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Reproductive biology of felids: implications for the conservation of endangered species

Bachelor Thesis

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Declaration

I declare that the Bachelor Thesis "Reproductive biology of felids: implications for the conservation of endangered species" is my own work and all the sources I cited in it are listed in Bibliography.

In Prague 17.4. 2019 _____

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Reprodukční biologie kočkovitých šelem: důsledky pro ochranu ohrožených druhů

Souhrn

Doposud byly asistované reprodukční technologie (ART) úspěšně aplikovány u několika druhů savců. U kočkovitých šelem však tyto technologie ještě zdaleka nedosahují standardů popsaných u jiných domestikovaných zvířat. Cílem této bakalářské práce je poskytnout aktualizovaný přehled o ART u kočkovitých šelem. Práce nejprve shrnuje taxonomii, rozšíření, stupeň ohrožení a biologii čeledi Felidae (kočkovití). Poté, s využitím domácí kočky jako hlavního modelu, práce popisuje kočičí reprodukční anatomii a fyziologii. Metody a postupy používané pro získávání a uchovávání gamet, pro provádění umělé inseminace a oplodnění in vitro jsou popsány jak u divokých, tak u domestikovaných koček. Výhody a nevýhody každé metody jsou uvedeny spolu s její proveditelností u divokých i domácích druhů. Úspěšná aplikace ART silně závisí na kočičí reprodukční biologii, jejíž znalosti jsou pro většinu druhů stále omezené a neúplné.

Klíčová slova: Riziko vyhynutí, získávání a uchovávání gamet, inbreeding, kocour.

Reproductive biology of felids: implications for the conservation of endangered species

Summary

To date, assisted reproductive technologies (ART) have been successfully applied in several mammalian species. In felids, however, these technologies are still far from reaching the standards described in other domestic animals. The aim of this bachelor thesis is to provide an updated review of the ART in felids. First, the thesis summarizes the taxonomy, distribution, conservation status, and biology of the Felidae family. Then, using the domestic cat as a main model, the thesis describes the feline reproductive anatomy and physiology. The methods and procedures used for obtaining and storing gametes and for performing artificial insemination and in vitro fertilization are described both in domestic and non-domestic felids. The advantages and disadvantages of each method are listed together with its feasibility in wild and domestic species. Overall, the successful application of ART strongly depends on the feline reproductive biology, the knowledge of which is still limited and fragmentary for most of species.

Keywords: Extinction risk, gamete collection and storage, inbreeding, tomcat.

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1 Introduction

Despite the fact that the family Felidae hosts a high number of threatened species, little is still known about the reproductive biology of many of them. The knowledge of feline reproductive anatomy and physiology represents crucial steps for defining and optimizing the assisted reproductive technologies (ARTs) for these species.

Assisted reproductive technologies use specific tools to conserve endangered wildlife, including felids. The most used tools among the mammals are artificial insemination and embryo transfer (commercially used primarily in cattle), *in vitro* fertilization (frequently used in human), and cryopreservation of sperm, oocytes and embryos. These tools have been promoted in many different species with many successful results. However, in wild felids it is different.

Assisted reproductive technologies are not as well studied in felines as in other mammals. Several factors as a complicated social and reproductive behaviour, anthropological pressure, which is considerably restricting wild felids and their habitat, complicates the obtaining of information required for further study. Another discussed phenomenon related to application of ARTs in felids is teratospermia in tomcats and induced ovulation in queens.

For this reason, a domestic cat (*Felis catus*) was chosen for the purpose of obtaining the basic knowledge of reproductive anatomy and physiology of felids. This basic knowledge improved the efficiency and feasibility of assisted reproductive technologies and helped with understanding of several key factors in feline reproduction. However, most of the wild felids considerably differ from domestic cat in many aspects and thus the studies about wild felids require further research. This research uses basic knowledge about the reproduction and application of ARTs in domestic cats and is also focusing more on several circumstances that might improve the future progress of ARTs in wild felids. Therefore, every upcoming study or research might be crucial for the conservation of endangered felids and its population management.

2 Objectives of work

This bachelor thesis aims to illustrate the felids' biology, mainly focused on the anatomy and physiology of the reproductive system. Several reproductive peculiarities of Felidae are described including, for instance, the phenomenon of induced-ovulation in females and the relatively frequent occurrence of teratospermia in males. Past and future goals of the assisted reproductive techniques (ART) are summarized in domestic and wild felids.

3 Taxonomy

Taxonomy tree	
Kingdom	Animalia
Phylum	Chordata
Class	Mammalia
Order	Carnivora
Suborder	Feliformia
Family	Felidae

The family Felidae can be divided into two subfamilies, 8 lineages, 14 genera, 41 species and 77 subspecies. The mentioned subfamilies are: *Pantherinae* (big cats) and *Felinae* (small cats). Both these subfamilies are divided into lineages. The *Pantherinae* subfamily consists only of one lineage called Panthera. Into this lineage belongs genus *Panthera* and genus *Neofelis.* The remaining 7 lineages belonging into *Felinae* subfamily are: caracal, ocelot, bay cat, lynx, puma, leopard cat and domestic cat. The frequently discussed cheetah is classified in subfamily *Felinae* in the Puma lineage (Kitchener et al. 2017).

4 Family Felidae

4.1 Distribution

The family Felidae is native almost worldwide. The only exceptions are Australia, Antarctica, Japan, New Zealand, Madagascar and many isolated islands. Twenty-one species, almost 60% of all living felids, occur on the Asian continent, 14 of them are endemic there. There is one exception, and that is a domestic cat (*Felis catus*). Domestic cat appears to be on every continent mentioned above - except Antarctica (Denis 1964; Kelsey-Wood 1989; Nowell & Jackson 1996; Feldhamer et al. 1999; Vaughan et al. 2000; Grzimek 2003). Domestic cats have been introduced around the world by humans, often causing loss of biodiversity.

Wild felids are found in almost all terrestrial habitats except treeless tundra and polar ice regions. Most species are habitat generalists – they can be found in a wide range of environments. This statement claims that most of the wild felids can very well adapt to a new

habitat. However, only few of them have adapted to a limited range of habitats. For example, optimal habitat for sand cats *(Felis margarita*) consists of sandy and stony deserts. Domestic and feral cats *(Felis catus*) are ubiquitous globally and are especially pervasive in urban and suburban areas (Grzimek 2003; Nowell and Jackson 1996). Some of the species are known to have large ranges spanning several continents. According to Macdonald et al. (2001), typical examples are leopards (*Panthera pardus*). They are found from the Russian Far East and parts of Eurasia through tropical Asia, the Middle East, and throughout sub-Saharan Africa. Another example is the wildcat (*Felis silvestris*) that is widely distributed in Africa, Asia, and Europe.

