules on the surface of the sperm and within the zona pellucida (Töpfer-Petersen, 1999). These molecules allow the sperm to attach to the egg in a lock-and-key manner. The sperm then undergoes the acrosome reaction. The acrosome is an enzyme-rich "cap" that covers the main body of the sperm (Fig. 4). In the acrosome reaction, the membrane that covers the acrosome fuses with the membrane that covers the underlying sperm proper. This fusion releases the acrosomal contents, allowing the sperm to penetrate the zona pellucida. The fusion, and subsequent fusion of the sperm membrane with the ovum membrane, requires interactions of many proteins, some of which have been identified in mouse, pig, rabbit, hamster, rhesus monkey, bull and human sperm (Burkin and Miller, 2000; Jungnickel *et al.*, 2001).

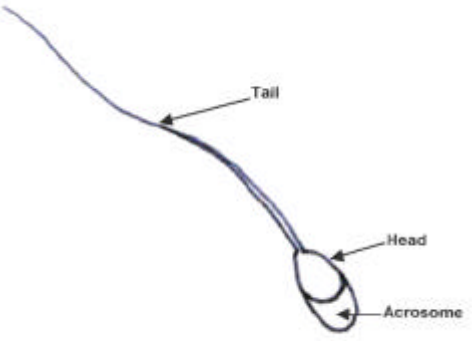

Fig. 4 Schematic of a canine sperm cell.

In some mammalian species, the state of knowledge allows assessment of sperm fertility by the presence or quantitation of molecular protein indicators or seminal fluid proteins. In some cases, chemical induction of the acrosome reaction, sperm capacitation (a process that allows the sperm to recognize the egg), and ability to penetrate zona pellucida-free hamster eggs can be assessed *in vitro*. An excellent review of the above-mentioned techniques is provided by Braundmeier and Miller (2001). Binding capacity tests and oocyte penetration tests that assay the ability of sperm to bind with or penetrate the egg have been done using canine sperm (Hewitt and England, 1997; Ivanova *et al.*, 1999). Knowledge of the biochemistry of sperm-egg interactions is not only important

for studying normal fertilization processes, but it is essential in understanding assisted reproduction methods.

According to Cornell's Meyers-Wallen, there has been little published material on basic sperm physiology in canines, although a number of papers have been published on sperm activation, and a recent paper described what occurs in the sperm during fertilization (Saint-Dizier *et al*., 2001). Nevertheless, much of the work that has been done on canine sperm physiology has been carried out by corporations and remains confidential. Because of this, biochemical tests of sperm function and potency are difficult to derive. Thus, it is essential that canine sperm physiology be studied and that results of this research are published in the peer-reviewed literature.

Advantages--Such tests may be useful adjuncts in determining whether sperm, especially frozen-thawed sperm, are capable of fertilizing eggs.

Disadvantages--Many of these methods to determine sperm viability are expensive and/or inaccurate. For example, flow cytometry—a mechanized method of examining single cells as they pass through a laser beam --allows for the characterization of thousands of sperm/second and is more accurate than simple light microscopic observation, but expensive (Rodriguez-Martinez, 2000).

Sexing of sperm—There are several methods in use to sex mammalian sperm—in other words, to determine if the sperm contains an X chromosome, which will yield a female offspring, or a Y chromosome, which will yield a male offspring. (The ovum contains only an X chromosome.) These methods are used in livestock. They may be applicable to canines, but are not yet used. Once sexed, by using a fluorescent stain in a flow cytometer, sperm are sorted according to an electric charge placed on them. One of the

methods used was developed by the U.S. Department of Agriculture and licensed to a private firm, XY, Inc., of Fort Collins, CO., which is affiliated with Colorado State University (Garner and Seidel, 2000).

Advantages-- Flow cytometer/cell sorters are fast and can process as many as 4,000 live sperm/sec of each sex (Garner and Seidel, 2000). After sorting, sperm are frozen and packaged in straws for AI.

Disadvantages--All sex-sorting methods are patented. Intellectual property issues may require licensing of techniques or purchasing pre-sexed spermatozoa from commercial entities.

Ethical Issues-- Some breeders question whether it is acceptable to select the sex of the litter. Certainly, preferences for one gender over another may lead to skewed sex ratios and such selection can be influenced by trends and fashion, rather than by logic.

Microencapsulation of Sperm—Research has been done on microencapsulation of bovine and porcine sperm to allow it to be preserved without freezing (Nebel *et al.*, 1985; Torre *et al.*, 2000; Watson, 1993). In microencapsulation, the sperm are enclosed in a polymeric material. Alginates have been tried and have been successful as slow-release methods for sperm. This has not been done in dogs.

Advantages-- Microencapsulation can extend the viability of sperm and could increase the period of time in which AI could result in pregnancy. It may substitute for freezing and could, potentially, result in higher fertility levels than freezing, as thawing causes loss of and damage to sperm.

Disadvantages-- Different species may have different physiological requirements for encapsulation materials and methods. This technique is not in use in canines.

Ethical implications-- Similar to those of AI with frozen sperm.

