Assisted Reproduction in Captive Wild Felids

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Introduction

Today many species of animals are threatened or endangered. The primary threat to their numbers is loss of habitat in the face of a rapidly growing human population. No real wilderness areas remain. There are only islands of rainforest or grasslands surrounded by grazing pastures, roads, and cities. These isolated breeding populations face the deleterious effects of inbreeding. As popular public attractions, species of the family felidae have become a center point for promoting conservation efforts. There are thirty-seven species in the family felidae. Only the domestic cat is not threatened or endangered. There are estimated to be only 300-400 Siberian tigers remaining in the wild with only 700-800 held in captivity. Fifty Florida panthers are all that remain isolated in the Big Cypress and Everglades region of southern Florida. In the face of dwindling numbers of non-domestic species, zoos have become a genetics sanctuary. It is now the mission of the American Association of Zoological Parks and Aquariums to protect, preserve, and propagate these populations of endangered animals. However, limited cage space, limited financial support, limited research, limited manpower, as well as small numbers of founder animals within the captive populations are obstacles to maintaining viable, growing, healthy populations. Further, research and development within the field of conservation biology is necessary to fulfill the goal. Integration of animal management and the techniques of assisted reproduction have met with some success and show promise for further and more consistent results.

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Some felid species have little trouble breeding in captivity. Lions reproduce so well in captivity that the lioness is often implanted to prevent conception. However, captive breeding for most felid species has not been successful. Factors often associated with failure to achieve a mating and/or pregnancy include: psychological distress following shipment or encountering of a new mate, pair incompatibility, reproductive disease of either or both animals, and lack of knowledge about species specific reproductive physiology and the effect of stress on it. Assisted reproduction techniques such as artificial insemination, in vitro fertilization, embryo transfer, and gamete collection and preservation will avoid breeding pair incompatibility and the stress of animal transport and pair introduction. Such techniques distribute the genetics of valuable animals more evenly, more rapidly, and more broadly than natural servicing alone. In the near future, assisted reproduction will be able to introduce new genetics from the wild into a stagnant captive population. The value of these rare animals, the inherent dangers of working with them, and the generally small numbers of animals from which to collect data in zoos limits the amount of research and data that can be collected from these species. Therefore, much of what has been achieved in the non-domestic felid is the result of techniques developed using the domestic cat as a model.

Inbreeding and Loss of Heterogeneity

Non-domestic felid populations in zoos are often small. Avoiding inbreeding and the resulting loss of heterogeneity is a challenge and incentive towards developing reliable management and assisted reproduction techniques. Natural disaster, illness, and inbreeding can severely diminish heterogeneity of a small population of animals. As the number of breeding animals becomes smaller, the genome of all animals in the population become more alike. This leads to unmasking and expression of recessive traits, which are often lethal, and the

appearance of congenital abnormalities. For example, the Florida panther is an isolated subspecies of the American cougar. It is estimated that only fifty Florida panthers are left isolated in southern Florida. A common feature of the Florida panther is a kinked tail, forty percent of the males are believed to be unilaterally or bilaterally cryptorchid,[20] and the spermatozoa may be up to 75% pleiomorphic.[18] These traits are all thought to be congenital and linked to loss of genetic diversity. The American cougar is spread through Western North America. While threatened, the American cougar's numbers are significantly greater than the Florida panther's. The appearance of kinked tails, cryptorchidism, and severe pleiospermia is significantly less in the American cougar population.

Loss of genetic diversity initially affects the reproductive system, newborns, and the immune system. Irregular estrus, repeat breeders, abortions, stillbirths, and decreased litter size are seen reproductively in the inbred female. Decreased birthweight, lack of vigor, failure to nurse, perinatal atelectasis, increased prevalence of disease, delayed puberty, and gonadal hypoplasia often occur in the inbred neonate.[20] With the inbred male, there is decreased spermatozoa viability, motility, concentration and increased structural abnormalities.[12] Further research needs to be done to understand the reproductive physiology involved and the mechanisms by which homogeneity affects this.

The immune system also suffers from lack of heterogeneity. Heterogeneity allows for coding of a polymorphic array of proteins including the immunoglobulins and the proteins associated with cell mediated immunity. Homozygous genomes produce monomorphic proteins. There is a lack of variety of immuno-proteins, including the MHC classes of proteins, so there is insufficient recognition of the antigens that will stimulate an adequate immune response. The cheetah is the model of species homogeneity. Somewhere in its evolution, the cheetah experienced a genetic bottleneck. The MHC complexes of each animal are so similar that skin grafts between unrelated cheetahs will not be rejected.[20] The entire cheetah population is therefore highly susceptible to the same infectious agents, especially diseases in which the immune system is directly affected. For example, the cheetah is highly susceptible to Feline Infectious Peritonitis (FIP). The entire cheetah collection at the Henry Doorly Zoo in Omaha, was lost to FIP in the late 1980's.[2] Research also needs to be done to understand the physiological interactions of the immune system and the reproductive system.