4.2 Conservation status

By the IUCN (International Union for Conservation of Nature 2019) the family of Felidae includes approximately 38 threatened species (Table 1). Thanks to the conservation efforts, in 2015 the IUCN downlisted the Iberian lynx, the most endangered felid worldwide, from the "critically endangered" to the "endangered" status. There are another 4 felids considered as endangered and also 13 considered as vulnerable and the wild populations of these felids are rapidly decreasing due to many threats.

Table 1 – *Endangered and vulnerable species of the family Felidae by the IUCN (2019)*

Name	Vulnerability	Estimated population (mature individuals)
Tiger (*Panthera tigris*)	Endangered	2,154-3,159
Borneo Bay Cat (*Catopuma badia*)	Endangered	2,200
Andean Cat (*Leopardus jacobita*)	Endangered	1,378
Flat-headed Cat (*Prionailurus planiceps*)	Endangered	2,499
Iberian Lynx (*Lynx pardinus*)	Endangered	156
African Golden Cat (*Caracal aurata*)	Vulnerable	N/A
Chinese Mountain Cat (*Felis bieti*)	Vulnerable	9,999
Black-footed Cat (*Felis nigripes*)	Vulnerable	9,707
Fishing Cat (*Prionailurus viverrinus*)	Vulnerable	N/A
Cheetah (*Acinonyx jubatus*)	Vulnerable	6,674
Guiña (*Leopardus guigna*)	Vulnerable	5,980-92,092
Southern Tiger Cat (*Leopardus guttulus*)	Vulnerable	6,047

Northern Tiger Cat (*Leopardus tigrinus*)	Vulnerable	8,932-10,208
Clouded leopard *(Neofelis nebulosa)*	Vulnerable	N/A
Snow Leopard (*Panthera uncia*)	Vulnerable	2,710-3,386
Sunda Clouded Leopard (*Neofelis diardi*)	Vulnerable	4,500
Leopard (*Panthera pardus*)	Vulnerable	N/A
Lion (*Panthera leo*)	Vulnerable	23,000-39,000

N/A = not available

There are many threats that are affecting populations of wild felids. The main threat is loss of the felid's habitat: - deforestation, extraction of rare woods, urbanization, all of which are anthropogenic. Large felids are often getting into conflict with humans and their livestock. These felids are usually shot or trapped. Spotted or stripped felids are usually killed by poachers for their fur.

Due to decreasing of felids populations and their habitat, there is bigger chance of inbreeding among related individuals. That might be a problem within a small population, as inbreeding causes decreasing of genetic variability that ends with loss of an ability to reproduce. This is especially true for cheetahs. A study of Dobrynin et al. (2015) analysed 7 cheetah genomes (4 from Namibia and 3 from Tanzania). The study found that cheetah genomes are on average 95% homozygous. This means that all cheetahs are genetically very close to each other, proving, that this is likely the reason causing the problems with reproduction and sensitivity to common viruses.

With decreasing genetic variability and impossibility of reproduction, every genetic disease can decimate already damaged population. Those diseases are then spreading deeper into population with every generation born, causing artificial methods of reproduction ineffective.

The decrease in habitat has also other negative effects. There are more individuals on a smaller area that have bigger chance meeting each other. In the first case, this may cause frequent fights for territories. In the second case, meeting with other individuals more often (or crossing an occupied territory), increases the chances of the felid being infected with a contagious disease.

4.3 Biology

4.3.1 Morphology

Due to the relatively recent evolution, the morphology of the most felid cats is visibly similar. Despite this fact, the body size of each specie varies. A good example can be the Rusty-spotted Cat (*Prionailurus rubiginosus)* and its opposite the Siberian tiger (*Panthera tigris altaica*). Rusty-spotted Cat with the weight of 1–1,6 kilograms and the height of 20 centimetres is considered as the smallest cat in the world (Mukherjee et al. 2016; Macdonald & Loveridge 2010). On the other hand, the biggest cat in the world – Siberian tiger can weight over 300 kilograms (Macdonald & Loveridge 2010). Despite their difference in body size, all of the wild felids are considered as a strictly carnivorous and thus they are very well specialized for pursuing, capturing and killing their prey.

For capturing the prey, specialized claws are used. These claws are fully retractable (with the exception of cheetah that has only semi-retractable claws) and are protected in sheaths when at the rest. The claws help the cat to catch and hold the struggling prey until the killing bite is delivered. Unlike other carnivores, felids have rounded heads and shortened faces. The mouth holds 28-30 teeth. Canines are sharp, pointed teeth adapted to dispatching prey and tearing/cutting flesh (Smithers 1983).

The body is as well adapted for hunting. One of the adaptations is represented by the limbs. Limb length in felids is a compromise between short and long length. Short limbs allow the cat to leap precisely on the prey and to accelerate maximum speed over a short distance. Longer length of limbs allows for greater speed over a long distance. The compromise in lengths ensures maximum speed over distance with a precise leap and powerful grasp for holding the prey (Macdonald & Loveridge 2010). The fastest felid in the world – cheetah (*Acinonyx jubatus*) has developed even more adaptations. Compared to other felids, cheetahs have smaller teeth, providing more space in the skull for bigger nasal cavities, which together with big lungs and heart, enable efficient air intake when sprinting (Bradford 2018).

Another adaptation is the tail. Despite that the length of a tail varies among felids, it has still the same purpose. Tail helps the cat with balance either while leaping, climbing or balancing on narrow objects. Some felids have their tails shortened because of many reasons. Felids living in a cold region (for example lynx) have their tails shortened, so they do not unnecessarily lose their body heat. Felids that hunt aquatic preys (for example fishing cat) have short tails, as it may be an obstacle in water conditions (Kitchener 1991).

Despite many similarities, there are also differences between domestic cats and wild felids. One of the differences, not visible at first sight, is brain. Both have nearly identical brain in structure, but not in size. Wild felids have slightly bigger brain for their size, than domestic cats. Other, more visible, difference is a shape of pupils. Domestic cats (and other small cats from *Felinae* subfamily) have their pupils shaped into vertical-slits, unlike the cats from *Pantherinae* subfamily that have their pupil round shaped. This is being explained by the nocturnal activity and hunting of the small cats (Johnson et al. 2006).