TIMING OVULATION

To increase the probability of fertilization—and also to aid in harvesting eggs or embryos for *in vitro* methods, it is essential to time ovulation. Although there are behavioral hints that the bitch is fertile, and vaginal cell cytology also indicates the stage of the estrous cycle (Feldman & Nelson, 1995b), this is more reliably done by using any of several available assays. One method depends on making vaginal smears on several days during what appears to be proestrus, and leading into estrus. The changes in structure in tissue lining the vagina indicate the approximate end of proestrus. At this point, blood or serum should be tested via enzyme-linked immunosorbent assay (ELISA) for progesterone levels, which begin to increase with the LH surge. An LH ELISA is also available.

Tests available:

Test	Test	Source
Progesterone ELISA	ICG Status Pro	Synbiotics Corp. 271 Great Valley Pkwy. Malvern, PA 19355 Tel.: 800-248-8099 www.Synbiotics.com
	PreMate	Camelot Farms P.O. Box 9191 College Station, TX 77842 Tel.: 979-690-0499 www.camelotfarms.com
LH ELISA	ICG Status LH	Synbiotics Corp. 271 Great Valley Pkwy. Malvern, PA 19355 Tel.: 800-248-8099 www.Synbiotics.com

Advantages-- The advantages of using these tests is that they give an accurate date to time the LH surge, and thus to determine when ovulation will occur. They give the breeder a greater degree of accuracy in determining appropriate dates for natural breeding or AI. In the research lab, they give dates that will aid in determining when eggs should be harvested or embryos removed from the uterus. These tests are relatively inexpensive (about $10/test).

Disadvantages--Taking multiple vaginal mucosa samples and multiple blood samples could be irritating to the bitch.

Ethical issues--Breeders with more dogs and fewer economic resources may find this method expensive, as multiple tests on a single bitch are necessary and can add up in costs. This may make it difficult for them to compete in breeding with someone who can afford this method and AI.

Technologies that Are Experimental in Canines

The most sophisticated assisted reproduction techniques rely on egg maturation *in vitro, in vitro* fertilization (IVF), fertilization techniques that result in clones (nuclear transfer), embryo manipulation, and placement of the early embryo into the uterus (embryo transfer). There are a number of basic physiological techniques scientists and veterinarians rely on to accomplish these goals. The knowledge gaps in canine reproductive physiology mean that any attempts that have been made to carry out any of these techniques in canines have had poor results. It is notable that the group at Texas A & M University that is involved in the major dog cloning project has, to date, produced a cat and not a dog (Fox, 2002; Shin *et al.*, 2002).

What Are The Problems?[1]

As stated previously, there are a number of places in the estrous cycle in which modern reproductive technologies can intercede. Researchers, however, do not yet know how to reliably regulate the reproductive cycle in the bitch. In 1980, Archbald *et al.* reported that they produced estrus in bitches by treatment with pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG). Kim (1994), too, induced estrus in about half of the bitches treated with PMSG or FSH. Thus, the initial problem in attempting IVF of any sort is the ability to have a bitch in the right stage of estrus for implantation. As this now stands, for research purposes, large numbers of bitches would have to be available. To put this in perspective, to produce the first cloned sheep, Dolly, 156 embryos were implanted into ewes (Varner, 1999). To obtain 24 viable cloned cattle, scientists at Advanced Cell Technology (Worcester, Mass.) and their colleagues needed to synchronize 247 cows and transfer 496 early embryos (Lanza *et al.*, 2001). To produce one cloned cat, the Texas A & M group produced 82 cloned embryos (Shin *et al.*, 2002)! Without the ability to manipulate the estrous cycle, a researcher would need access to 124 bitches in one year to have 247 estrous cycles available for implantation. This requires a large number of research animals in a large facility and entails significant expense, not to mention the concern of animal rights activists regarding treatment of these animals.

MATURATION OF CANINE EGGS *IN VITRO*

Prior to doing IVF, researchers need to study the physiology of canine eggs and their *in vitro* maturation (IVM). Some research uses ovaries from bitches that were just spayed

[1] The following information is from conversations with Drs. Barbara Durrant, head of the reproductive physiology division, Center for Reproduction of Endangered Species, Zoological Society of San Diego; Vicki Meyers-Wallen, associate professor of veterinary anatomy, Cornell University College of Veterinary Medicine; and Mark Westhusin, associate professor, department of veterinary physiology and pharmacology, College of Veterinary Medicine, Texas A & M University. Durrant has worked on dog reproduction as a model for endangered canids and Westhusin is principal investigator in the Missyplicity Project, a privately funded project to clone a wealthy donor's pet mutt, Missy.

(Bolamba *et al*., 1998; Durrant *et al*., 1998). Other research projects require the animals to be anesthetized for removal of eggs from the ovaries or oviducts (Westhusin *et al*., 2001). Because canids, unlike other mammals, ovulate eggs that have not completed meiotic division, their eggs may require different strategies for *in vitro* maturation than those used in other species (Bolamba et al., 1998; Durrant et al., 1998). There has been some success in maintaining and maturing these eggs *in vitro* (Hanna *et al*., 2002; Saint-Dizier *et al*., 2001; Westhusin *et al*., 2001; Yamada *et al*., 1992, 1993), but even when fertilized *in vitro*, to date, none has developed beyond the earliest embryonic stages.