Species Survival Plans

The AAZPA has developed a system of Species Survival Plans (SSP) in an effort to manage captive populations. SSPs are organized plans that define breeding and management strategies for the benefit of the species. Sixty-one SSPs have already been established. Table 1 lists the SSPs currently established for felid species. By the year 2000, it is hoped that SSPs will be established for over 150 species. Each SSP is directed by a nine member propagation group that is annually elected and headed by a project coordinator. Each SSP is centrally located at a facility with expertise in the specific species. Individuals considered experts on the species may serve on SSP advisory boards and research teams. This includes veterinarians, wildlife workers, and zoo curators among others.

The SSP maintains a studbook listing of all members of the species present in North America and handles the separate zoo populations as one population. Each participating zoo or organization has signed a memorandum of participation. The group evaluates each animal's genetic quality and the distribution of their genetics in the population, then suggests optimal breeding pairs. The SSPs also make recommendations about husbandry and management techniques to help the species do well in captivity. Their objective is to use as large and diverse of a founder population as possible and distribute the genetics as evenly as possible to achieve the maximal genetic diversity. The long range goal is to increase the captive population to zoo carrying capacity and, depending on the species, establish a reintroduction program. Unfortunately, it requires at least 250 founder animals to provide the genetic base necessary to sustain a healthy, genetically diverse population.[2] Many captive populations have numbers below this level.

SSP success has yet to be fully evaluated as many programs are still in initial stages. It appears to have had early success with the black-footed ferret. On the verge of extinction, the last 100 black-footed ferrets were brought in for captive management and breeding in the

late 1980's. An SSP was developed for them. Re-introduction of selected ferrets to the wild has begun in the last year.[2] There have even been preliminary reports of births in the wild. Re-introduction of many of the cats will likely not happen despite any successes with SSP or assisted reproduction. The competition for land coming from the rapidly growing human population has destroyed most of the habitat necessary for these animals to live freely. The optimum SSP program will have worldwide cooperation. Early steps in this direction have already been taken with Japan and Australia.[13]

Semen Collection/ Evaluation/ Processing

The poor breeding history of non-domestic felids in captivity is due, in part, to the poor semen quality. Little is known about the species specific physiology of spermatogenesis or the capacitation requirements in the non-domestic felids. It is known that non-domestic felid semen viability depends on careful handling and good processing techniques. Procedures have been developed to reliably collect, evaluate, and process semen to maximize utilization of the sample to the highest degree.

Collection: Reliable semen collection techniques are essential for the success of an assisted reproduction program in nondomestic felids. Semen is collected for several reasons: to evaluate the breeding potential of the male; to use fresh or extended samples with artificial insemination or *in vitro* fertilization procedures; for cryopreservation of valuable genetics; and to obtain new genetics from the wild. Several techniques are available to collect semen: post-mortem, manual manipulation, artificial vagina, or electroejaculation. Because of the danger posed by the conscious animal to personnel, semen is collected from captive felids almost exclusively using electroejaculation or post-mortem collection.

Electroejaculation is relatively easy and reliable to do and has the advantage of requiring general anesthesia of the animal. The basic equipment required for electroejaculation includes: an electrostimulator, a rectal probe with three longitudinal electrodes on one side; a 110 volt AC 60 Hz sine wave generator with variable voltage and amperage control; and a receptacle.[9] Preparation for electroejaculation requires that the rectum be evacuated of feces. The probe is inserted up to nine centimeters into the rectum with the electrodes toward the ventral rectal wall. There should be enough lubricant present to create good contact with the mucosal surface of the rectum. The penis should be exposed, cleaned, and the receptacle held in place before stimulation begins.

There are a number of protocols available describing stimulation of the animal. The protocol used should be standardized for all males to reduce variability and allow comparison to other collections. Howard suggests a series of three sets of stimulations with gradual increases in voltage. The first two sets contain thirty stimulations each. The third set has 20 stimulations. For the first set, the voltage is held at zero then raised quickly to 2 volts, held at 2 volts for two to three seconds, returned to zero for three seconds, and repeated for a total of ten stimulations. The eleventh through twentieth stimulations are increased to three volts. The final ten stimulations of the first set are raised to 4 volts. After 2-3 minutes of rest, the second set follows the same pattern as the first, but it begins at 3 volts and is raised to 5 volts. The final set involves ten stimulations at 4 volts and 10 stimulations at 5 volts. An ejaculate should be obtained in 15-20 minutes.

Everything that comes in contact with the semen should be pre-warmed to 37 °F to prevent cold shock which will reduce motility. Urine is a possible toxic contaminant of electroejaculated samples. It occurs when the rectal probe has been positioned too far cranially or too high of a voltage has been used. This problem can be avoided by catheterizing the bladder and removing the urine prior to electroejaculation.

Another spermatozoa collection technique is post-mortem following an acute death. Sterile technique is necessary. The tail of the epidiymus and the ductus are dissected free of the testis. The ductus lumen is flushed with a rinsing medium such as physiologic saline through the epididymal tail and body. The tail is then minced in 10 to 20 milliliters of the rinsing medium in a collection dish. This suspension is filtered through a sterile gauze, or a 0.4 to 0.75 micron filter, to remove the ductus debris. Collect the filtrate in a sterile tube. This is the sperm rich fraction. Centrifuge at 300 x g for five minutes. Remove the supernatant. The pellet can be resuspended in the medium it is to be used in. Collection needs to be done within twelve hours of death. Epididymal spermatozoa have poor motility when first collected. However, incubat-

ing them in culture medium or physiologic phosphate buffered saline for fifteen minutes will improve motility. The cause of death should be determined to avoid transmitting any infectious diseases. After collection, the semen needs to be evaluated before its intended use.