4.3.2 Social behaviour and lifespan

Wild felids are considered as solitary, but two species are capable of living in pairs or larger groups. These species are lions and cheetahs. The lions form a matriarchal group called pride. Pride consists of a mother, her daughters and also several mature females (usually sisters), joined usually by two males (Schaller 1972; Bertram 1975). The number of members in the pride varies form 4-37 (Schaller 1972). The cheetahs form a family group consisting of one female and her offspring, accompanied by group of a few non-breeding males. These males do not last permanently (Kleiman & Eisenberg 1973).

Although the lifespan of wild felids considerably varies among the species, the fact that wild felids live much longer in captivity remains the same for all of them. Differences between average lifespans of felids in wild and these held in captivity are listed in the Table 2.

Table 2- *Average lifespan of non-domestic felids in wildness and captivity (Sunquist & Sunquist 2002)*

Name	Lifespan in wild	Lifespan in captivity
Cheetah (*Acinonyx jubatus*)	6-9 years	up to 16 years
Lion (*Panthera leo*)	13-17 years	up to 30 years
Tiger (*Panthera tigris*)	14-15 years	over 20 years
Leopard (*Panthera pardus*)	12-17 years	up to 21 years
Snow leopard (*Panthera uncia*)	12-17 years	up to 21 years
Jaguar (*Panthera onca)*	11 years	up to 25 years
Ocelot (*Leopardus pardalis*)	12-13 years	up to 20 years
Caracal (*Caracal caracal*)	9-11 years	up to 16 years
Serval (*Leptailurus serval*)	10 years	up to 20 years

4.4 Biology of the feline's reproductive system

For better clarification, this chapter will mainly describe reproductive biology of a domestic cat (*Felis catus*). The differences between reproductive biology of domestic cats and wild felids will be mentioned at the second place.

4.4.1 Anatomy of the reproductive system

4.4.1.1 Male

Testes are the male gonads and in the adult tomcat they are located outside from the abdominal cavity, in a pouch of furred skin called the scrotum. Testes usually descend to the scrotum by the third month of the tomcat's life. The scrotum lies ventral to the anus, close to the ischial arch. Because testicular temperature is physiologically lower than the body temperature, several anatomical structures guarantee the proper thermoregulation of the testes: the dartos, the cremaster, and the pampiniform plexus. The dartos regulates the temperature of testes by contracting the skin of the scrotum. On the other hand, the cremaster lowers/raises the testes depending on the temperature of environment. Cold temperatures cause contraction of the cremaster, helping the testes getting closer to the warm of the body. On the other hand, in warm temperatures, the cremaster relaxes and pull the testes further from the body allowing them to remain cool. The pampiniform plexus is a network of small veins located within the spermatic cord. It regulates the temperature of the testes by cooling the arterial blood entering the testicle and transferring its heat to the venous blood leaving the testicle (Graça et al. 2012).

The testicular tissue consists mostly of coiled seminiferous tubules lined by spermatogenic cells and Sertoli cells. Long, coiled tube lying within a sheath along the dorso-lateral border of the testis is called epididymis. The epididymis is divided into three regions: *caput* (head), *corpus* (body) and *cauda* (tail) (Hamilton et al 2002). From there the epididymis continues as the deferent duct. The deferent duct passes out of the scrotum into the peritoneal cavity via the inguinal ring. Both deferent ducts then join urethra. From this point the track is shared by both the urinary and reproductive system. The tract is supplied by the prostate gland lying close to the neck of the bladder and the bulbo-urethral glands. These lie close to the tip of the penis. Outside the pelvic cavity, the urethra becomes surrounded by cavernous erectile tissue. This tissue consists of "caverns" of connective tissue lined with endothelium. This tissue is filled with blood during any sexual excitement causing penis to be erected. Ventral to

the urethra, within the erectile tissue is a bone, the *os penis*. The final part of the penis is called *glans penis*. The surface of this part is covered with backward-pointing barbs called penile spines. The spines stimulate the queen, as the tomcat withdraws his penis from the vagina after coitus. The mechanical stimulation of the vagina during the coitus provokes the ovulation. According to Aronson & Cooper (1967) the growth of the spines is related with the tomcat's level of androgen. When the level of androgen decreases (for example because of castration, illness etc.), the spines regress. Last part of the reproductive system is the prepuce. Prepuce is a skin that covers penis in relaxed state. The outside is covered with hairy skin and the inside consists of a mucous membrane which is supplied with lubricating glands (Aspinall 2011).

4.4.1.2 Female

The female reproductive gonad is the ovary. Ovaries are situated caudally to the kidneys and dorsally suspended by the mesovaria. They are attached proximally by the suspensory ligament.

The ovary is composed by two parts: the cortex (externally) and the medulla (internally). The cortex contains ovarian follicles that host germ cells (oocytes or ova) at different stages of development. The ovaries are surrounded by the ovarian bursa, that is closely attached to the oviduct. From there the oocyte leaves the ovary and gets through the oviduct into the uterus (Aspinall 2011). The uterus is composed of three parts: the *cornua* (horns), the *corpus* (body), and the *cervix* (neck). The horns of the bicornuate uterus are suspended dorsally by the mesometrium. The body of the uterus is partially divided by a septum. Cervix is short, connecting the body of uterus with the vagina. Vagina extends from the cervix to the external urethral orifice. It lies within the pelvic cavity and is surrounded by connective tissue. Vestibule is continuation of a vagina that leads to the vulva. Vulva marks the external opening of the tract. Below the anus are located lips of the vulva.

4.4.2 Physiology of the reproductive system

Individual is able of reproduction when puberty is reached. Puberty is a time period of individual's life, when necessary anatomical and physiological changes related to reproduction happen. It is defined by many actions and interactions that occur. During this period the sexes become fully differentiated, secondary sexual characteristics become conspicuous, the neural tissues that mediate mating behaviour are activated and mainly - the

individual is able to create gametes. Thus, females begin the oestrous cycle, which leads to oocytes production and males begin spermatogenesis, which produces sperm (Broom & Fraser 2007). The age of reaching the puberty is different for every species and even for male and female and the onset can be influenced by many factors. These factors are not the same for male and female. The onset of puberty in females is mainly determined by their weight. If the female will not gain at least 75% of their adult weight, the onset of the puberty might delay. In the opposite case, if the female is malnourished, the puberty might onset much later. Any occurring male around the female might also influence the onset of puberty. In males, the main factor influencing the onset of puberty is level of their testosterone. Testosterone is produced by Leydig cells in testes. Thus, any anatomical/physiological problem affecting the testes (cryptorchidism, anorchism…) might delay the onset of puberty. Other factors like breed, malnutrition or length of the daylight might also influence the onset of puberty in both males and females.