Durrant has found that in cell culture conditions, very few canine eggs mature to the point at which they would be useful in IVF. In fact, most undergo degenerative changes (Bolamba *et al*., 1998). Thus, methods for harvesting eggs from ovaries would not, at present, yield useful eggs for IVF, as Westhusin *et al*. (2001) indicate. Furthermore, collection of eggs requires invasive surgical procedures in the bitch.

Harvesting Eggs--Among the techniques that are used in other species—but rarely in canines—for harvesting eggs is superovulation of the female. This stimulates production of eggs by giving the female hormonal stimulants that cause greater than normal numbers of ovarian follicles to develop. Most researchers of canine reproduction do not induce ovulation through hormone dosing, although Yamada *et al*. (1992), stimulated beagle ovaries using equine and human chorionic gonadotrophin administration, and Kim (1994) treated bitches with FSH or PMSG. There are several methods of harvesting these eggs. One method used in cattle is ultrasound-guided transvaginal oocyte pick-up (OPU), in which a probe that can pick up the ova is guided to the ovary through visualization using a transvaginal ultrasound probe. Another method used in animal husbandry is

laparoscopic ovum pick-up (LOPU) (from mature and immature females). This method uses a laparoscope inserted through a small incision, which guides a probe that will pick up the ova. Ova also may be harvested through full open surgery. The latter technique was reported as being used by researchers in the Missyplicity Project (Graeber, 2000). Westhusin, who is the principal investigator for that research, says they are using ovaries from spayed animals.

In some domestic species, eggs harvested from immature females are matured *in vitro.* They then undergo IVF and embryo transfer into a surrogate dam. This method is called juvenile *in vitro* embryo transfer (JIVIT). This method is very new and has had little success to date.

Advantages--Superovulation will produce more than sufficient eggs for research purposes and for advanced reproduction techniques such as *in vitro* fertilization and cloning. Once the technique is perfected, laboratories could induce estrus at will and maintain fewer dogs in their research animal colonies.

Disadvantages-- Requires use of hormones to regulate the female's estrous cycle, which has not been perfected for dogs or requires appropriate timing of estrus. Egg harvesting techniques are invasive, with open surgery being the most invasive. In mice, superovulated females often are sacrificed; this is not appropriate in dogs. In addition, exposure to hormones to induce superovulation may result in ovarian superovulation syndrome.

Ethical Issues-- The egg donor may require surgery that otherwise would be unnecessary, putting her at risk for infection, complications, or even death. The animal induced may not be the one to incubate the embryo. Thus, assuming recovery from the

surgery is less than the 66-day gestation period, a bitch could have her eggs removed surgically, have them fertilized *in vitro,* and produce more young—while not carrying them to term. The bitch could be maintained in competition once she recovers from the surgery, and never be taken out of competition in order to produce offspring.

Oocyte (Egg) Culture—As indicated above, to date, culture of canine eggs has not been very successful. Although Westhusin and colleagues (2001) have been able to fertilize cultured eggs and produce embryos, and Saint-Dizier and colleagues (2001) have also been able to fertilize canine eggs in culture—and induce them to mature, culture of oocytes has been more successful in other species (Shin *et al.*, 2002). Gutierrez *et al.* (2000), maintained follicles sliced from bovine ovaries in culture for as long as 28 days. The Roslin Institute researchers, who cloned Dolly the sheep and have since cloned other sheep and cows, treated the follicles with hormones and growth factors over this time, and the follicles grew and matured. The oocytes within the follicles also grew. The hope is that such follicular cultures could, in the future, provide large numbers of eggs for IVF and cloning.

Advantages-- Once perfected, this technique will yield much larger numbers of fertilizable eggs than harvesting naturally ovulated or superovulated eggs directly from animal ovaries. Thus, it should decrease the number of animals needed as egg donors. Ovaries from spayed animals could be used and could produce a long-term supply of many eggs for research purposes.

Disadvantages--Requires special laboratory conditions, aseptic technique, and skilled technicians.

***In Vitro* Fertilization (IVF)**-- Begin with the caveat that this has not yet successfully produced viable young in dogs because eggs are very difficult to mature *in vitro* and the bitch's cycle cannot be controlled to the point at which successful implantation is likely. IVF has been used in domestic livestock for many years and was first successfully accomplished in humans in the late 1970s. The usual method is to expose ova to sperm in a Petri dish, allow any fertilized eggs to undergo several cleavages (cell divisions), maintain the embryos in culture for several days, and to implant the resulting early embryo into the mother or a surrogate dam. Modern techniques allow embryos to be brought to the blastocyst stage (an early stage) of development. At this point, they can be implanted or frozen for future use.