Evaluation: A semen sample collected by electroejaculation versus AV collection has a greater volume, lower sperm concentration, and increased pH. Electroejaculation produces a higher volume of sperm free seminal plasma due to overstimulation of the prostate and bulbourethral glands. The parameters used to evaluate semen include: volume, percent motility, progressive motility, morphology, concentration, pH, and acrosomal integrity. All evaluation is done on slides prewarmed to 37 °C.

Motility is evaluated by placing a drop (5ul) of the semen sample on the warmed slide. A cover slip is placed overtop. At low power, the general percentage of motile sperm is estimated. At 250X-5400X the percentage of four fields are estimated for a better idea of the motility. At this power, the quality of motility progression can be graded on a scale of 0-5. Table 2 describes the scoring. Briefly, zero is non-motile while five is good motility with rapid forward progression. The spermatozoa need to have forward progression to be functional.

Morphology is evaluated by fixing 20ul of semen with 0.5ml of glutaraldehyde in 1% physiologic saline, then staining with silver nitrate or green/rose bengal. Using 1000X and phase contrast microscopy, 100 spermatozoa are categorized as normal and abnormal. Abnormal are further classed as having a primary defect or a secondary defect. Primary defects include: abnormal head size, bicephalic, tricephalic, abnormal acrosome, tightly coiled flagellum, and multiple flagella. Secondary defects include: bent mid-piece with or without cytoplasmic droplet, and a bent flagellum with or without a cytoplasmic droplet. The primary defects originate within the testis and are more detrimental to fertilizing ability. The morphologic abnormalities of an animal are consistent regardless of the collection method, ie. electroejaculation or AV, or the frequency of collection. Generally, non-domestic felid semen has a high degree of pleomorphism averaging 40%.[8] The cheetah has up to 80% pleospermia. The exact reason for this is not known, but inbreeding and/or the stress associated with a captive situation could be underlying factors.

Acrosome evaluation should be performed because an intact normal acrosome is necessary for fertilization. However, acrosomal evaluation in non-domestic felids is difficult because of the normally thin acrosome structure. Gross changes can be discerned with light microscopy at 1000X. However, evaluation of the felid's acrosomal ultra-structure requires the use of electron microscopy. The acrosome's ultra-structural integrity is sensitive to freeze/thawing. Therefore, the acrosome should be evaluate whenever frozen sperm is utilized. Post-thaw acrosomal integrity has been used to evaluate new freezing protocols in many species of animals.

Functional test: No single evaluation parameter can determine the functional fertility of spermatozoa. Other factors such as season variability and stress levels should be considered. The male should be collected and evaluated three to four times at three week intervals before declaring him infertile or unsatisfactory. *In vitro* functional tests are available to evaluate penetrating and fertilizing ability of spermatozoa. These function tests can be valuable in demonstrating the fertility of a male before using the semen on the valuable oocytes of endangered animals. However, the expense and labor involved have made the tests underutilized.

The zona pellucida penetration test evaluates the ability of heterologous capacitated spermatozoa to penetrate a fresh or salt stored domestic cat ova with an intact zona pellucida. The capacitated spermatozoa are incubated with the intact domestic cat ova in an appropriate cell culture medium. Functional sperm will be able to attach and penetrate the zona pellucida and perhaps even get into the perivitelline space. Those with deformities may be able to attach but will not penetrate. After incubation, the ova and sperm are washed with media. The number of sperm in the zona pellucida and the depth reached reflects the penetrating ability of the spermatozoa. This test can also be used as an index of capacitation success and evaluation of semen post-thawing. The penetration test is made more practical by the readily available source of domestic ova from ovariohysterectomized cats.

Fresh or salt-stored hamster ova with the zona pellucida removed can be used to evaluate the ability of the spermatozoa to fertilize an ovum. The ova and the heterologous spermatozoa are incubated together for three hours in cell culture media. The ova are then collected and

flattened under a coverslip to better display oocyte detail. The functional sperm will insert into the ova cells. This will be evidenced by swelled, darkened vitelline cells, and decondensation of the spermatozoa nuclear material. This test can be done *in vitro* and utilizes a readily accessible supply of domestic ova. Non-domestic felid semen *in vivo* fertility has been shown to correlate well with this *in vitro* test.[7]

Processing and Capacitation: Factors affecting sperm viability after collection include: culture conditions, medium composition, pH, and temperature. Seminal fluids can compromise sperm viability. Raw non-domestic sperm remains motile less then two hours post-collection.[12] Motility can be extended by diluting the semen with standard tissue culture medium such as Krebs-Ringer bicarbonate, or Ham's F10. The semen needs to be washed before use to limit transmission of infectious disease and to prolong the spermatozoa viability. The semen is spun into a pellet with low speed centrifugation at 300g for 5-10 minutes. The seminal plasma is poured off. The pellet can than be resuspended in the media.

