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Wildlife Cats Reproductive Biotechnology

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1. Introduction

The techniques used in assisted reproduction for wildlife or domestic animals are similar, and consist in the collection, evaluation, and cryopreservation of semen; artificial insemination (AI), *in vitro* fertilization (IVF), and embryo transfer (ET).

Considering that the maintenance of genetic diversity is reproduction dependent, reproduction technologies are important tools for the preservation of endangered species, throught the development of methods to increase the fertility in these animals (Howard, 1993).

Several studies show that hormone therapies may induce ovulation and/or superovulation in wild animals (Pope, 2000). The artificial induction of ovulation and superovulation are important components in assisted reproduction techniques, especially in cats, because their ovulation is induced by external stimulations (Hamner et al., 1970).

According to The Word Zoo Conservation Strategy ([IUDZG/CBSG], 1993), some of the many benefits from using assisted reproduction techniques in conservation management programs are:

1. To enable the exchange of genetic material between two or more zoos collaborating in the same program, including animals in captivity and in the wild. Semen transportation is economical and reduces the risks involved in animal transfers and consequently diseases transmission;
2. To enable reproduction in animals with physical and reproductive behavioral disabilities. This is important for animals that are representative of genetic lines that cannot be lost;
3. To enable rapid population growth when only a small founder population is available;
4. To assist in maintaining the ratio between males and females by selectively transplanting embryos of one sex;
5. To determine the number of offspring per individual;
6. To promote the formation of databases for gametes and embryos from species of interest.

The reproductive techniques used for domestic animals are gradually being used for zoo animals (Comizzoli et al., 2000; Dresser et al., 1986).

Artificial insemination has been conducted in different species of carnivores such as cougar (*Felis concolor*), leopard (*Panthera pardus saxicolor*), cheetah (*Acinonyx jubatus*), tiger (*Panthera tigris altaica*), ocelot (*Leopardus pardalis*) tigrina (*Leopardus tigrinus*), and jaguar (*Panthera onca*) (Dresser et al., 1982; Donoghue et al.; 1993, Howard et al., 1992a; Jimenez et al., 1999; Moore et al., 1981; Moraes et al., 1997; Silva et al., 2000; Swanson et al., 1996a).

In vitro fertilization (IVF) has also been performed in captive wild cats such as tiger (*Panthera tigris altaica*), jaguar (*Panthera onca*), ocelot (*Leopardus pardalis*) and tigrina (*Leopardus tigrinus*) (Donoghue et al., 1990; Morato et al., 2000; Swanson & Brown, 2004).

The application of artificial reproductive methods in wild animals has not being successful, showing low reproductive rates. Some of the several reasons for these low reproductive rates are lack of knowledgement on the species' physiology, poor sperm or oocytes quality, and difficulties in adapting from the methodologies used in experimental models.

Considering the zoos' limitation to maintain genetically viable populations of threatened species, the establishment of genetic banks containing semen, oocytes, embryos and cells emerge as a strategy to ensure the genetic diversity of populations (Lasley et al., 1994). The potential of assisted reproduction for endangered species should be emphasized by the possibility of semen and embryos cryopreservation, which are genetically valuable for the future populations.

Although there are some records related to the female reproductive physiology (Table 1) and semen characteristics for various Neotropical felids species (Table 2), there is a little knowledge about the fertilization ability using artificial methods (Table 3) in these animals. Studies about the application of assisted reproduction techniques in Neotropical felids showed limitations because of the lack of basic knowledge on the physiology of the species.

2. General reproductive characteristics in wildlife cats

The large number of felines and their wide geographical distribution determine many of the species' particularities, mainly in the reproductive aspects. The reproductive seasonality is one of the most variable aspect between species.

In domestic cats (*Felis catus*), the reproductive seasonality is related to photoperiod (Johnston et al., 1996; Shille et al., 1979; Tsuitsui & Stabenfeldt, 1996), whereas in wild animals, it is also related to high or low food supply during the seasons (Ewer, 1975).

The ovulation mechanism is another variable aspect among cat species. Studies evaluating serum hormone levels confirmed that some wild cats like tiger (*Panthera tigris*) (Seal et al., 1985), snow leopard (*Panthera uncia*) (Schmidt et al., 1993), jaguars (*Panthera onca*) (Wildt et al., 1979) and cougars (*Puma concolor*) (Bonney et al., 1981), have induced reflex ovulation, similar to the domestic cat (Johnston et al., 1996; Shille et al., 1979; Tsuitsui & Stabenfeldt, 1996).

However, female leopards (*Panthera pardus*) presented two ovulation mechanisms in two different situations. When kept isolated, they showed typical hormonal profile for ovulation reflex mechanism; but, when housed in pairs with another female, the ovulation was probably stimulated by physical contact (Schmidt et al., 1988).

Lionesses (*Panthera leo*), when isolated from the males, showed an ovulation pattern distinct from other cats. Schmidt et al. (1979), using serum hormone levels and corpus luteum

visualization, demonstrated that this species presents spontaneous ovulation in a higher frequency compared to what is described for other felines.

The genus Leopardus is polyestral and can cycle all year round (Morais et al., 1996; Moreira et al., 2001); the margay (*Leopardus wiedii*) is the only species in this genus presenting spontaneous ovulation (Moreira et al., 2001).