To assure fertilization, eggs can be directly injected with sperm in a process called intracytoplasmic sperm injection (ICSI). Fulton (1998) and colleagues were able to fertilize canine eggs with canine sperm via this method, although the experimental protocol did not allow the fertilized eggs to develop into embryos. This method has been successfully used to produce cats (Gomez *et al.*, 2000). ICSI, however, prevents the sperm from undergoing the normal capacitation and acrosome reactions (see p.22) that occur when it naturally encounters an egg (Terada *et al.*, 2000). This, in turn, is thought to result in sex chromosome abnormalities in the offspring. Additional research is necessary to determine the causes of these chromosomal defects and to learn more about the molecular biology of sperm-egg interactions in ICSI.

Advantages-- Both IVF and ICSI may allow bitches that are infertile due to uterine, Fallopian tube, or other health problems, to produce their own offspring—albeit a surrogate dam will be necessary.

Disadvantages--In vitro culture systems have a negative effect on fetal growth after embryo transfer (Sinclair *et al.* 1999). Sheep fetuses that were reared in culture for six days—although they were produced by laparoscopic intrauterine AI and then removed from the ewes surgically—showed effects of the culture system employed. The affected fetuses were large, developed abnormally, and, in some cases, were bathed in excessive amounts of amniotic fluid. Organs and tissues also developed abnormally. Sinclair and colleagues (1999) pointed out that they were not the first to observe such abnormalities in fetuses derived from embryo culture, and that this has been seen in cattle and sheep.

In 2001, the Roslin group (Young *et al.*), identified the problem as fetal overgrowth, called "large offspring syndrome" LOS, in sheep as a result of epigenetic (developmental) changes that occur during culture and affect later stages of fetal growth. They found decreased concentrations of the protein insulin-like growth factor 2R (IGF2R), which resulted from suppression of the gene for *IGF2R.* They hypothesized that this may be responsible for LOS. Recently, Tamashiro *et al.* (2002) recognized a similar situation in cloned mice and mice produced from *in vitro* cultured embryos. As the mouse clones did not transfer the overweight condition to their naturally conceived and born offspring, the researchers assumed the problem was related to the effect of the conditions in which the embryos are raised.

Thus, there are many unknowns that can affect IVF, including embryo storage and maintenance methods.

EMBRYO TRANSFER

Whether the embryo is implanted in the biological mother or in a surrogate dam, the female to receive the embryo must be hormonally stimulated so that she is at the correct time in the estrous cycle to receive the embryo. In attempts to save endangered species, the embryo may be implanted into a member of another, related domestic species. This is called transspecies implantation. Zoo and corporate researchers have used transspecies implantation between several species: the Indian desert cat and the domestic cat, the bongo (a rare African antelope) into an eland (another, more common African antelope), the mouflon sheep and a domestic sheep, a red deer into a common white-tailed deer, and a Southeast Asian gaur and a cow (Lanza *et al.,* 2000).

Dogs are so closely related to gray wolves that they can interbreed. Because of this close relationship, domestic dogs may serve as a model system for understanding canid reproductive physiology and may be used in assisted reproduction for endangered species of canids. Oddly, there has been little success in embryo transfer in dogs; one pup was delivered from an estrus-induced female (Kim, 1994), and Tsutsui *et al.* (2001b), produced 10 pups through embryo transfer. But an experiment in embryo transfer in the silver fox (*Vulpes vulpes*) yielded two pups (Jalkanen and Lindeberg, 1998).

Advantages-- Embryo transfer would allow a bitch that conceives early embryos to produce young, yet not carry her embryos to term, as they can be flushed out or surgically removed and transferred into another dam. This could be used in a situation in which a pregnant bitch develops a health problem that must be treated. It also could be used to keep a prize-winning show dog in contention during a time she otherwise would be pregnant.

Disadvantages-- The success rate of this method has been very low. As a commercial technique in canines, it would be expensive. There also would be risk of embryo loss (which can occur during transplantation, see Tsutsui *et al.*, 2001a) and injury to the mother and surrogate, both of which need to be anesthetized for the procedure.

Ethical issues-- Is it ethically acceptable to breed a bitch, yet show her at the same time because a surrogate dam is carrying the offspring? Just as frozen semen from one champion stud can be used too frequently, if a particular bitch becomes popular, her eggs could be much in demand for breeding. This may lead to excessive inbreeding and increase the number of genetically defective animals in a breed. Decreasing the diversity of the gene pool for a particular breed is another ethical issue.

Frozen embryos— Although frozen embryos are used extensively in human assisted reproduction, this technique has not yet had much success in canines (Farstad, 2000a). Use of frozen embryos would have similar advantages and disadvantages to embryo transfer using fresh embryos (see above).

CLONING

Cloning is the production of a genetically identical organism or organisms. Because with our current technology, an adult cell cannot simply be cloned on its own, there are two methods in use: nuclear transfer (NT) and parthenogenesis. More than 30 organizations worldwide are working on NT and cloning. The list is on the Roslin Institute's website (see Appendix).