As mentioned before, non-domestic felid semen has high proportions of abnormal spermatozoa which may be a factor in poor pregnancy rates. The proportion of normal sperm can be concentrated on the basis of motility and morphology, thereby improving the quality of the sample. A commonly used process to improve sample quality is the Swim-up procedure. The raw semen is diluted with culture medium, then centrifuged at 300g for ten minutes. The supernatant is removed. A layer of fresh media is gently layered onto the pellet. Incubate the tube for 1-2 hrs at 37 °C. Motile sperm will migrate into the media. Evaluate the supernate as previously described. The result should be increased proportions of motile and morphologically normal spermatozoa.

Capacitation is accomplished by incubating fresh collected and/or washed spermatozoa in Tyrodes solution, or another cell culture medium, containing bovine serum albumin (BSA, 4.0mg/ml) for one hour. These spermatozoa can then be co-cultured with zona pellucida intact domestic cat oocytes, as previously described, to index the capacitation. BSA facilitates the *in vitro* capacitation process. The mechanism is not known. Domestic cat spermatozoa and Leopard cat spermatozoa penetration rates increased from 14% and 7% respectively in BSA free Tyrodes solution to 60% and 58% when BSA was included.[1]

Cryopreservation: Published reports of cryopreservation protocols in non-domestic felids are few. Specific basic research into cryopreservation techniques have not been done for each species. Techniques developed for domestic cats may be applicable to the nondomestic felids. However, no one protocol is satisfactory for all species. Therefore, before beginning cryopreservation of non-domestic felid semen on a large scale, the protocol to be used should be thoroughly evaluated with relation to effect on motility, structure, and overall fertility post-thaw as compared to pre-preservation values. In general, semen should have at least 40% motility and progressive motility of 3 or better (0-5) pre-preservation.[17] The use of specific cryprotectants in felids has also not been adequately documented.

Monitoring/Influencing Female Reproductive Physiology

Females of the felidae species each have unique, subtle differences in their reproductive physiology. For example, the length of estrus in the African wild cat is 2 to 3 days while that of the jaguar is 6 to 17 days. Insufficient research has been done to broadly define these differences across species lines. Monitoring the physiology and endocrine status of the captive felid has been an obstacle to properly defining species specific estrous cycles. Daily serum samples are the best method to monitor hormonal changes. However, this would require daily immobilization of the non-domestic felid because of the danger of working with these animals. Estradiol and progesterone metabolite levels have been successfully monitored using daily urine samples via radioimmunoassay. To account for daily differences in urine volume and concentration, the urine metabolite levels need to be compared to the urine creatinine concentration. Creatinine is excreted at a constant rate and serves as a constant comparative.[15] Currently, work is being done at the National Zoo in Washington to read estradiol and progesterone metabolite levels in feces.[2] This would greatly facilitate the monitoring of estrous cycles. Daily sample collection could be easily incorporated into the animal keepers routine. Such a system would be invaluable towards broadly defining the hormonal schemes of non-domestic felids.

For success of assisted reproduction techniques, the stage of estrus needs to be known. Therefore, ovarian activity is often induced with exogenous gonadotropins to allow assisted reproduction techniques to proceed with the best chance for success. The gonadotropins currently used are PMSG and FSH-P which induce follicular development, and hCG which induces ovulation. There is insufficient data on the rare animals to determine what an adequate regimen for each species is. A regimen developed in the domestic cat has effectively induced follicular development and ovulation in Siberian tiger, puma, and cheetah.[5,10,18] It consists of one injection of 1000-2000 IU PMSG intramuscularly followed 84 hours later with one injection of 75-100 IU hCG intramuscularly. Ovarian activity can be induced year round, however, it is often done in the non-breeding season. At this time, there will be less endogenous hormonal activity to interfere with the administered hormones. Concern about development of antibodies and resulting refractory responses to these exogenous gonadotropins needs to be kept in mind with repeated use.

Artificial Insemination

Artificial insemination (AI) increases the efficient use of valuable male genetics, ensures reproduction between two valuable but behaviorally incompatible animals, eliminates the risks of animal transport, and can infuse new genetics into a stagnant population from semen collected in the wild. Surgical and non-surgical AI methods are available. Vaginal deposition of semen has not succeeded in consistently establishing pregnancy in non-domestic felids. Laparoscopic transabdominal AI has shown promise as a reliable technique to assist reproduction in non-domestic felids. Factors associated with failure of vaginal deposition include the high degree of pleiomorphic sperm in non-domestic semen, cervical filtering of these spermatozoa, altered uterine motility and tone due to exogenous gonadotropins, and evidence that anesthesia inhibits ovulation and uterine motility.[11] Laparoscopic AI bypasses the cervix and places the spermatozoa in close proximity to the site of fertilization. Successful pregnancies have been established with this procedure in the puma, cheetah, and tiger.[5,10,19] Failure associated with laparoscopic AI may be due to lack of knowledge of the species specific reproductive physiology, improper timing of estrus with relation to time of insemination, improper site of insemination, alteration of endocrine scheme with use of exogenous gonadotropins.