4.4.2.1 Male

The tomcats reach puberty at an average age of 8-12 months, resulting in start of spermatogenesis. Spermatogenesis is a cyclic process in which spermatogonia (immature sperm cells located in the seminiferous tubules of testes) differentiate into mature spermatozoa. Approximately 4.5 cycles are needed to complete the whole process of differentiation (Amann & Schanbacher 1983). Thus, the total length of spermatogenesis takes 46.8 days in domestic cat (Franca & Godinho 2003). Spermatogenesis can be divided into three stages: spermatocytogenesis, meiosis, and spermiogenesis.

During spermatocytogenesis stage, spermatogonia firstly go through asymmetric mitotic divisions, which maintain a self-renewing stem cell population, following with symmetric mitotic divisions, which produce daughter cells that will undergo a differentiation. The results of this differentiation are primary (first) spermatocytes that proceed to the next stage. In the meiotic stage, the primary spermatocytes go through two meiotic cell divisions, resulting in haploid spermatids. The final stage is called spermiogenesis. During this stage, spermatids differentiate into mature spermatozoa (Ramm & Schärer 2014). Mature spermatozoa are then ejaculated during coitus.

Ejaculation is defined as a process of seminal fluid expulsion from the urethral meatus. This process is formed by coordinated series of reflexes activated during two phases: emission and expulsion. Seminal emission involves secretion of seminal plasma from both epithelial cells and the accessory sexual glands. This phase also moves spermatozoa with seminal

plasma to the proximal urethra via contractions of the vas deferens. Simultaneously with these actions, urethral smooth muscles contract and close the neck of the bladder, preventing retrograde ejaculation. Seminal expulsion then follows. The semen is rapidly advanced forward through the urethra and springs from the penile meatus. This is caused by the coordinated contraction of the muscles surrounding the urethra, called urethral sphincter and the bulbocavernosus (Lucio et al. 2011).

Twenty-eight species of the Felidae family can be classified as teratospermic (Wildt 1994). The ejaculates of teratospermic felids usually contain more than 60% of pleiomorphic (morphologically abnormal) spermatozoa. Thus, those abnormal spermatozoa are not capable of participating in fertilization. Studies proved that these deformities are not influenced by methods of semen collection and that gametes collected by electroejaculation (Neubauer et al. 2004), an artificial vagina or by epididymal recovery (Pukazhenthi et al. 2006) have the same proportions of pleiomorphic spermatozoa. The causes underlying the phenomenon of teratospermia are still not completely elucidated. There are several opinions that relates with this problem. Pukazhenthi et al. (2006) pointed out a positive relationship between pleiomorphic sperm production and genetic uniformity. In other words, that semen from homozygous donors usually contain high proportions of pleiomorphic spermatozoa. However, the teratospermia occurred in individuals which are bred in ZOOs but are not homozygous. This fact proved that genetic uniformity is not the only factor that affects the spermatozoa. In this case, nutrition played a role. It is important to provide balanced and vitamin/mineral-enriched food for optimal spermiogenesis and epididymal maturation. A study in rats demonstrated a relation between flagellar defects and selenium deficiency in both spermatids and epididymal spermatozoa (Swanson 2003).

4.4.2.2 Female

Females of the domestic cats reach puberty between 4-12 months of age (average 6–9 months), at approximately 75% of their body weight (Tsutsui & Stabenfeldt 1993; Wildt et al. 1998). Puberty in the females initiates oestrous cycle. The oestrous cycle is composed of proestrus and estrus. If ovulation occurs, estrus is followed by diestrus, otherwise interoestrus. Anestrus is the phase of sexual inactivity that occurs during short daylight or lactation (Figure 1).

Proestrus has the shortest period that lasts usually less than one day. This period is associated with the growth and maturation of the ovarian follicles, increasing circulating estrogens and occasionally male interest excluding copulation. The growth and maturation are

caused by follicle-stimulating hormone (FSH) that is excreted from the pituitary gland. Advanced follicular development and the highest concentrations of estradiol are typical for estrus and it is usually accompanied by male mounting, coitus and depending on the species, behaviours such as vocalization, lordosis, rolling, rubbing and foot treading. Estrus lasts three to 10 days. Felids are traditionally classified as induced ovulators (Shille et al. 1983) – they need mating to stimulate ovulation. However, felids exhibit a range of ovulatory patterns from strictly induced to different combination of induced and spontaneous. These differences happen not only across species, but also between individuals within a species (Brown 2006). It is also known that, due to individual-specific responses to physical and psychosocial stimuli, many domestic cats have ovulations regularly without mating (Brown 2011). To stimulate the release of gonadotropin-releasing hormone (GnRH) from the medial basal hypothalamus and subsequent upsurges of luteinizing hormone (LH) from the anterior pituitary gland, it is believed that multiple intromissions are needed, often over several days. This process inflicts final follicular and oocyte maturation and results in ovulation after mating. Diestrus is characterized by high blood progesterone levels secreted by one or more corpora lutea. The amount of progesterone stays high for varying lengths of time depending on whether conception occurs or not. The last phase, anestrus is a period of sexual inactivity, till the next estrus. Anestrus occurs when the length of the day is short (in the Northern Hemisphere it is from September to January). When the daily lighting time is reduced to approximately 8 hours, the estrus is inhibited, and the anestrus is induced (Kutzler 2007).

According to Shille V.M. (1979), the domestic cat is seasonally polyestrous under natural photoperiods, but will cycle year-round when housed on a 12-14 h light cycle. In general, ovarian cyclic activity is reduced under decreasing photoperiod and resumes after exposure to increasing light. Melatonin appears to regulate photoperiod-induced seasonality in the cat, with concentrations being highest during the dark phase (Leyva et al. 1989). It is similar with many non-domestic felids like the tigers, clouded leopards, lynxes and snow leopards whose reproduction is somewhat seasonal. By contrast, follicular activity in lions, leopards, bobcats, pumas, margays, ocelots, tigrinas, jaguars and fishing cats is not influenced by season (Brown 2006).