Nuclear transfer (NT)-- In NT, in its simplest form, the nucleus is removed from an oocyte and discarded. The oocyte is then referred to as enucleated. A somatic (nonreproductive) cell (that has been maintained *in vitro* in tissue culture) or, in some cases, a cell from a developing early embryo, is inserted into the enucleated egg and the

resulting cell is subjected to an electrical pulse in a process called electrofusion to fuse the cells together. Viruses or chemicals can also be used to fuse the cells. These embryos may be grown *in vitro* for several days until they have grown to blastocysts, at which point they contain 100+ cells. The blastocyst is implanted into a female that has been hormonally treated to regulate her estrous cycle. It then undergoes gestation. These techniques are described in Campbell *et al.* (1996) and Wilmut *et al*. (1997). The Wilmut paper describes the cloning of Dolly the sheep, the first livestock animal cloned from an adult somatic cell. This success spurred on many other cloning projects worldwide in efforts to produce not only animals that are genetically identical to existing animals, but humans that are genetically identical to already living humans. (Gene banks are encouraging people to preserving tissue samples so that dead pets and people can, eventually, be cloned.) The history of the field and the inherent problems are reviewed in Jones (2001). NT has been used to successfully clone sheep, cows, goats, mice, monkeys, a cat, and, an early human embryo. A second method of NT that has been successfully used to clone pigs uses two fusion steps. A cultured adult pig cell is inserted into a pig egg from which the nucleus has been removed (Polejaeva *et al.*, 2000). The cell undergoes electrofusion. Then a very early embryo, which has had its nuclear material removed, is transferred into the electrofused cell. This cell again is treated by electrofusion. The resulting zygote is grown in culture to the blastocyst stage.

Advantages-- Cloning ostensibly will give an exact genetic replica of another animal.

Disadvantages--Although the nuclear DNA in the clone is identical to that of the nucleus donor, the mitochondrial DNA (which has the major role in cellular metabolism)

is that of the egg donor (Evans *et al.*, 1999). Thus, if the egg donor has a metabolic disease, this will be passed on to the clone. In addition, the environment, i.e., the surrogate, exerts effects on the embryo. This concept was nicely demonstrated in CC the cloned cat whose coloration is not identical to that of the "donor nucleus" cat because pigmentation is not purely a result of genotype, but a result of interplay between genes and the environment (Shin et al., 2002). This phenomena has been previously noted in other cloned animals with multicolored coats.

There is a fear that if cloning became commonplace it may result in a decrease in the gene pool.

Cloning is not at a point where it is in "mass production." It requires significant laboratory time and is expensive. Cell cycles of the egg and the donor cell must be synchronized. A dam must be ready for embryo implantation. Literally, hundreds or thousands of eggs must be used to produce a handful of viable offspring. Polejaeva *et al*. (2000) reported using 2,101 eggs to produce 586 embryos that were transferred into females. There were five live births.

Cloned animals have shown genetic anomalies—such as shortened telomeres (the ends of the chromosome) and abnormal numbers of chromosomes. Although Lanza *et al*. (2001) optimistically reported that 80 percent of the cloned cattle they produced—those between one and four years of age--remained healthy, Dolly, the cloned sheep is arthritic (Dyer, 2002; Naik 2002b). Other cloned animals may have abnormal organs, such as the lung or heart. Many suffer from large-offspring syndrome (Travis, 2001). Placental problems that cause many fetuses to die predominate *in utero* (Travis, 2001). Current

thought is that these abnormalities result from defects in reprogramming the somatic cell nucleus so that it resembles the nucleus in an embryo.

Ethical Issues—Although it currently is not possible to clone a dog, the ethical issue of whether dogs should be cloned must be addressed. Ethicist Dr. Montague Brown, professor of philosophy at St. Anselm's College in New Hampshire, believes that there should be a good reason for cloning a dog. He does not consider cloning one's beloved pet to be acceptable. He does see the value of cloning dogs to produce guide dogs or dogs for use in medical research. Brown also expresses concern about the flawed animals that have been cloned. He believes that humans should take responsibility for the flaw. There is some concern that a cloned dog will take a home that could have been available to a stray or shelter dog that needs a home. The contrasting argument is that this is not an issue, as cloning is extremely specialized and very few dogs will be cloned.

There are religious objections to cloning of all organisms, and the animal rights movement considers it unnecessary and objectionable. Ethical issues involved in cloning canines are discussed in Varner (1999), who is quite positive about the level of care and ethical code followed by the researchers of the Missyplicity Project. But there is no guarantee that other laboratories will follow in such ethical footsteps.

In terms of dogs used for laboratory research, clones will aid in fulfilling the animal rights' objectives of reducing numbers of dogs needed for laboratory research projects, as cloning will eliminate genetic variability between animals, yielding more accurate results with fewer animals.

Intellectual Property Issues—Although not all the advanced techniques are protected by patent, some are. For example, some of the cloning methods used by

Advanced Cell Technology Inc. are licensed to the Australian company, ProBio Inc. (Naik, 2002a). Embryo culture methods may be patented. Patents may be held in several countries, making it difficult to carry out specific procedures anywhere in the world.