Technique: The laparoscopic AI procedure has been previously described.[11,21] Initially, the ovaries are stimulated to produce follicles using the gonadotropin regimen previously described. General anesthesia using ketamine and xylazine is performed 25-50 hours after the hCG injection. Anesthesia is maintained chemically or with gas. The animal is clipped ventrally and surgically prepared for the laparoscopic procedure. The equipment necessary includes: verres needle, insufflator, trocar cannula (710mm diameter), laparoscope with light source and 180 degree field of vision, accessory forceps, indwelling feline catheter, sterile polyethylene tubing, 30 gauge needle, 1ml syringe, and spermatozoa suspension. The verres needle is inserted lateral to the midline and is attached to the insufflator. Its purpose is to create a pneumoperitoneum using either 100% CO_2 or room air. The trocar cannula is inserted into the peritoneal cavity through a one centimeter skin incision located three centimeters cranial to the umbilicus. The trocar is removed and replaced with the laparoscope of similar diameter.

Post-ovulation ovaries are desirable to increase success of the AI procedure. The ovaries are observed for the presence and number of follicles, and the presence and number of corpora lutea and corpora hemorrhagica. Mature follicles are clear and two to four millimeters in diameter. They are spherical or slightly raised. The corpora lutea are generally four millimeters in diameter, opaque, dark red, and raised two to three millimeters. The animal is considered post-ovulatory if there is a minimum of one CL present.

The accessory forceps are placed 3 cm caudal and 4 cm lateral to the umbilicus. The forceps are used to manipulate and stabilize the reproductive tract. The uterine horn is elevated towards the ventral body wall. It is cannulated using the sterile feline catheter. The catheter is placed into the uterine lumen in the proximal one-third of the uterus. It is important to make certain the end is in the lumen and not in the submucosa. Once in place, the stylet is removed and replaced with sterile polyethylene tubing that has a 30 g needle attached. The other end has a 1ml syringe with 100ul of suspension containing 0.1 x 106 to 0.2 x 106

capacitated spermatozoa. The sample may be a raw collection, extended, or frozenthawed. The tubing is advanced into the lumen. An assistant slowly and gently expels the inseminant. The same technique is done with the opposite horn. The incisions are sutured and the animal is awakened. Total elapsed time from electroejaculation to artificial insemination should take 30 min. to one hour. The animals are evaluated 45 and 60 days later, either ultrasonically or radiographically, for pregnancy. Further research needs to be done to define the reproductive physiology of the female and the male to make success using laparoscopic AI a routine occurrence.

Embryo Manipulation

Embryo manipulation techniques show promise towards greatly advancing conservation biology. Zoo researchers employ several techniques for successful embryo manipulation including: laparoscopic transabdominal oocyte aspiration, oocyte *in vitro* maturation, oocyte *in vitro* fertilization (IVF), embryo assessment, and embryo transfer (ET). Embryo manipulation provides many advantages. It allows for more efficient use of the genetics of a valuable female that may be physically incapable of reproducing due to injury, disease, or age. The genetics from valuable females can be proportionately and rapidly distributed through the population by transfer to recipients from a closely related, yet more abundant species. The oocytes can be recovered and utilized following the acute death of an adult or a prepubertal animal. The need to detect overt estrus is eliminated as the gametes are cultured and brought together *in vitro* under optimal conditions. Embryo cryopreservation will allow future use of genetics.[24] These embryo manipulation techniques have already had preliminary success. The birth of a Siberian tiger cub was accomplished at Omaha's Henry Doorly Zoo.[4] Successful interspecific embryo transfer has been accomplished with the transfer of Indian desert cat embryos to a domestic cat.[22] Embryo manipulation procedures in non-domestic felids is still in preliminary stages. More research is needed to make successful pregnancies routine. The various embryo manipulation procedures used in zoos are briefly described here.

Oocyte collection: Oocyte collection is often performed via transabdominal laparoscopic aspiration as described.[18] Direct laparoscopic aspirations of oocyte from pre-ovulatory follicles provides an efficient and thorough method to maximize the oocyte harvest from the female. It also allows for the *in vitro* evaluation and sorting out of the higher quality oocytes more likely to survive to fertilizition and transfer.

The domestic cat again serves as the model for the procedure. The ovaries are stimulated with exogenous gonadotropins following the same regimen as described for AI. However, laparoscopic examination of the ovaries is done 24-26 hours post hCG injection to find the follicles in a pre-ovulatory state. General anesthesia and laparoscopic preparation is the same as previously described for AI. The verres needle is again used to create a pneumoperitoneum as well as to manipulate the organs. The ovaries are evaluated with the laparoscope. The number of follicles are counted for later assessment of the completeness of collection. Next, a 4 cm long, 22g needle is placed on the end of 100 polyethylene tubing (id .86 mm). The tubing is rinsed with two to three ml of mKRB that has had 4mg bovine serum albumin/ml and 40 units heparin/ml added to it. A similar preparation of Ham's F10 would also work. At the opposite end of the tube, a 7ml siliconized collection tube, containing one of the previously mentioned cell culture mediums, is attached. The system is driven by a vacuum pump which will apply a negative pressure of 100mmHg for aspiration. The reproductive tract is stabilized with the verres needle. The aspiration needle is then inserted through the abdominal wall ventral and medial to the ovary. Follicles measuring at least 2mm diameter are perforated with the needle. Negative pressure is applied. Each visible follicle is perforated on each ovary.