According to Tebet (1999), the estrous cycle in ocelots is characterized by the presence of serum estradiol peaks associated with relatively low levels of serum progesterone (<2.61 ng/mL). This demonstrates the polyestral characteristic of this species, similar to previously observed for other species such as cats (Shille al., 1979; Tsuitsui & Stabenfeldt, 1996; Verhage et al., 1976), tigers (Seal et al., 1985), and cheetahs (Brown et al., 1996).

Ocelot females that ovulated and were not fertilized showed a period of increased progesterone serum concentration and estrus inhibition (Tebet, 1999), called pseudopregancy or diestrus, similar to domestic cats (Feldman & Nelson, 1996) and leopards (Schmidt et al., 1988).

Under an evolutionary analysis and among other factors, the process of spontaneous or induced ovulation may be related to the sociability of the species. Thus, solitary cats would require a longer estrus period, extended viability of oocytes, and extended time for the ovulation to occur, after the couple meet in the wild (Ewer, 1975).

The detection of fecal estrogens and progestins, throught the analyses of fecal metabolites in domestic and wild cats such as the leopard cat (*Felis bengalensis*), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), and snow leopard (*Panthera uncia*) was successfully performed by Brown et al. (1994). Likewise, this methodology has been widely used to monitor ovarian function in Neotropical felines such as the ocelot and margay (Morais et al, 1996; Moreira et al., 2001).

In this context, noninvasive methods such as the quantification of fecal hormonal metabolites are increasingly being used in wildlife animals.

The estrus cycle, gestational time, and number of pups observed in different species of Neotropical wild cats are presented in Table 1.

Common Name	Scientific Name	Estrus (days)	Estrous Cycle (days)	Gestation (days)	Pups (n°)
Ocelot	*Leopardus pardalis*	4.63±0.63[1]	16.5 ± 1.5[2] 18.4±1.6[4]	70-85[6]	1-4[6]
Tigrinus	*Leopardus tigrinus*	3.0-9.0[5]	15.8 ± 1.5[2] 16.7±1.3[4]	73-78[6]	1-4[6]
Margay	*Leopardus wiedii*	4.0-10.0[5]	19.5 ± 2.1[2] 17.6±1.5[4]	81-84[6]	1[6]
Geofroy's cat	*Oncifelis geoffroyi*	2.5±0.5[1]	20.0[5]	72-76[6]	1-3[6]

Common Name	Scientific Name	Estrus (days)	Estrous Cycle (days)	Gestation (days)	Pups (nº)
Pallas cat	*Oncifelis colocolo*	-	-	80-85[6]	1-3[6]
Jaguarondi	*Herpaelurus yaguarundi*	3.17±0.75[1]	53.63±2.41[1]	72-75[6]	1-4[6]
Puma	*Puma concolor*	8.0[5]	23.0[5]	84-98[6]	1-6[6]
Jaguar	*Panthera onca*	12.0±1.0[3]	47.2±5.4[3]	90-111[6]	1-2[6]

Mellen 1989[1]; Morais et al., 1996[2]; Morato & Paz, 2001[3]; Moreira et al., 2001[4]; Oliveira, 1994[5]; Oliveira & Cassaro, 1997[6].

Table 1. Neotropical wildlife cats reproductive characteristics.

3. Sperm collection

Two methods are used for the collection of semen from wild animals: the first is the epididymis' semen collection from dead animals or after castration or vasectomy. The second is through an artificial vagina, electroejaculation, or digital manipulation.

3.1 Post mortem sperm collection

In the post mortem sperm collection, immediately after death or castration, the testes should be kept cold at 5°C (Howard, 1993). Generally, postmortem epididymidal spermatozoa remain viable for several days after the animal's death. However, this period depends on the species, the storage conditions of the testes, and methods used for sperm collection. Several techniques could be used including: totally cutting (homogenization) the cauda epididymis; washing out or aspiration (flushing) through sperm duct; making cuts (mincing) in the cauda epididymis and squeezing of content; or just squeezing (pressing). The first two techniques are the most frequently used (Maksudov et al., 2008).

For cats, the epididymis is washed and homogenized in HEPES medium plus Ham's F10, and the sperm is recovered after centrifugation. Live sperm can be recovered within the first twelve hours of the epididymis isolation using this technique (Howard, 1993). In vasectomized animals, the semen is collected by aspiration from the epididymis' tail using syringe and needle. The syringe should contain HEPES medium plus HAM'S F10 and the sperm is recovered after centrifugation.

Sperm recovered from the epididymis of cats are known to be motile, viable, and capable of penetrating oocytes (Goodrowe & Hay, 1993; Hay & Goodrowe, 1993). However, epididymal sperm have naturally more cytoplasmic droplets, which normally are lost during transport through the duct (Briz et al., 1995).

According to Tebet et al. (2006) there were no significant differences between the fresh or frozen-thawed domestic cat spermatozoa for the variables: sperm motility, plasma membrane integrity and morphology of electroejaculated and epididymal spermatozoa analyzed immediately after collection, and after freezing and thawing. Jewgenow (Jewgenow et al., 1997) reported motility of frozen-thawed epididymal sperm of lions (*Panthera leo*), tigers (*Panthera tigris*), leopards (*Panthera pardus*), pumas (*Felis concolor*) and jaguar (*Panthera onca*).

Epididymal spermatozoa were collected from immobilized adult male lion by caudal epididymectomy, cryopreserved, and used for IVF of *in vitro* matured lionesses' ova. The post-thawed motility ranged from 55-65%, and the percentages of fertilized ova were 12.7% and 11.5% for 30 and 36 hours of *in vitro* maturation, respectively (Bartels et al., 2000).