Parthenogenesis-- In parthenogenesis, the unfertilized oocyte is activated and begins to divide, forming an embryo. Recent news reports of parthenogenetically derived macaque monkey embryos indicated that they were produced as potential sources of stem cells for tissue or organ transplantation (Anon., 2001; Cibelli *et al.* 2002). A recent patent for a technique to activate oocytes parthenogenetically allows these activated cells to develop as far as blastocysts. These activated cells could then be enucleated and used as egg donors that will receive a donor nucleus from another cell (Machaty & Prather, 2001). These technologies are so new that it is impossible to project their potential successes and problems.

Disadvantages-- At present, no one expects parthenogenesis to create a fully developed animal.

Intellectual Property-- Various patented methods exist, including the Machaty and Prather (2001) patent, which covers use of their method for stimulating an unfertilized dog oocyte.

STATUS OF THE MISSYPLICITY PROJECT

To date, researchers working on a project to clone dogs have not had any success, due to the difficulties inherent in applying the techniques of superovulation, estrous synchronization, IVM of oocytes, IVF, and embryo transfer to canines. Recently, the investigators of this project reported that enucleated dog oocytes were successfully fused with donor cells from adult dogs. Twenty-three percent of the resulting embryos cleaved at least once after culture *in vitro*. Five

cloned embryos were transferred into three bitches but this did not result in any pregnancies. However, when enucleated bovine oocytes were used for fusion with adult dog cells, 43% cleaved to the eight-to-sixteen-cell stage. Forty-seven embryos were transferred into four bitches resulting in a single conceptus, which was lost after 20 days of gestation (Westhusin *et al*., 2001). A recent news article on cross-species cloning indicates that signaling problems between the nucleus of one species and the mitochondria of another prevent development (Regalado and Song, 2002). Westhusin and colleagues (2001) indicated the need for advances in research in the areas of IVM of oocytes and regulation of the estrous cycle in canines to make progress in developing successful methods for cloning dogs.

DNA MANIPULATION

Manipulation of an organism's DNA or genetic material may be attempted to correct a genetic defect (gene therapy) or to create a transgenic organism. The value of transgenic organisms is that they have specific genes either inserted into their genome (knock-in) or specific genes removed from the genome (knock-out). Such animals are valuable for research purposes, as they serve as animal models for human or animal diseases. Insertion of DNA may be done with a vector—oftentimes a virus--or through use of "Gene Guns," which inject DNA directly into a cell, or electroporation, which uses an electric charge to insert DNA into a cell.

Gene Therapy-- Gene therapy may be used to correct genetically based diseases. There are two types of gene therapy: somatic cell and germ cell. In his 1999 book on canine genetics, Lowell Ackerman (1999) stated that he expects the next major breakthrough in canine breeding will be somatic cell gene therapy—the introduction of new genetic material, probably by a viral vector—into the nonreproductive cells of the body to correct a hereditary disease or defect.

There has been a successful experiment in gene therapy for hemophilia B in dogs (Mauser *et al.*, 1997), and gene therapy has been successfully employed in dogs suffering from genetically mediated Leber congenital amaurosis, a form of congenital blindness (Acland *et al.*, 2001). Gene therapy has also been used to introduce a gene for a white blood cell stimulating factor in healthy dogs. This may be the beginning of gene therapy against cancers (Hogge *et al.*, 1998, 1999). As a result of the Canine Genome Project, which is an outgrowth of the Human Genome Projects, scientists can now identify the genetic bases for many conditions in dogs, such as progressive retinal atrophy, hemophilias, and many others (Ackerman, 1999; Acland *et al.*, 1998; Meyers-Wallen, 2001). Widespread genetic screening of some dog breeds for particular conditions may occur in the not-too-distant future. Information and lists of genetics tests available may be found at http://www.optigen.com , www.akcchf.org and www.vgl.ucdavis.edu/research/canine. Genetic testing requires a small tissue or blood sample—some hairs may suffice—to allow analysis of the DNA. Some of these genetic diseases, such as progressive retinal atrophy, may be amenable to gene therapy. Genetic changes made via somatic cell gene therapy cannot be passed on to offspring. They are active in the individual only.

Germ cell gene therapy is correcting the genetic defect in the germ line—either in the egg or sperm or in the very early embryo. At this time, germ line gene therapy is unlikely to be accomplished in the canine embryo in the near future because of the difficulties involved in *in vitro* fertilization in dogs. Even if it could be accomplished, to implant the embryo into a bitch for gestation would require a bitch that is naturally in the correct phase of the estrous cycle for implantation or one that has been hormonally

stimulated to that stage. The former is difficult, but not impossible, to arrange. The latter is extremely difficult to accomplish.

Advantages-- Any kind of gene therapy could correct a genetic defect either permanently or long-term. Some somatic cell therapies that have been tested have worked for months. The goal is to have therapies that will work for the lifetime of the animal.

Disadvantages--This is purely experimental. It is risky as the methods are so new. There is the risk it may not work, and there is a risk that the method may kill the animal or make it sick. Currently, costs would be phenomenally high.

Ethical Issues-- Germ line gene therapy would permanently change the individual and all of its offspring. We do not know what effect permanently changing the genome may have on the species.