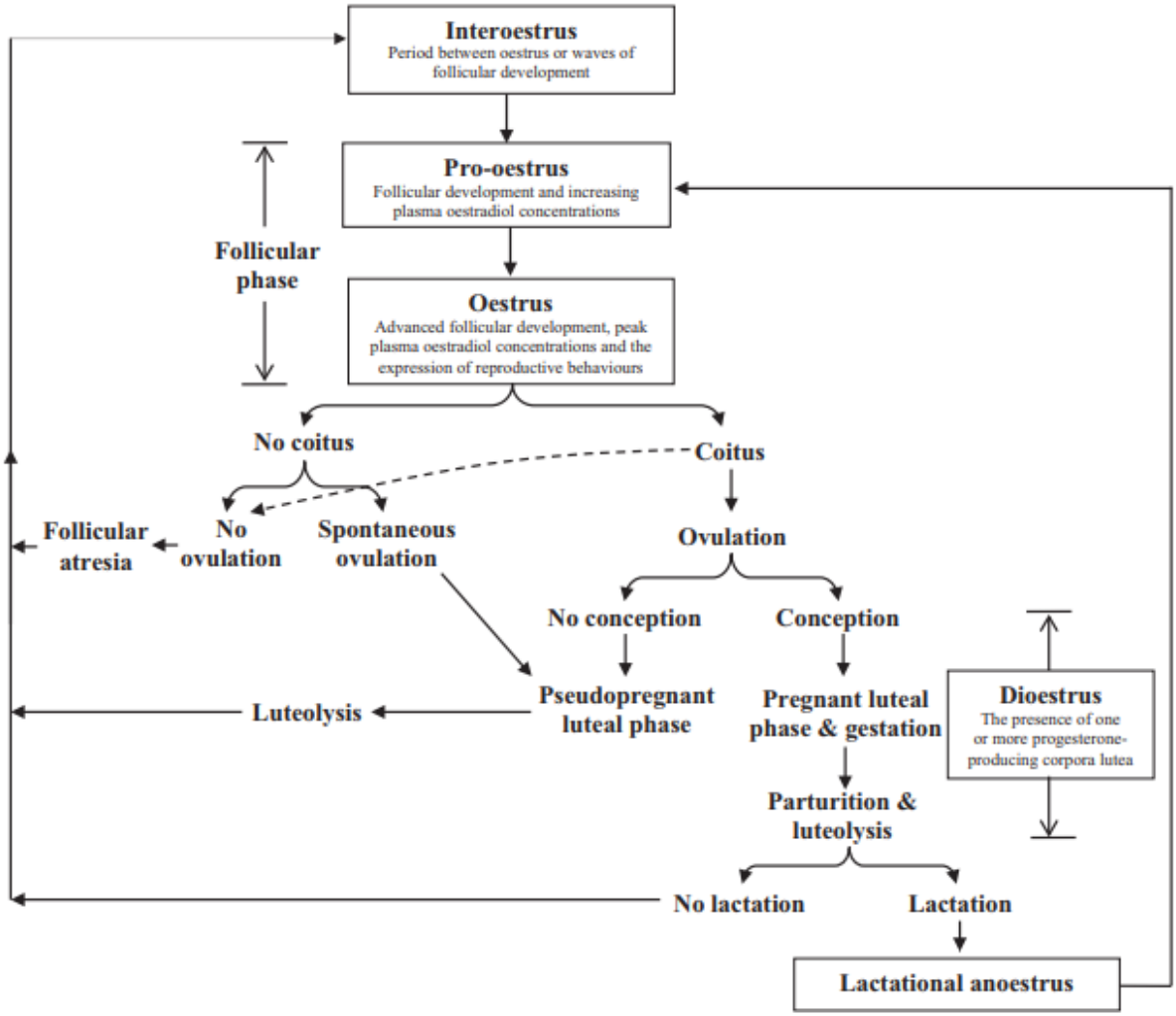

Figure 1 *- Generalised oestrous cycle of non-seasonal felids (Andrews et al. 2018)*

The oestrous cycle is described for only 24 species of the Felidae family. The estrus duration does not significantly vary between lineages or species. Despite there is some variation, the estrus duration generally ranges from 2-10 days. In some species, longer periods of estrus have been observed (Table 3), but the mean durations of estrus still fall within 2–10 days range.

Table 3 - *Wild felids with longer periods of estrus (Andrews et al. 2019)*

Name	Weighted mean	Range	Sample size
Sand cat (*Felis margarita*)	2.9 days	**1–11**	109
Rusty-spotted cat (*Prionailurus rubiginosus*)	5.6 days	**1–11**	50
Jaguar (*Panthera onca*)	6.5 days	**<15**	201
Snow leopard (*Panthera uncia*)	4.3 days	**1–19**	145
Clouded leopard (*Neofelis nebulosa*)	5.2 days	**1–17**	237

If the queen ovulates but fails to conceive, the pseudopregnancy occurs. Pseudopregnancy may occur when queens are mating with an infertile male or with a male that is fertile, but the fertilization will not happen for reasons like illness, stress, defect of reproductive tracks/organs, among others. It can also be a result of a spontaneous ovulation or it may be induced by progesterone injections during the estrous cycle to achieve a longer period without estrus (Shille & Stabenfeldt 1979; Brown 2006). The duration of the pseudopregnancy has been established to approximately 40 days by many studies (Paape et al. 1975; Verhage et al. 1976; Wildt et al. 1981). This duration is close to half the duration of pregnancy, giving queens (unlike other mammals) an advantage of reproducing quickly, because they can re-enter proestrus sooner and breed again.

In the opposite case, the queen gets pregnant after a successful mating. The duration of a gestation in the queen lasts 61-69 days (Verhage et al. 1976) and after 2-8 weeks following normal lactation and weaning, the cycle returns to estrus. The study of Feldman & Nelson (2004) showed that removing the kittens from the queen early after parturition, shorten the time to enter new cycle to 6-8 days after the separation.

After a successful conception, the corpus luteum changes into the corpus luteum graviditatis, which continues in releasing progesterone during gestation. Progesterone is needed for the development of embryos and also together with oxytocin (released from hypothalamus) ensures uterine contractions during birth. Another group of hormones participating in the gestation are estrogens. Estrogens are produced by follicles, evolving embryos and placenta. During gestation, the estrogens primarily prepare queen for birth by growing the uterine mucosa and muscles. Estrogens also ensure development of mammary glands. Relaxin hormone, released from corpus luteum and placenta, relaxes birth paths right

before and during parturition. Corpus luteum graviditatis then gradually regresses into corpus albicans, stops releasing progesterone and prepares queen to a new estrous cycle.

Gestation usually results in the size of a litter from 1 to 6 kittens (*Felis catus*). Kittens are born blind and helpless but are often haired and also spotted. As long as they cannot hunt for their own, they remain with their mother.

5 Assisted reproductive technologies in felids (ARTs)

We can notice a diversity in the reproductive cycles of felids. Unlike the domestic cat, the success of such techniques is low in wild felids. Especially the endangered ones have their own individual or even specific reproductive cycles. This is the reason why it is hard to use assisted reproductive techniques among the endangered felids. Despite the fact that monitoring of the ovarian cycle can improve natural breeding and also can maximize the reproductive success of wild felids, it has been studied in two-thirds of the feline species with thorough reports on 16 species only – nine species were born by artificial insemination, another nine by *in vitro* fertilization and three by somatic cell nuclear transfer (Thongphakdee et al. 2017). Knowledge of each feline hormonal patterns can offer new opportunities to develop ARTs - there still is a need for better knowledge of feline reproductive biology. Several ovarian activity control regimens have been established with higher precision for **artificial insemination, *in vitro* fertilization, oocyte aspiration, somatic cell nuclear transfer and embryo transfer**.