It has been shown, for several species, that the methods developed for ejaculated sperm are effective for freezing epididymidal spermatozoa. However, the physiology of ejaculated and epididymidal spermatozoa are different thus, it can be assumed that optimum methods of freezing and thawing may be different.

Postmortem material that can be retrieved in zoos usually belonged to the aged animals with different diseases or that died because of accidents (stress, fights). Moreover, some species display seasonal breeding. All these factors can influence on the spermatozoa's presence and quality in epidydimis, at the collection time (Maksudov et al., 2008).

Analysis of different factors showed that the concurrency of death with breeding season has the strongest affect on the spermatozoa content in testicles of dead animals. The spermatozoa content and quality were almost equal in males that died of sickness (cancer, chronic cardiovascular or excretory system disorders) and in males that died in accidents (stress, fights). However, the quality of postmortem semen of animals that died of natural death is worse than that of animals that died accidentally (Maksudov et al., 2008).

The energy invested by a male with copulation is minimal compared to that of the female, for whom the costs continue throughout pregnancy and lactation. Therefore, it is a significant genetic advantage for cats to be reproductively active throughout the year, or at least to remain active well outside of the usual female breeding season (Spindler & Wildt, 1999).

This strategy is apparent in wild felids. The female Siberian tiger exhibits more estrual activity in January-June than at any other time of the year (Seal et al., 1985), however, the sperm quality is fairly consistent throughout the year (Byers et al, 1990). The quantity and quality of the sperm from some felids can vary when a distinctive female reproductive seasonality is know, as in the snow leopard (Johnston et al., 1994) and Pallas' cat (Swanson et al., 1996), the males remains reproductively active for longer periods than the females of the same species.

The collection of the postmortem sperm of recently dead animals belonging to endangered species can be of substantial importance, and therefore, the method of choice for the preservation of reproductive cells, in the wild, at zoos, and in national parks. The preservation and utilization of postmortem represent the last chance to obtain offspring from the dead males in cases of unexpected loss of valuable animals (Maksudov et al., 2008).

3.2 In vivo sperm collection

The collection of semen, through digital manipulation or using an artificial vagina are indicated because they promote a natural and normal ejaculation, however, these methods require intensive animal training. These techniques are effective for wild dogs, but no routinely used in wild cats.

The electroejaculation is the most used method in wild cats because it can be performed in anesthetized animals. However, one of the disadvantages of using this method in cats is the urine contamination of the semen. The urine contamination occurs when the voltage exceeds the minimum necessary level for ejaculation or when the electrode is positioned cranially. One alternative to minimize this problem would be the catheterization or cystocentesis before start procedure.

The Tiletamine-Zolazepam combination has been the most used anesthetic protocol for this procedure because it produces insignificant changes in the ejaculate. The ketamine hydrochloride and xylazine association in the same syringe has been used for semen collection in small cats, but according to Dooley et al. (1991), these drugs, in combination, seems to be related to retrograde ejaculation.

The electroejaculator should indicate both the voltage and amperage. The voltage should reach up to 12V, controlled by the command button, which should provide smooth control and gradual increase in power output.

The rectal bipolar electrode used for electroejaculation should have three longitudinal strips of copper. The copper strips must have a 0.4 cm apart and protruding approximately 0.2 cm. The electrode, previously lubricated with mineral oil, should be introduced into the rectum with the longitudinal strips ventrally positioned, applying light pressure to increase contact with the pelvic plexus region. The diameter of the electrode should be specific to each species (Table 2).

The electrical series follows a specific protocol with 80 stimuli divided into three series: 30 (series 1: 10 stimuli at 2, 3 and 4V), 30 (Series 2: 10 stimulations in 3, 4 and 5V) and 20 (series 3: 10 stimulations in 4 and 5V) (Howard 1993). In jaguars, the last series of stimuli can reach up to 6V to achieve ejaculation (Paz et al., 2000).

The stimulation cycle starts at 1 second from 0 voltage to the desired voltage, 2 to 3 seconds at the desired voltage, and 3 seconds returning to 0 voltage. An interval of 10 minutes should be used for resting between sets.

Before the start of the series, the penis must be exposed, examined, and washed with saline solution and gauze. The semen collection should be performed in plastic tubes that are maintained warm at 37° C water bath. For each series, the tubes should be replaced in order to avoid urine contamination. All ejaculates should be used; the total volume of semen is the sum of each ejaculate's volume (Figure 1).

4. Sperm evaluation

The appearance is the first evaluation of the semen: changes in color may be associated with diseases in the accessory organs and testes. The ejaculate's volume is the second aspect to be evaluated and must be determined immediately after collection. The volume provides information about the semen production in different species. The pH determination is important because it may indicate urine (acidic pH) or bacteria contamination (basic pH).

For sperm evaluation, an aliquot of the ejaculate is placed on a microscope slide warmed at 37°C, covered with a warmed glass coverslip, and examined at 400 X magnification. The

semen should be evaluated for motility and progressive sperm motility. Motility is expressed in percentages, with 0% being the value for immobile spermatozoa and 100% for maximum spermatozoa performance. The sperm type of movement is evaluated by the progressive sperm motility in scale from 0 to 5 (0 - no motility, 1 - poor lateral movement with some progression, 2 - moderate lateral movement with occasional progression, 3 - slow progression, 4 - progression, 5 - rapid progression) (Howard, 1993).