Oocyte evaluation: Once the aspirations are complete, the collection tubes are emptied into plastic 35mm culture dishes which are then examined through a stereomicroscope. Preliminary evaluation of the oocytes is necessary to separate degenerated and immature oocytes from the mature ones. The oocytes are removed from the debris using a micropipette with suction provided by an attached mouth pipette. They are placed in a new culture dish with fresh cell culture medium. The oocytes are then examined and assessed. They are categorized as mature when the corona radiata and the cumulus oophorus cells are loosened in appearance and expanded, immature when the corona radiata cells are tightly compacted around the

oocytes, and degenerate when the oocyte is abnormally pale and /or lacks any corona radiata cells around it. The mature and immature oocytes are placed in separate culture dishes. All oocytes are washed three times with fresh medium while under light weight paraffin oil. The overlying paraffin oil maintains the oocytes within cell culture medium droplets that form when the oil is layered atop. The immature oocytes will need to be cultured in cell culture medium for another 24 hours and then reassessed. All oocytes are incubated in 5% CO2 at 38 °C. The oocytes can tolerate lower temperatures for short periods of time, however, they are very susceptible to higher temperatures. Mature oocytes are prepared for IVF. After 24 hours, the culturing immature oocytes are re-evaluated. If they have matured, they are inseminated as will be described.

In vitro **fertilization:** The IVF procedure has been described.[18] No more than ten mature oocytes should be within a medium droplet under the paraffin oil. The inseminant could be from any of the previously mentioned sources, fresh or frozen. Each medium droplet is inseminated with $0.1X10^6$ to $0.2X10^6$ viable spermatozoa in 100ul of medium. The oocytes and spermatozoa are co-cultured at 38 °C in humidified 5% CO2 for 18-20 hours.

After the co-culture period, the oocytes are removed from the co-culture medium and washed three times in .2% hyaluronidase solution, for three minutes each time, to remove residual cumulus cells and loosely attached spermatozoa. They are returned to the incubator in fresh equilibrated culture medium and examined 24 hours post-insemination to evaluate fertilization success. The criteria for determining if fertilization did occur is the presence of two polar bodies, two pronuclei, or cleavage to at least the two cell stage. If there are more than two nuclear structures in the cytoplasm, the oocyte is considered polyspermic and discarded. The cytoplasm of felid oocytes is often very dark and opaque making it difficult to assess the nuclear structures. These oocytes can be spun at low speed centrifugation (200-300 g) for five minutes. This will cause the opaque cytoplasmic material to shift to one side, while the internal structures remain in place. This does not damage the oocyte function.[17]

Embryo transfer: Embryos are examined under the stereoscope. They are assessed as good or excellent if they are symmetrical, spherical, and uniformily dark. A fair to poor rating is given if the embryo has degenerated somewhat and appears pale, misshapened, and/or has fragmented cells within it. The good to excellent embryos are collected in cell culture medium at the two to four cell stage for transfer into the oviducts of the recipient. This recipient may be the same individual or another female of the same species. Work is being done to develop techniques for interspecific transfer. Such techniques would allow for the widespread transfer of embryos from rare, severely endangered animals to more abundant recipients. Thus, speeding the regeneration time of the rare species. Whatever the origin, the recipient has undergone simultaneous gonadotropin treatments to synchronize her estrous cycle with that of the donor. Estrous cycle synchronization can be achieved using the previously described gonadotropin regimen.

The transfer technique discussed here has been described.[4] General anesthesia is required so the reproductive tract can be bilaterally exposed via ventral midline laparotomy . The procedures for anesthesia and surgical preparation have been described elsewhere. Once the reproductive tract has been exposed, the ovaries are examined for CL development and the presence of pre-ovulatory follicles. The uterine horn ipsilateral to the ovary with the most CLs is used for transfer. The embryos are aspirated from the culture dish using a syringe attached to 50 polyethylene tubing into two milliliters of physiologic saline. Twelve to sixteen embryos are transferred into the horn. The tip of the tubing is placed into the fimbriated end of the oviduct. Gentle pressure on the syringe expels the embryos into the horn. Once done, the tubing should be checked with the stereoscope to ensure that all the embryos have been expelled.

Theoretically, the synchronized estrous cycle of the recipient and the presence of the CL should be sufficient for pregnancy to establish. The recipients are checked 45 and 60 days later with ultrasound or radiography to determine if the animal is pregnant. In the study performed by Donoghue, et al, the donors were also used as the recipients with the exception of another tiger that served only as a recipient. Successful pregnancy was only achieved in the tiger that had not served as a donor and had been supplemented at the time of transfer with a silastic progesterone implant containing 35 mg crystalline progesterone. An explanation provided was that the aspiration process may

have removed too many of the cells necessary for adequate CL formation. Therefore, the progesterone output of these animals, may have been insufficient to maintain the pregnancy in the early stages. Interspecific embryo transfer was successful from the Indian desert cat to a domestic cat recipient.[22] These recipients were not supplemented with progesterone. Further research is necessary to determine the response of the embryo donor to exogenous hormones, to establish methods to assess and adjust recipient estrus to prevent embryo rejection, to develop safe and effective transfer techniques, and more fundamental research into basic embryology is necessary to make embryo transfer a successful and valuable tool in conservation biology and species preservatlon.