The ARTs such as artificial insemination, in vitro fertilization and also somatic cell nuclear transfer have potential for conserving endangered species, but on the other hand, most of these techniques can also have negative effects like ovarian hyperstimulation, premature luteolysis and impaired female reproductive function - for example oocyte maturation, embryo development and implantation, all of which causes abnormal placenta/fetus development and low pregnancy rate.

Inter-species hybridization has been yet recognized in many wild animals. In felids, hybrids have been bred only in captivity using the ARTs such as artificial insemination or embryo transfer. Recently known hybrids of wild felids are: liger, hybrid between male lion and female tiger (Gray 1972), the reversed hybrid (male tiger × lioness) is called tigon and hybrid of male leopard and lioness is called leopon (Li et al. 2016). Despite the fact that the ethical question of inter-species hybridization is still highly discussed topic, it will not be included in this thesis.

5.1 Gamete collection and preservation

5.1.1 Sperm collection, evaluation and storage

There is one precondition to begin any method of artificial fertilization. The precondition is a quality sperm from a male individual. Sperm samples can be collected *in vivo* via **artificial vagina, urethral catheterization and electroejaculation** or after castration and *post mortem* via **epidydimal cutting or flushing of the deferens ducts**.

5.1.1.1 *In vivo* sperm collection

The sperm collection via urethral catheterization should be started with anesthesia and a proper examination of the reproduction paths. For this purpose, ultrasound is used. If there seems to be no issue with the paths, then the region around penis is cleaned with water and dried. The penis is then subsequently extended beyond the prepuce and a shaft is fixed with surgical forceps. After that is the penis disinfected and wiped with sterile gaze. The catheterization of the urethra is done with a suitable urinary catheter that is lubricated on the tip with non-spermicidal sterile lubricant. Then the catheter is inserted into the external urethral opening. A second examiner then palpates the urethra transrectally to check for catheter passage at the point of the pelvic flexure. The catheter is being advanced until reaching the prostate and then is immediately slowly retracted and checked for semen content. Semen is ejected from the catheter with a syringe directly into a prewarmed plastic tube (Lueders 2012).

The process of sperm collection into artificial vagina is not as common as other methods used with felids. Due to the temperament of wild felids this method is not as effective and successful as other methods of sperm collection. Main disadvantage is that trained male is required for successful collection and in most cases a “teaser” queen (female in heat or spayed queen treated with estrogen) is needed too (Sojka 1986). Despite these disadvantages, with proper handling and patience, this method may also offer advantages. The artificial vagina can be procured at low cost and only one technician can perform the whole process of sperm collection. On top of that, none physical and chemical restraints are needed. The technician then only needs to slip an artificial vagina over an erected penis when the male mounts the “teaser” queen. After 1-4 minutes the sperm collection is completed (Zambelli & Cunto 2006).

The most used method of sperm collection is electroejaculation. Electroejaculation is more preferred than artificial vagina because of many reasons. Not every male is usable for

natural breeding or sperm collection (usually due to physical/psychological reasons) and also "teaser" queen and male training is not needed. This means that any male under anesthesia can be used for this method (Sojka 1986). However, this method has its disadvantages. The equipment for this method is expensive. There is also a chance of sample contamination with leaked urine after a stronger stimulus. Despite general anesthesia animals usually show strong muscular contraction (Lueders et al. 2012). The process starts with general anesthesia. Then an electrode is lubricated and inserted in the rectum of the tomcat. Electrical stimulation then affects the sympathetic nerves which cause ejaculation (Johnstone 1984).

5.1.1.2 *Ex vivo* and *post mortem* sperm collection

Sperm can be collected even *post-mortem*, which means after the death of a male. Two *post-mortem* methods are described. One of these methods is called flushing of deferens ducts. Testes, together with epididymis and deferens ducts are firstly removed from the male. Cauda epididymis and vas deferens is then isolated from each testicle. Flushing consists of catheterizing the lumen of the deferens ducts with a tubing adaptor and retrograde flushing of the deferens ducts and cauda epididymis with 5 mL of warmed (37°C) milk-based semen extender. The resultant mixture is then collected into a warmed sterile tube (Dooley et al. 1991).

The other method is called floating. Several horizontal incisions/cuts are made in the removed epididymis and vas deferens with a scalpel blade. These incised parts are then placed in a tube or glass Petri dish and covered with the semen extender (Dooley et al. 1991, Filliers et al. 2008). Sperm are then gradually released from the incisions into the surrounding semen extender, from where are extracted and submitted to analysis.

In felids, the floating method is usually more preferred, because more sperm can be obtained through this method than by the flushing method. On the other hand, the flushing provides more concentrated volume of sperm cells, resulting in less contamination with tissue or blood (Pickett et al. 1989).

5.1.1.3 Evaluation and storage

After a successful collection of a sperm sample, the sperm must be analysed. Semen have different parameters that can be examined. The most common are: concentration, total and progressive motility, sperm morphology, membrane integrity, and acrosomal status. Sperm velocity parameters include: percentages of slow, medium, and rapid spermatozoa, linearity of the sperm movement, beat cross frequency, and amplitude of lateral head

displacement, among others (Verstegen et al. 2002; Rijsselaere et al. 2005). Wildt et al. (1988) evaluated several semen parameters mentioned above in four selected felids (Table 4).

For this purpose, CASA (Computer-Assisted Sperm Analysis) is mostly used. This measuring device consists of phase-contrast microscope, a camera, a minitherm stage warmer, an image digitizer and a computer that saves and analyses the data. All of these devices then work together as a cell analyser that reconstruct sperm trajectories from the position of sperm heads in successive frames and calculating various motility parameters simultaneously.