Transgenics—Generally, the creation of transgenic animals is for research purposes. Animals may have genes removed. In that case, they are called "knock-out" animals. "Knock-in" animals have additional genes inserted. These "designer" animals are not, at this point, likely to be family pets. Knock-out and knock-in animals are laboratory animals developed for research purposes. Knock-out and knock-in technologies can be used in conjunction with IVF and cloning, so that all the animals are genetically identical. A brief review of methods for modifying germ lines is given in Perry *et al.* (1999), followed by a report on use of ICSI to produce transgenic animals.

RELATED TECHNOLOGIES

Cell or Tissue Banking—Cell or tissue banking saves part of an animal for future use, usually for reproduction. A number of companies have been started, especially to

preserve tissue samples for future cloning. Sperm banks have been in existence for years and allow dog breeders to use frozen semen to breed their bitch to a long-dead champion. Unfertilized eggs can also be stored by freezing, as can fertilized embryos.

Advantages--The advantages of storing sperm have been described on pages 17 and 18. The advantages of storing unfertilized eggs allow eggs to be available for *in vitro* methods without the need for a currently active canine research colony. Saving tissue samples may allow for cloning of a sick or dead pet, or research animal or guide dog with specific genetic traits.

Disadvantages--Storing eggs at this stage of our knowledge may not be useful, as researchers do not yet have a reliable method for maturing canine eggs *in vitro.* Storing tissue samples for possible cloning may be futile, as, once the technique is perfected, few dogs will be cloned. Individuals whose much beloved dog has just died may feel pressured to deposit a tissue sample in a tissue bank. They also may falsely believe that any cloned dog will be an exact replica of the "nucleus" donor, not understanding that any animal is a product of nature and nurture, and that the exact environment in which the first dog lived cannot be replicated. Furthermore, as indicated previously, there are questions about abnormalities found in animals that were maintained *in vitro* as embryos.

Ethical Issues-- Storing tissue samples can be very expensive, running as much as several hundred dollars for the initial storage and processing, followed by an annual fee. Grief-stricken dog owners who have just lost their beloved pets are ripe for pushing into expensive arrangements to maintain a dog's tissue so it may, at some future date and for some even larger amount of money, be cloned. Consumers need to understand how rare

pet cloning is likely to be and the small likelihood of their pet's tissue ever being used to produce a clone. Information on canine genome banking is in the Appendix.

Litter Size Determination–A technique recently patented for pigs allows screening of the sow's genome prior to mating to search for particular alleles (genes) that will determine if the sows will produce large litters (Li *et al*., 2001). At present, researchers have not identified the genes that control litter size in dogs. Research on the canine genome, however, may yield this information. Meanwhile, the best but not particularly reliable method for determining canine litter size is an ultrasound examination of the pregnant bitch.

Advantages-- If such a method were available, breeders could select for bitches that can carry the optimum litter size for the breed.

Disadvantages-- Breeders could select for dogs that, genetically, produce litter sizes that are too large for the bitch and could be dangerous to the bitch's health. Also, unscrupulous breeders (such as those who run "puppy mills") could breed large numbers of dogs—many defective—and maintain them in substandard conditions until they are sold to pet stores.

Ethical issues-- See "disadvantages" above.

Summary and Conclusions

Although there are near-weekly reports of a new species being cloned or new method for cloning, assisted reproductive technologies are, in general, much tougher for canines than for other species. The reasons are twofold: the difficulty in maturing canine eggs *in vitro* and the difficulty in regulating the bitch's estrous cycle to allow harvesting of eggs, and implantation of embryos produced by IVF or cloning.

Researchers recommend that significant additional funds be made available for long-term research into canine reproductive physiology, especially the reproductive physiology of the bitch. Additionally, funds are needed to devise methods to extend the viability of canine sperm for AI or IVF. Studies on IVM of eggs are also required.

As there are many different breeds of dogs, research will have to extend to as many breeds as possible; most breeds are not obtainable for research purposes. Whatever is true for the beagle may not be what occurs in the golden retriever. Researchers are confined to using Class A breeders (breeders certified by the U.S. Department of Agriculture as a source for laboratory dogs), who raise only a few breeds of dogs for research. There needs to be a solution to the problem of obtaining various breeds for this research.

The next likely area for research breakthroughs in canine genetic technologies is the use of molecular screens for genetically mediated diseases and abnormalities, followed by somatic cell gene therapy to correct the problem. This is a direct result of the research done in the Canine Genome Project, an adjunct to the Human Genome Project.

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Glossary

Acrosome: an enzyme-rich "cap" that covers the main body of the sperm.

Acrosome reaction: the fusion of the membrane that covers the acrosome with the membrane that covers the underlying sperm proper. This releases the acrosome contents and allows fertilization to proceed.

Allele: a variation of a particular gene.

Artificial insemination (AI): the introduction of semen from a male animal into a female without the act of mating.

Blastocyst: an early mammalian embryo that is at the stage in which it is ready for implantation into the uterine wall.

Cleavage: cellular division in the early embryo.

Cloning: production of a genetically identical organism or organisms.