Fig. 1. Ocelot's penis exposed before semen collection; the penis' spines can be observed (left). Ocelot's electroejaculation procedure (right). Pictures: Regina Paz.

The sperm morphology and concentration can be evaluated by fixing an aliquot of semen (1:3 dilution) in a 10% formaldehyde saline solution or in a 2.5% glutaraldehyde solution after the preparation of samples in a humidified chamber.

For the determination of the sperm morphology, 200 cells per slide were counted at 1000 X magnification under light microscopy; the abnormalities were classified as primary or secondary defects expressed as percentages. According to the primary defects presented in the sample, the sperm can be classified as macrocephalic, microcephalic, bicephalic, pyriform head, rounded head, abnormal acrosome, abnormal midpiece, no midpiece, tightly coiled tail and biflagelat. According to the secondary defects presented in the sample, the sperm can be classified as bent midpiece with or without droplet, bent tail with or without droplet, and proximal or distal droplet.

The concentration can be evaluated using a Neubauer chamber at 400 X magnification under ligth microscopy. The volume, concentration, motility, vigor and abnormal sperm data in different species are presented in Table 2.

Species	Probe (cm)	Nº Ejaculates	Volume (mL)	Concentr. (x10⁶/mL)	Motility (%)	Vigour (0-5)	Normals (%)
Ocelot	1.0[1]	5[1]	0.3±0.1[1]	28.0±17.0[1]	72.0±12.5[1]	4.0±0.5[1]	80.8±0.9[1]
(L. pardalis)		38[2]	0.62±0.08[2]	53.8±17.8[2]	70.4±2.3[2]	-	58.4±5.8[2]
Tigrinus (L. tigrinus)	1.0[1]	18[2]	0.11±0.02[2]	78.5±33.8[2]	62.1±5.7[2]	-	35.6±6.0[2]
Margay	1.0[1]	11[1]	0.2±0.1[1]	79.9±28.1[1]	86.0±3.3[1]	4.6±0.2[1]	48.5±6.1[1]
(L. wiedii)		27[2]	0.31±0.05[2]	14.2±5.3[2]	62.8±5.3[2]	-	39.5±7.7[2]
Jaguarondi (H. yagouarundi)	1.0[1]	3[1]	0.1±0.1[1]	12.5±9.4[1]	50.0±9.9[1]	3.5±0.4[1]	35.4±14.3[1]
		21[2]	0.08±0.02[2]	7.2±4.0[2]	57.8±2.5[2]	-	25.7±4.6[2]
Pampas cat	1.0[1]	5[1]	0.3±0.1[1]	10.8±5.7[1]	36.7±6.6[1]	2.8±0.2[1]	65.9±23.8[1]
(O. colocolo)		2[2]	0.08±0.01[2]	364.0±326.0[2]	81.3±6.3[2]	-	56.5±0.5[2]
Geofroy's cat	1.0[1]	8[1]	0.2±0.1[1]	300.0±233.2[1]	73.0±4.4[1]	4.0±0.3[1]	29.0±11.5[1]
(O. geofroy)		24[2]	0.21±0.03[2]	66.5±24.4[2]	64.0±4.7[2]	-	46.9±5.0[2]
Puma (P. concolor)	1.6[1]	12[1]	2.8±0.5[1]	20.2±4.7[1]	52.0±8.0[1]	3.5±0.2[1]	23.4±3.7[1]
Jaguar	3.0[1]	5[1]	2.7±0.6[1]	12.0±1.9[1]	82.0±5.8[1]	4.1±0.3[1]	58.2±11.1[1]
(P. onca)	2.3[3]	38[3]	5.7±1.71[3]	13.16±10.76[3]	56.9±9.35[3]	3.02±0.77[3]	65.73±6.7[3]

Howard, 1993[1]; Morais, 2001[2]; Paz et al., 2000[3];

Table 2. Neotropical wildlife cats seminal characteristics.

The poor semen quality in carnivores may be related to the nutritional status of the animals. Rodrigues da Paz et al. (2006), studying the reproduction of jaguars, observed a positive correlation between the improvement in the semen quality and the decrease of primary defects, after diet supplementation with vitamins and minerals. Ocelots, tigrinus and margays showed an increase in the number of ejaculates and 20-30% improvement related to sperm defects after receiving vitamin and mineral supplementation (Morais, 2001).

The seminal plasma constituents compromise the sperm viability in some species. Thus, washing the ejaculates in culture media (HEPES, HAM'SF-10) by centrifugation is efficient in removing the seminal plasma, which could contain bacteria and other undesirable microorganisms, especially when the semen will be used for intrauterine artificial insemination.

5. Sperm cryopreservation

The semen cryopreservation procedures should be initiated only after the centrifugation at 300g for 10 minutes in culture medium or HEPES HAM'SF-10. This procedure is essential for eliminating microorganisms and seminal plasma remove.

The "Double Step" cryopreservation method, using glycerol as cryoprotectant, is in general, used for the semen of most carnivores. This method use two fractions: fraction A containing nutritional constituents and antibiotics; and fraction B containing nutritional constituents, antibiotics, and the cryoprotectant. The PDV medium is used for the semen cryopreservation of the majority of wildlife cats. The fraction A contains 20% egg yolk, 11% lactose, 1000 IU penicillin/mL, 1000 mg streptomycin/mL; the fraction B contains 20% egg yolk, 11% lactose, 8% glycerol, 1000 IU penicillin/mL and 1000 mg streptomycin/mL.