Conclusion

Assisted reproduction techniques have proven successful in our domestic livestock industry. The basic research has been done to define the physiology of the animals, the financial support is available, and the data base is extensive. Lack of basic research due to lack of money, lack of interest, and the difficulty of working with the rare, non-domestic felids has been the primary obstacle to the widespread successful utilization of assisted reproduction technologies in non-domestic felids. Preliminary successes have been achieved. However, more research needs to be done to define the physiology of male gamete production and capacitation, to develop safe and effective gamete recovery protocols, to define oocyte maturation requirements, to define the effect of exogenous hormones on oocyte maturation and the effects of exogenous hormones and stress on reproductive physiology, and to establish the placental-uterine relationships within individual species.

This paper has briefly discussed the assisted reproduction techniques commonly used in zoos today. Much more work is ongoing in several areas including interspecific embryo transfers and gamete cryopreservation. While the challenge to preserve genetic lines is great, advances in non-domestic animal assisted reproduction continue to come from a few dedicated researchers. With more research and support, the routine propagation of animals through the use of AI, IVF, and embryo transfer will become a reality and help to preserve the wild felids.

References

1. Andrews J. C., et al.Sperm Capacitation in the Domestic Cat *(Felis catus)* and Leopard Cat *(Felis benalensis)* As Studied With a Salt-Stored Zona Pellucida Penetration Assay. *Molecular Reproduction and Development.* 31: 200-207. 1992.

2. Armstrong D L. Personal communication. Omaha Henry Doorly Zoo. Nov. 28, 1993 to Jan. 28, 1994.

3. Boever W J. Medical Input into Species Survival Plans. Zoo and Wild Animal Medicine: Current Therapy. vol. 3. Murray E. Fowler, ed. Saunders, Philadelphia. 11-14. 1993.

4. Donoghue A M., et al. *In vitro* Fertilization and Embryo Development *in vitro* and *in vivo* in the Tiger *(Pantheris tigris). Biology of Reproduction.* 43: 733-744. 1990.

5. Donoghue A. M., et al. Birth of a Siberian Tiger Cub (*Fanthera tigris altaica*) Following Laparoscopic Intrauterine Artificial Insemination. *Journal of Zoo and Wildlife Medicine.* 24(2): 185-189. 1993.

6. Englert Y., et al. Comparative Auto-Controlled Study Between Swim-up and Percoll Preparation of Fresh Semen Samples for *in vitro* Fertilization. *Human Reproduction.* 7(3): 399-402. 1992.

7. Goodrowe D. L., Howard J. G., Wildt D E. Embryo Recovery and Quality in the Domestic Cat: Natural Versus Induced Estrus. *Theriogenology.* 25(1): 156. 1986.

8. Howard, et al. Comparison of Ejaculate Characteristics in Non-Domestic Felids with Emphasis on Sperm Morphology. *Abstracts of Papers of the Annual Meeting of the AAZV,* Louisville. 168-169. 1986.

9. Howard JoGayle, Bush M, Wildt. D E. Semen Collection, Analysis, and Cryopreservation. In: Nondomestic Mammals. in Theriogenology: Current Therapy, doasiu. l047-1053. 1986.

10. Howard JoGayle, et al.Successful Induction of Ovarian Activity and Laparoscopic Intrauterine Artificial Insemination in the Cheetah *(Acinonyx jubatus). Journal of Zoo and Wildlife Medicine.* 23(3): 288-300. 1992.

11. Howard J G, et al. The Effect of Pre-Ovulatory Anesthesia on Ovulation in Laparoscopically Inseminated Domestic Cats. *Journal of Reproduction and Fertility.* 96:175-186. 1992.

12. Howard JoGayle. Semen Collection and Analysis in Carnivores. Zoo and Wild Animal Medicine: Current Therapy. vol. 3. Murray E. Fowler, ed. Saunders, Philadelphia. 390-399. 1993.

13. Hutchins M, Wiese R J. Beyond Genetic and Demographic Management: The Future of the Species Survival Plan and Related AAZPA Conservation Efforts. *Zoo Biology.* 10: 285-292. 1991.

14. Killen I. D., Caffery G J. Uterine insemination of Ewes with the Aid of a Laparoscope. *Australian Veterinary Journal.* 59: 95. 1982.

15. Lasley, B.L., et al. An update on Endocrine Monitoring of Reproductive Physiology. *Abstracts of Papers of the Annual Meeting of the AAZV,* Louisville. 165. 1986.

16. Lasley B L., Shidler S E. Methods for Assessing Reproduction in Non-Domestic Species. Zoo and Wild Animal Medicine: Current Therapy. vol. 3. Murray E. Fowler, ed. Saunders, Philadelphia. 79-86. 1993.