DNA (deoxyribonucleic acid): the genetic material.

Electrofusion: Fusing cells by subjecting them to an electrical charge**.**

Embryo transfer (ET): inserting the early embryo into the uterus of a female that is not the egg donor.

Enucleated: condition of being a cell from which the nucleus has been removed.

Epigenetic: related to the sequential development of the fetus.

Estrous cycle (n. estrus): The reproductive cycle in the mammalian female (except for great apes and humans). During this cycle, there are times when the female is receptive to copulation (called "estrus") and times when she is nonreceptive (anestrus).

Flow cytometry: a method used to separate cells that are stained with a fluorescent dye, using a microscope and a laser beam to detect the dye.

Follicle: the saclike structures in the ovary that include the developing ovum and the cells surrounding it.

Germline or germ cell: cells that can pass on their DNA to the next generation of organisms. This includes sperm, eggs, or embryos.

***In vitro* fertilization (IVF)**: fertilization of an ovum (egg) by sperm, outside the body of the female, such as in a Petri dish or in a test tube. *In vitro* means "in glass," and refers to chemical

or biological interactions that occur in the laboratory and outside the body of a living organism. (Opposite, *in vivo*, or inside a living organism.)

Intracytoplasmic sperm injection (ICSI): an *in vitro* fertilization technique by which eggs can be directly injected with sperm.

Juvenile *in vitro* embryo transfer (JIVIT): A technique used in some domestic species, in which the ova have been harvested from immature females, matured *in vitro,* and then undergone IVF and embryo transfer into a surrogate dam.

Laparoscope: An endoscope specially designed to examine the abdominal cavity.

Laparoscopic ovum pick-up (LOPU): a method for harvesting eggs from the ovary. It uses a laparoscope inserted through a small incision, which guides a probe that will pick up the ova.

Laparoscopy: examining the abdominal cavity with a laparoscope.

Large offspring syndrome (LOS): fetal overgrowth found in some mammals produced by IVF, embryo transfer, or cloning. It is assumed that this is due to growth conditions of the early embryo when it was maintained *in vitro* prior to transfer.

Luteinizing hormone (LH): a hormone produced by the pituitary gland in the brain. In the female, its production spikes just before ovulation and, in concert with ovarian hormones, it stimulates ovulation and production of the corpus luteum, the wall that remains of the follicle immediately after ovulation.

Meiotic division (Meiosis): this is called reduction division. It occurs in the production of eggs and sperm. It reduces the number of chromosomes from two full sets—one from the father and one from the mother of the animal that produces the sperm or egg—to one set of chromosomes, or from diploid to haploid.

Morphology: the science that deals with the structure of the organism.

Oocyte: An immature egg or ovum.

Ovulation: release of eggs from the ovary.

Ovum: Egg. Plural, ova.

Palpate: touching and feeling the body to examine it.

Parthenogenesis: development of an embryo from an egg without activation by a sperm.

Reprogramming: a series of chemical changes that must occur in a programmed adult cell so that it can become totipotent and produce any cell in the body.

Somatic cell: cell that is neither an egg nor a sperm (e.g., skin cell, liver cell, etc.).

Superovulation: extrinsic hormonal stimulation of the ovary leading to development of greater than normal numbers of ovarian follicles and greater than normal numbers of eggs.

Surrogate dam: female that is not the egg donor, but carries the developing fetus.

Telomere: the far ends of the chromosome.

Theriogenology: the study of veterinary reproduction. A theriogenologist is a veterinarian or veterinary researcher who specializes in veterinary reproduction.

Transspecies implantation: implantation of an embryo from one species into a member of another, related species. This is usually used for gestation of an embryo from an endangered species.

Ultrasound-guided transvaginal oocyte pick-up (OPU): a technique in which a probe that can pick up the ova is guided to the ovary through visualization using transvaginal ultrasound.

Vector: an agent, such as a virus, that can carry foreign DNA and insert it into the cell of another organism.

Zona pellucida: the transparent layer that surrounds the ovum. Sperm must get through the zona pellucida to fertilize the ovum.

Appendix

Recommended Websites

British Medical Journal: bmj.com

International Embryo Transfer Society: www.iets.org

International Veterinary Information Service: www.ivis.org

Nature: www.nature.com

Roslin Institute Online: www.roslin.ac.uk (extensive links)

The American Kennel Club Canine Health Foundation: www.akcchf.org

The Canine Genome Project: www.fhcrc.org/science/dog_genome/dog.html
_mendel.berkeley.edu/dog.html

The Kennel Club U.K.: www.the-kennel-club.org.uk

The Missyplicity Project: www.missyplicity.com

University of California, Davis, Veterinary Genetics Laboratory:
www.vgl.ucdavis.edu/research/canine/
www.vgl.ucdavis.edu/research/canine/links.htm (good links, some old)

Information on Tissue Banking

www.advancedcell.com/

www.caninecryobank.com/

www.lazaron.com/

www.perpetuate.net/

www.savingsandclone.com

Resources

Dr. Montague Brown
Chair
Department of Philosophy
St. Anselm College
Manchester, NH

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