After being removed from liquid nitrogen, the semen straws should be immediately thawed for 1 min in waterbath at 37°C, evaluated for total motility (%) and progressive sperm motility (scale, 0-5) before use.

The first step in the process of freezing semen is the removal of the supernatant after centrifugation of the semen collected in HAM'S F-10 or HEPES culture media, and the subsequent ressuspension of the pellet in PDV fraction A at 37 °C. This mixture should be kept in the refrigerator for 2 hours followed by a subsequent slow addition of the PDV fraction B. The material is them transferred to cooled 0.25 mL straws and kept in the refrigerator for 30 minutes. Afterwards, each straw is placed in liquid nitrogen vapor for 20 min, immersed in liquid nitrogen, transferred to the racks, and loaded into the canisters for long-term liquid nitrogen storage at -196°C.

The straws, racks and canister identification are extremely important, being the determining factor for the germplasm bank establishment and successful operation. The material collected might be extremely valuable for populations in the future and the safety use of this material depends to the correct identification.

The straws identification must contain the animal species (scientific name), tattoo or microchip number, the institution to which the animal belongs, and the date. For free-living animals, the straws must contain the species, the location where the animal was captured, and the date. The racks may be identified by numbers or if applicable, by species. A registry, which can be computerized, is essential to record all of the straws, racks, and canisters, thereby facilitating the location of the material.

The reasons for the poor quality of wildlife semen after thawing are still unknown and involve a range of information and specific characteristics for each species, which are also not yet clearly understood. New tests with different protocols and different cryoprotectors for each species of interest are required in order to maximize the spermatozoa viability after cryopreservation procedures.

6. Ovarian activity induction and superovulation

The currently used ovarian stimulation and superovulation protocols require injections of exogenous gonadotropins, which consist of large complexes of glycoproteins. Equine Chorionic Gonadotropin (eCG) and Human Chorionic Gonadotrophin (hCG) are frequently used due to their long half-life in circulation (24-48h) and good ovarian response with a single application. Other hormones used are the porcine Follicle Stimulating Hormone (pFSH) and porcine Luteinizing Hormone (pLH), these hormones are characterized as short half-life (~ 2h) gonadotropins, therefore, they present the disadvantage of requiring multiple applications to produce a good ovarian response (Crichton et al., 2003; Dresser et al., 1988; Pope, 2000; Wildt et al., 1981).

Studies on wild cats report the use of eCG/hCG in combination, mainly to avoid the stress associated with multiple injections of FSH (Roth et al., 1997). However, the use of porcine FSH/LH determined equivalent number of oocytes compared to the established protocol for eCG/hCG used in tigers (*Panthera tigris*) (Crichton et al., 2000), ocelot (*Leoparuds pardalis*) and tigrinas (*Leopardus tigrinus*) (Paz et al., 2005, 2006), demonstrating that the stress caused by daily injections did not influence the ovarian response.

The eCG/hCG combination has been used successfully in tigers (*Panthera tigris*), cheetahs (*Acinonyx jubatus*), clouded lepards (*Neofelis nebulosa*), pumas (*Puma concolor*), ocelots (*Leopardus pardalis*) and tigrinas (*Leopardus tigrinus*) (Barone et al., 1994; Donoghue, 1993; Donoghue et al., 1990; Howard, 1992b; Moore et al., 1981; Moraes et al., 1997; Morato et al., 2000; Swanson, 1996a). The FSH and hCG combination was used successfully in the Indian desert leopard (*Felis sylvestris ornata*) (Pope et al., 1989), and the pFSH and pLH combination in tigers (Crichton et al., 2000, 2003) jaguars (*Panthera onca*) (Morato et al., 2000), ocelots (*Leopardus pardalis*) and tigrinas (*Leopardus tigrinus*) (Paz et al., 2005, 2006).

Swanson et al. (1995, 1996a) suggest that the repeated administration of exogenous gonadotropins, within short time intervals is a problem because it causes a reduction in the ovarian stimulation which is immunologically mediated. The repeated administration of exogenous gonadotropins has been associated with the production of neutralizing immunoglobulin, which prevents the ovarian response to superovulation protocols.

Alternating gonadotropins regimens in sequential treatments are indicated because of variable immunoglobulin affinities to different exogenous gonadotropins (Maurer et al., 1968; Swanson et al., 1995).

Ocelots and tigrinas treated four to six times, at 4-month intervals, with alternating exogenous gonadotropin regimens (eCG/hCG and pFSH/pLH) did not show a reduction in ovarian response (total follicles and Corpora Lutea), oocyte maturation or exogenous gonadotropins antibodies production over time (Paz et al., 2005, 2006). The findings suggest that, these endangered cat species may be managed intensively with the use of alternating exogenous gonadotropin regimens in assisted reproduction procedures without compromising ovarian responsiveness to these hormones.