Table 4 - *Semen parameters in cheetah, tiger, leopard and puma by Wildt et al. (1988)*

Semen parameters	Cheetah	Tiger	Leopard	Puma
Ejaculate volume (ml)	1.8	7.0	5.1	3.4
Sperm concentration ($\times 10^6$/ml)	27.3	31.9	46.2	22.0
Sperm motility (%)	69.0	81.5	43.8	64.3
Motile spermatozoa/ejaculate ($\times 10^6$)	30.9	230.6	108.0	44.2
Morphologically abnormal spermatozoa (%)	64.6	37.5	79.5	73.5

The analysed sperm has to be used immediately or it can be preserved with cooling and/or freezing. Both ejaculated and extracted sperm sample can be used for the cooling process. First of all, the sperm is diluted and subsequently cooled and stored at +5°C. This will provide a short-term storage for about 24h (Glover & Watson 1985; Pope et al. 1989, 1991) and sometimes even up to 4-7 days (Glover and Watson 1987, Harris et al 2001). To protect spermatozoa against cold shock, egg yolk is included in semen diluents – effect of the lipoproteins included in the low-density fraction. The sperm is then used for insemination or is frozen for later use.

According to Buranaamnuay (2017) there are three methods of cryopreserving sperm samples. First method is done with dry ice. This method uses dry ice to freeze samples loaded in ampules/straws. These samples are exposed to dry ice for 10 min and then are stored in liquid nitrogen (Nelson et al. 1999). The second method uses vapor of liquid nitrogen. To freeze the semen with this method, it is firstly needed to equilibrate the temperature of the sample at 4-5°C. Cooled semen is packaged in ampules or straws and then exposed to liquid

nitrogen vapor by suspending 4–5 cm above the liquid nitrogen. This process decreases the temperature of the sample as required. The commonly used temperatures are: −40 (Zambelli et al. 2002), −100 (Wood et al. 1993), −110 (Baran et al. 2004), −120 (Siemieniuch & Dubiel 2007) or −130 (Schafer & Holzmann 2000) °C. The sample is then plunged into liquid nitrogen and stored in temperature-controlled freezer (Buranaamnuay 2017). Third method uses programmable freezing machine. This method initiates with relatively slow cooling of the sample (i.e. −1 °C/min; from 4 °C to −1 °C or −25 °C), preventing ice crystals form in the cells. After this process, the cooling of the sample is accelerated to (i.e. −30 °C/min) until the final temperature is reached. The temperatures tested by various studies are: −100 °C (Lengwinat & Blottner 1994) or −196 °C (Mizutani et al. 2010). After final cooling, the samples are subsequently submerged into liquid nitrogen.

5.1.2 Oocyte collection, evaluation and storage

5.1.2.1 *In vivo* oocyte collection

The oocyte collection in *in vivo* is done via laparoscopic ovum pick up (LOPU). The females undergoing this procedure are under general anesthesia. The abdomen area is shaved, disinfected and small incisions (2 or more) are made in the abdominal wall. Filtered air is then blown into the abdominal cavity, which helps with separating organs. Through the one of incisions, a laparoscope is inserted in the cavity. Laparoscope is an optical instrument used to examine visually the interior of the abdominal cavity. The laparoscope is then moved closer to the ovary, exposing follicles for puncture. Follicles are then punctured by the aspiration needle. The aspiration needle should avoid any vascularized area and should be entered by the side of the follicle. Oocytes from the punctured follicles are then collected via suction system that is inserted through another incision. The suction system is composed of a suction pump connected to a tube that is connected to the pipette (Jorge Neto et al. 2018).

5.1.2.2 *Ex vivo* and *post mortem* oocyte collection

Ovaries may be removed from living animal via surgery (for example castration) or from dead animal.

Two common surgery methods of removing ovaries are ovariohysterectomy (OHE) and ovariectomy (OE). Both of these methods use similar surgical techniques. In both methods the ovarian pedicle is ligated and severed. The only difference in ovariohysterectomy is, that the

uterine vessels are also ligated, and the uterus is severed (DeTora & McCarthy 2011). The ovary can be then removed and used for collection of oocytes.

One of the used methods to collect oocytes after the OHE/OE is collection of cumulus oocyte complexes (COC). The cumulus oocyte complex is an oocyte surrounded by specialized granulosa cells, called cumulus cells. These cells ensure healthy oocyte and embryo development. To collect COC, the ovary is sliced with a scalpel blade. COCs are then evaluated according to morphological criteria (Wlodarczyk et al. 2009) and used in ARTs.

Another method is called aspiration of antral follicles. This method is based on collection of oocytes using a syringe and needle. The needle punctures the antral follicles allowing collection of follicular fluid together with the oocytes surrounded by cumulus cells. Two procedures are used to collect oocytes. These procedures are called mincing of the ovarian cortex and follicle dissection. Mincing of the ovarian cortex is done by cutting the ovarian surface repeatedly with a surgical blade and the ovaries are then washed in a culture medium. The procedure of follicle dissection firstly divides ovaries into small pieces. The follicles located on the pieces are then punctured with a needle causing releasing of the oocytes (Luvoni 2013).

5.1.2.3 Storage of oocytes

The oocytes have different physical characteristics compared to sperms. The oocyte cell is much bigger, has lesser tolerance to low temperatures and needs longer time to reach osmotic balance with the cryoprotectant (CPA) solution. Despite the oocytes are particularly vulnerable to cold (Saragusty and Arav 2011), there are two methods that can be used safely in felids to cryopreserve oocytes – slow freezing and vitrification.

The method of slow freezing is based on water crystallization. Oocytes are exposed to solutions of cryoprotectants (the most common are e. g.- ethylene glycol, dimethylsulphoxide, 1,2-propanediol). These protectants prevent intracellular ice crystal formation through dehydration. Oocytes loaded into straws are then slow cooled via programmable freezing unit. This procedure ensures that the oocytes are minimally damaged. The oocytes are then submerged into liquid nitrogen for storage. To use frozen oocytes in ARTs, the oocytes have to be rapidly warmed to allow a rapid scatter of intracellular ice crystals. The cryoprotectant is then removed by washing the oocytes in medium with a decreasing concentration of cryoprotectants (Luvoni 2013).

The other method is vitrification. Vitrification consists of forming samples into glassy vitrified state without ice crystallization. This state is obtained by rapidly freezing the oocytes,

embedded into small volumes of cryoprotectant solutions, via immersion into liquid nitrogen. The correct viscosity of this state is obtained with high cryoprotectant concentrations or with a combination of different cryoprotectants in low concentrations. When needed, the samples are then rapidly warmed and washed in decreasing concentrations of cryoprotectants (Luvoni 2013).

5.2 Artificial insemination (AI)

Artificial insemination is necessary when natural mating fails. The main role of this technique is in aiding the animals that are for example aggressive, housed in distant location or just unsuccessfully paired.