17. Luskotuff N M. Personal communication. Omaha Henry Doorly Zoo. Jan. 17, 1994.

18. Miller A M, et al. Oocyte Recovery, Maturation, and Fertilization *invitro* in the Puma *(Felis concolar). Journal of Reproduction and Fertility.* 88: 249-258. 1990.

19. Moore H. D. M, Bonney R C, Jones D M. Successful induced ovulation and artificial insemination in the puma *(Felis concolor). The Veterinary Record.* 108: 282-283. 1981.

20. Munson L. Inbreeding and disease in Captive Wild Animals. Zoo and Wild Animal Medicine: Current Therapy. vol. 3. Murray E. Fowler, ed. Saunders, Philadelphia. 73-78. 1993.

21. Phillips L. G., et al. (1982). Gonadotropin Regimen for Inducing Ovarian Activity in Captive Wild Felids. *Journal of the American Veterinary Medical Association.* 181(11): 1246-1250. 1982.

22. Pope C. E., et al. Successful Interspecies Transfer of Embryos From the Indian Desert Cat *(Jelis silvestris omata)* to the Domestic Cat *Felis catus). Biology of Reproduction.* 40 (suppl. 1): 61 (abstract 40). 1989.

23. Post G. S., et al. Evaluation of Siberian Tiger Sperm Using Zona-Free Hamster Ova. *Abstracts of Papers of the Annual Meeting of AAZV.* Atlanta. 153-154. 1984.

24. Summers P., HearnJ P. Embryo Manipulation for the Regulation of Reproduction and Disease. *Symp. Zool. Soc. Lond.* 60: 119-134. 1988.

25. Wildt David E., et al. Adrenal-Testicular-Pituitary Relationships in the Cheetah Subjected to Anesthesial Electroejaculation. *Biology of Reproduction.* 30: 665-672. 1984.

26. Wildt David E., et al. Developing Animal Model Systems for Embryo Technologies in Rare and Endangered Wildlife. *Theriogenology.* 25(1): 33-51. 1986.

27. Wildt D. E. Reproductive Research in Conservation Biology: Priorities and Avenues for Support. *Journal of Zoo and Wildlife Medicine.* 20(4): 391-395. 1989.

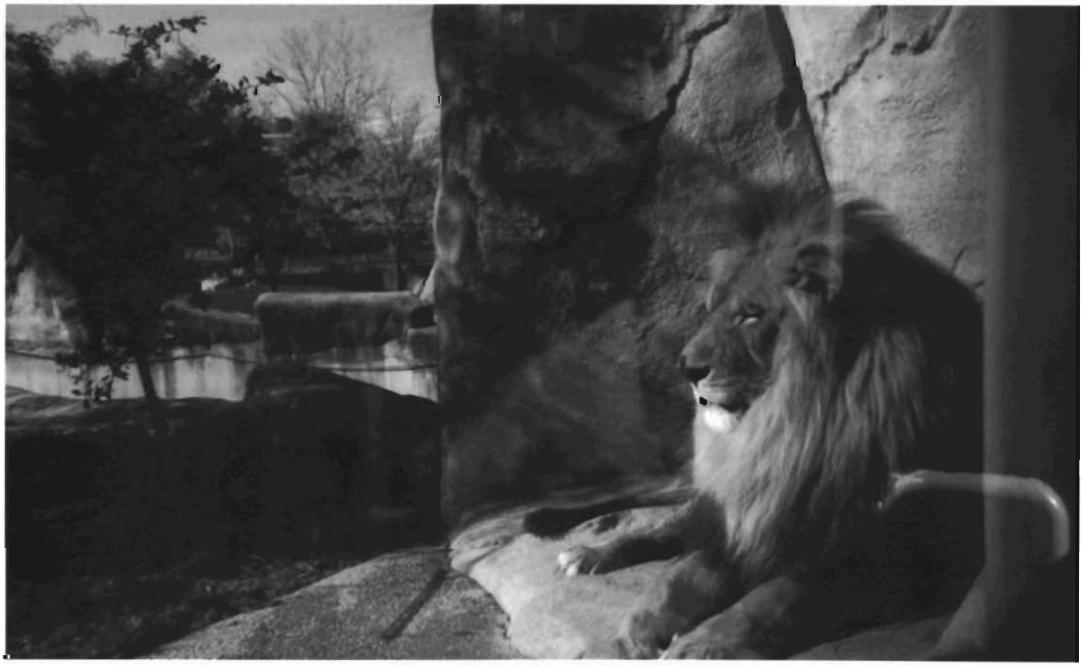
28. Wildt D. E., et al. Embryogenesis in Conservation Biology-or, How to Make an Endangered Species Embryo. *Theriogenology.* 37(1): 161-184. 1992.

Table 1: Felid Species with Established Species Survival Plan, (Boever, 1993)

Cheetah	Generic tiger
Clouded leopard	Siberian tiger
Snow leopard	Sumatran tiger

Table 2: Progressive Motility Grading (Howard, 1986)

0—no motility
1—slight motility, no forward progress
2—moderate side-to-side progression, occasional forward progression
3—slow forward progression
4—steady progression
5—rapid forward progression



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