Specie	Procedure	Treatment 1		Treatment 2	
		ECG (UI)	hCG (UI)	pFSH (UI)	pLH (UI)
Ocelot *(Leopardus pardalis)*	AI[6] IVF[4,5]	400[6] 500[4,5]	200[6] 225[4,5]	- 50[4,5]	- 20[4,5]
Tigrinus (*Leopardus tigrinus)*	AI[4,5] IVF[6]	75[4,5] 200[6]	100[4,5] 150[6]	30[4,5] -	10[4,5] -
Gato mourisco *(H. yagouarundi)*	AI[7] IVF[7]	100[7] 200[7]	75[7] 150[7]	- -	- -
Puma *(Puma concolor)*	AI[1] IVF	200[1] -	100[1] -	- -	- -
Jaguar *(Panthera onca)*	AI[2] IVF[3]	200[2] -	150[2] -	- 50[3]	- 25[3]

Barone et al., 1994[1]; Jimenez et al., 1999[2]; Morato et al., 2000[3]; Paz et al., 2005[4]; Paz et al., 2006[5]; Swanson et al., 1996a[6]; Swanson (Personal Communication)[7].

Table 3. Ovarian stimulation with exogenous gonadotropins used in wildlife cats reproduction (AI= Artificial Insemination and IVF= *In vitro* fertilization).

The exogenous gonadotropins dosage used for ovarian stimulation is another important factor to the fertilization rate and subsequent embryonic development (Donoghue et al., 1993). Species with similar size and weight may require varying dosages, possibly because

they have different sensitivities to exogenous gonadotropins (Roth et al., 1997; Swanson et al., 1996b). The nutritional status of the animal also influences the fertilization success rate. Swanson et al. (2002a), studying ocelots and tigrinas in Brazil, observed better quality of oocytes and increase fertilization rates after supplementing the diet with vitamins and minerals.

7. Artificial insemination and oocytes collection

The rate of artificial insemination success in carnivores is influenced by the localization of the semen deposition. Non-surgical methods of semen deposition in the vagina has shown inferior results compared with the surgical method, with semen deposition directly into the uterus. This can be explained by the chemical restraint need in wild animals, with the anesthesia compromising the sperm transportation in non-surgically insemination (Howard, 1993).

The artificial insemination success with semen deposition in the uterine horn is described in several species of wild cats such as puma (*Puma concolor*) (Moore et al., 1981); leopard (*Panthera pardus saxicolor*) (Dresser et al., 1982); cheetah (*Acinonyx jubatus*) (Howard, 1992); tiger (*Panthera tigris altaica*) (Donoghue et al., 1993); ocelot (*Leopardus pardalis*) (Swanson, 1996a) and tigrina (*Leopardus tigrinus)* (Moraes et al., 1997*)*.

Artificial insemination using video-laparoscopy technique has been developed for the semen deposition directly into the uterine horn, close to the oviduct where fertilization occurs, in addition to being a less invasive method. In this procedure, the ovaries and uterine horns can be accessed and evaluated for thickness, consistency and color in all species. In cats, the ovaries are easily observed, facilitating the counting and characterization of pre-ovulatory follicles (brighter small elevated areas) and post-ovulatory corpus luteum (yellow-red area).

According to ovarian stimulation protocols, the animals should be inseminated within 24 to 48 hours after hCG or pLH administration, or after the ovulation process. Inhalatory anesthesia is necessary to perform this procedure (Figure 2).

Fig. 2. Anesthesia with isoflurane gas mask (left) and intubation (right). Pictures: Regina Paz.

The anesthetized cats should be secured in dorsal recumbency with the use of leg ties on a tilting surgical table, and the abdominal region of each female should be clipped and prepped

with alternating applications of Betadine scrub and alcohol. A pneumoperitoneum should be created by means of CO_2 gas introduced through a *Verres* needle inserted transcutaneously into the central abdominal cavity. A 7-mm-diameter laparoscope should be inserted through a 1cm skin incision slightly cranial to the umbilicus. The ovaries could be manipulated with the *Verres* needle probe, and each ovary should be closely examined to determine the number of mature follicles (≥ 2 mm diameter), recently-formed corpora lutea, and corpora albicans.

To stabilize the uterine horn, where the cannula will be introduced to deposit the semen, grasping forceps is inserted laterally, 4 to 5 cm of the umbilicus. This procedure maintains the uterus close to the abdominal wall. The horn to be inseminated is the ovary that shows the corpus luteum after ovulation. The procedure should be performed in both uterine horns if both ovaries present the corpus luteum.

For the semen deposition, the uterine horn is cannulated using a 20G sterile needle catheter inserted through the abdominal cavity, near the uterine lumen. As soon as the needle pierces the uterine horn, it is removed, keeping the catheter in place. Inside the catheter, a sterile polypropylene tube must be inserted, which will be connected to a syringe containing the semen. (Figure 3).

The intrauterine artificial insemination by laparoscopy is less invasive because the semen deposition ocurrs directly in the uterine horn without laparotomy. This methodology resulted in a 46.2% increase in cheetah's pregnancy rates (Howard, 1992).

Similarly, Donoghue et al. (1993) reported the first birth of a tiger cub (*Panthera tigris altaica*) in Siberian Tiger Species Survival Plan (SSP Program) after intrauterine insemination by video-laparoscopy in females stimulated with eCG and hCG. This result demonstrates the importance in using assisted reproduction methods in the production of genetically viable population with recommended breeding by a management program.

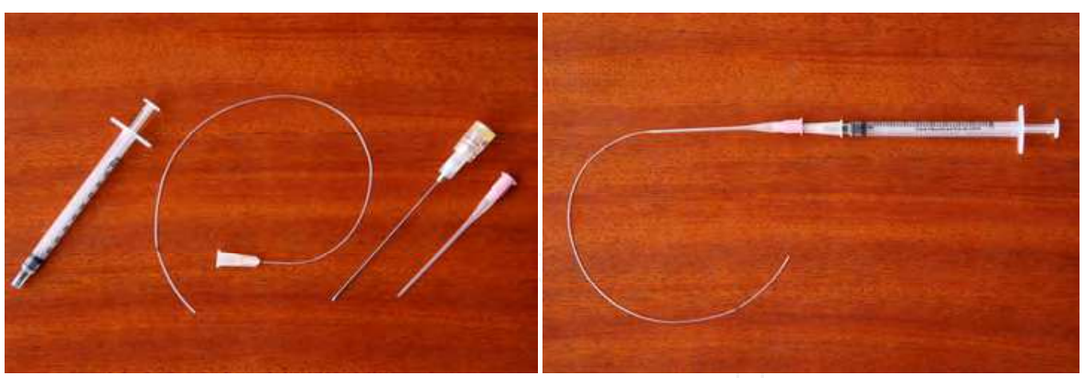

Fig. 3. Intrauterine insemination set: 1mL syringe, polypropylene tube and 22G needle (left). Conected set (right). Pictures: Regina Paz.

The procedures for oocyte retrieval in *in vitro* fertilization are performed using the laparoscopy technique. The ovaries' visualization and the oocyte retrieval in carnivores are species-specific. Cats' ovaries are easily accessed and the follicles easily aspirated.

General anesthesia is needed to perform this procedure; isoflurane inhalation anesthesia is generally used. After anesthetized, the animals are placed in the supine position (45 degrees) and pneumoperitoneum is created with the *Verres* needle, which can be coupled to a CO_2 gas automatic insulflator or a manual pump.

The endoscope is placed near the umbilicus for the evaluation of ovaries and observation of the follicles. Only mature follicles, larger than 2 mm, should be aspirated. The *Verres* needle is used for the follicles' size determination and for the maintenance of the ovary in an adequate position, near to the abdominal wall.

The mature follicles are aspirated with a 22G needle attached to polypropylene tubing connected to a sterile collection tube (15mL) containing M199 culture medium and heparin, which is attached to a vacuum aspiration pump (Figure 4). After the collection, the oocytes are placed in petri dishes with culture medium and observed by stereomicroscopy at 400 X magnification for their classification.

The oocytes from carnivores are dark and contain lipidic drops. The maturation status is characterized in I, II and III according morphological aspects. Oocytes I are of excellent quality, characterized by a uniformly dark cytoplasm, nucleus with a distinct corona radiate, and an expansive cumulus cell mass. Oocytes II are of regular quality, characterized by a non-uniformly cytoplasm, nucleus with an indistinct corona radiate, and a non-expansive cumulus cell mass. Oocytes III are degenerated and characterized by an abnormal cytoplasm, nucleus without corona radiata or cumulus cell mass (Goodrowe et al., 1988; Johnston et al., 1989).

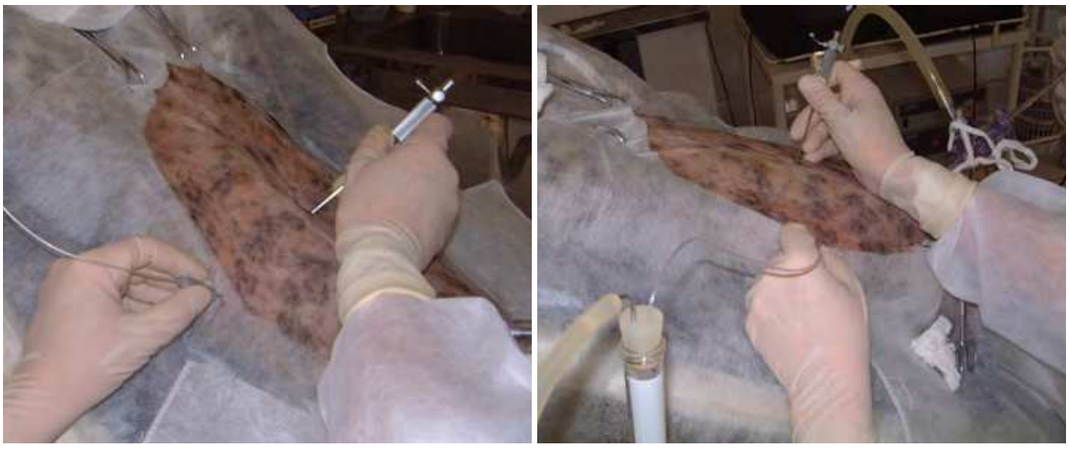

Fig. 4. Follicular aspiration using video-laparoscopy and *Verres* needle in ocelot (left). Aspiration follicular system (right). Pictures: Regina Paz.

8. *In vitro* fertilização and embryo transfer

The *in vitro* fertilization technique has been applied in wild animals after follicular aspiration using laparoscopy tecnhique, oocyte retrieval post-mortem, or after ovariohysterectomy. According Swanson (1998), oocytes collected from ovaries can be refrigerated at 5°C for 24 hours without maturation and changes in the fertilization potential.

Oocytes recovery from refrigerated ovaries can be achieved using the follicular aspiration technique with a syringe and needle, or through ovary laceration and oocyte harvest using stereomicroscopy. The second technique is used in small animals, which present ovaries with small diameters because the follicle aspiration would be difficult. The M199 culture medium is used for follicular aspiration and ovaries laceration.

However, laparoscopic follicular aspiration is the most used oocyte retrieval technique for IVF, which should be preceded by hormonal treatment. According Howard (1999), the

treatment with exogenous gonadotropins and laparoscopy are the basic requirements for ovarian stimulation and oocyte retrieval for IVF procedures.

The immature oocytes collected (≅ 60%) in cats become mature in 24-32 hours in culture media. These about 70% are fertilized, however, only a small percentage, from 20 to 30% develops in blastocysts (Johnston et al., 1989).

Donoghue et al. (1990) reported the birth of the first wild cat from IVF after an embryo transfer. Tiger cubs (*Panthera tigris*) were produced *in vitro* using excellent quality embryos, containing two to four cells, and surgically transferred to the oviducts of two females. Pregancy was successful in one of these females and three kittens were born after 107 days.

The first embryos produced by IVF in Brazil were jaguar (*Panthera onca*) embryos. The ovarian stimulation with pFSH/LH produced ≅ 25 follicles/female (> 80%), however, despite the recovery of high quality oocytes, the fertilization percentages were low (> 25%) (Morato et al., 2000).

In a Project involving the São Paulo University/Brazil (USP), the Mata Ciliar Association/Brazil (AMC) and the Cincinnati Zoo/USA (CREW), 128 ocelot (*Leopardus pardalis*) and tigrina (*Leopardus tigrinus*) embryos were produced. The animals were treated with eCG/hCG and produced ≅ 10 follicles/female. Follicles were aspirated by laparoscopy and 7-9 of excellent quality oocytes/female were recovered. Of these, 60% were fertilized *in vitro*, resulting in 76 ocelot and 52 tigrina embryos (Swanson & Brown, 2004). Two ocelots became pregnant with normal pregnancy development and birth, one from the Cincinnati Zoo/USA and another from the Sao Bernardo do Campo Zoo/Brazil. However, only the offspring born to the American female survived after birth (Swanson, 2002b).

Among all the Neotropical cats, the ocelot is the only species that produced offspring after the transfer of frozen embryos (Swanson, 2001, 2002b). These births were the result of a cooperative effort between Brazil and the USA for the development of adequate management programs for this species in captivity, and for the establishment of genetically viable populations between the Brazilian and American populations.

Based on the percentage of successful embryonic cleavage after thawing domestic cat embryos, which is less than 70% (Pope, 2000), the difficulties to develop feline embryos used post-freezing is recognized. The difficulties may be related to inappropriate timing for the embryo implantation or fetal survivel (Swanson & Brown, 2004) and not only related to the quality of the embryos. Thus, it is necessary to detect the female natural receptor estrus in order to perform the transfer of thawed embryos. This determination is achieved through measurement of fecal steroid levels according to the enzyme-immunoassay (EIA) technique for the fecal estrogens metabolites.

After the determination of the natural estrus, the ovulation should be induced with GnRH (Gonadotropin Releasing Hormone) and the embryos should be transferred by video-laparoscopy directly in the ostium of the oviduct.

9. Transmission of reproductive diseases

The methods used for assisted reproduction in wild animals should be free of contamination or diseases, therefore, some measures must be taken to avoid compromising the procedures. The semen centrifugation and seminal plasma removal are essential for artificial insemination with fresh or frozen semen and *in vitro* fertilization procedures.

The methods for processing semen without centrifugation and removal of the seminal plasma were responsible for the development of pyometra in 40% of inseminated domestic cats, regardless of the semen being diluted in culture medium containing antibiotics penicillin and streptomycin (Howard, 1993). It is believed that the donors might carry bacteria in their normal flora, eg *E. coli*, which would cause infection in the females.

In a study conducted by Paz et al. (1999), aiming at determining preputial microbiota in nine adult male jaguars (*Panthera onca*), the most frequently observed microorganism were *Staphylococcus sp* (40%), followed by *Streptococcus sp* (30%), *Escherichia coli* (20%) and *Corynebacterium sp* (10%).

The preputial microbiota in the genus Leopardus was assessed by Guido et al. (2000), and the results were *Escherichia coli* (40%), *Proteus rettgeri* (40%) and *Yersinia psedotuberculosis* (20%) in tigrina (*Leopardus tigrinus*) (n = 5), *Staphylococcus sp* (42.9%), *Escherichia coli* (28.5%), *Streptococcus sp* (14.3%), *Staphylococcus sp* + *Streptococcus sp* (14.3%) in ocelots (*Leopardus pardalis*) (n = 6) and only *Staphylococcus sp* in margay (*Leopardus wieddi*) (n = 1).

The feline immunodeficiency virus is present in the semen of domestic cats and can be transmitted to females by AI (Jordan et al., 1995, 1996). This aspect should be taken into consideration during assisted reproduction procedures performed in wild cats.

In addition, a reproductive evaluation and clinical examination should be performed in wild cats before the animal inclusion in the management programs using assisted reproduction.

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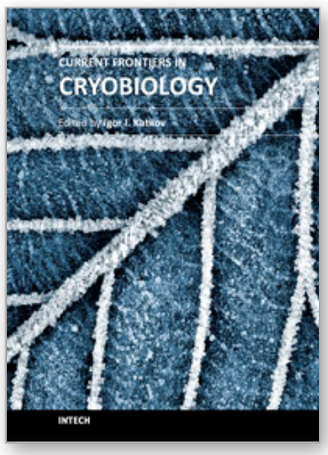

Current Frontiers in Cryobiology

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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