There are several circumstances that can influence artificial insemination to be more successful. These are for example: exact time of insemination, the number of sperm that will be inseminated, techniques of the sperm preservation, the site of sperm deposition, and the efficiency of ovarian induction protocol (Thongphakdee A. 2017). Another factor that may affect successful AI is age of a female. This may be observed for example in the cheetah. The nulliparous females of cheetah older than 7 years are poor AI candidates because of their low insemination success. Cheetahs commonly live 12-15 years in ZOOs, to guarantee the best results, AI should be used in relatively early ages in felids. Younger females have much better reproductive fitness compared to the older nulliparous females, whose effectiveness in AI is not that high (Grisham 2004).

There are three types of artificial insemination in the felids according to the site of insemination: intravaginal insemination, intrauterine insemination, and intraoviductal insemination. Each method has different success rate. To achieve a conception rate of **>80%** by AI with fresh semen in domestic cats, 80×10^6 sperm are required for intravaginal AI, and 8×10^6 sperm are required for unilateral intrauterine AI (Tajima H. 2016).

Intravaginal insemination has been successfully done and described in the tiger (Chagas 2000) and domestic cat (Tanaka 2000). To achieve pregnancy in these species, very high sperm numbers (10^7 – 10^8/ml) have been required. Studies of Howard et al. (1992) identify that anesthesia interferes with both sperm transport and ovulation in the domestic cat. The effects of anesthesia slow tract contractility and inhibit ovulation. This might be the reason why success in other felids is so low. Tsutsui (2006) achieved a conception rate of 54% using fresh spermatozoa ($5–50 \times 10^6$) and 11% using frozen-thawed spermatozoa (50–

100×10^6) in domestic cats. Chagas et al. (2000) reported birth of three Siberian tiger cubs using intravaginal insemination with diluted fresh semen (100×10^6).

Intrauterine insemination in cats is done by laparoscopy and/or mid-line laparotomy. Laparoscopy use minimally invasive surgical techniques – only small opening is made in the abdominal wall. Semen is then injected into the uterine lumen of females that are treated with gonadotrophin to increase the chance for fertilization. For maximum effect, high numbers of spermatozoa are used (8-10 million motile spermatozoa). Mid-line laparotomy is done by depositing semen directly into the uterine lumen or oviducts of females that are naturally in oestrus (Swanson 2019). The number of required spermatozoa is not as high (4–10 million spermatozoa per AI) as in the laparoscopy method, but the degree of surgical intervention that is needed for this method restricted its wider applicability (Tsutsui et al. 2000, 2001; Toyonaga et al. 2011). Tsutsui et al. (2000) achieved different conception rates using various amount of spermatozoa in domestic cat. The reported conception rates of fresh spermatozoa are: 13% with 2×10^6 spermatozoa, 31% with 4×10^6 spermatozoa and 80% with 8×10^6 spermatozoa. The conception rate of frozen/thawed ejaculated spermatozoa is 57% with 50×10^6 spermatozoa used. Cryopreserved epididymal spermatozoa (5×10^6) was also used for this method, but the conception rate was only 27%. Intrauterine AI done by laparoscopy has been successful in 8 wild felid species (leopard cat, cheetah, tiger, puma, snow leopard, clouded leopard, ocelot, tigrina), but the conception rates have been low (Swanson 2012).

Intraoviductal insemination is similar to the intrauterine one. This method is also done by laparoscopy, but the difference is, that AI needle is passing right through the oviductal ostium and the semen is deposited about 2 centimetres into the oviduct (Tipkantha et al. 2017). Due to the oviductal deposit of spermatozoa, this method bypasses most anatomical barriers and reproductive tract pathologies (Wildt et al. 1977). The conception rates for domestic cats were estimated by the study of Tsutsui et al. (2001) to 25% (using 2×10^6 spermatozoa) and 43% (using 4×10^6 spermatozoa). Swanson (2012) reported one pregnancy and one viable kitten in the ocelot and one pregnancy and three viable kittens in the Pallas' cat in the US ZOOs using this method.

5.3 *In vitro* oocyte maturation and fertilization

Oocytes collected via *post mortem* methods are generally immature and need to be cultured *in vitro* to develop into mature oocytes. Thus, immature oocytes have to undergo a process of in vitro maturation (IVM). *In vitro* oocyte maturation is a sophisticated process in which the effort is to imitate the dynamic changes occurring in the preovulatory ovarian

follicle and in the oviduct (Luvoni 2000). Studies of Pope et al. (1997) and Wood & Wildt (1997) confirmed an important role of cumulus cells in IVM. Oocytes with intact cumulus cells obtained the highest percentages of maturation and fertilization *in vitro*. All components of the cumulus-oocyte complex (cumulus cells, nuclear material, cytoplasm) are supported by the maturation system. Cultural conditions are also important. It has been reported that gonadotropins and antioxidant components (for example cysteine) considerably improve maturation rates that reach 70% (Luvoni & Colombo 1995).

After the maturation of immature oocytes in a culture medium, cultured oocytes are washed and maintained in fertilization drops. The oocytes are then fertilized with processed sperm samples that was added to the drops. After coincubation (18 hours), cumulus cells and loosely attached spermatozoa are removed and returned to incubation. At 30 hours after *in vitro* fertilization (IVF), the cleavage to the two-cell stage can be seen (Goodrowe et al. 1988; Johnston et al. 1991) proving the success of the fertilization. Oocytes are then physically denuded of cumulus cells and fixed in buffer.

One technique of producing embryos *in vitro* have been recently applied and reported in cats. This technique is called intracytoplasmic sperm injection (ICSI) and consists of microinjection of sperm cells in the cytoplasm of the *in vitro* matured oocytes. This method resulted in cleavage rates ranging from 40 to 60% (Gomez et al. 2000; Pope et al. 2000; Bogliolo et al. 2000). Pope et al. (1998) reported born kittens after transfer of the embryos obtained by ICSI, using only *in vivo* matured oocytes. One pregnancy has been reported by Gomez et al. (2000) after transfer of the embryos produced by ICSI of *in vitro* matured oocytes.

6 Conclusion

Many successes in assisted reproductive technologies have been reported in mammals over the past decades by several studies and authors. Felids considerably differ from other mammals in many factors – anatomy, physiology, social behaviour, all of which may influence the success of ARTs in most of them. Another factor influencing especially wild felids is human. Anthropological pressure on felids also significantly affects ART success and if the situation does not improve or even get worse, further successes may not occur.

However, this does not mean that it is impossible to use these methods in felids. The success of these methods lies in the complete understanding of each species in Felidae family. Several studies highlight the importance of understanding the feline reproductive biology and demonstrate that, after fulfilling some of the conditions and circumstances related to each species, the assisted reproductive technologies can be successfully applied in this mammalian family.

Regardless of all findings brought by many studies, the practical application of assisted reproductive technologies is still not as efficient as might be expected, especially in felids. Admittedly, many successful attempts were made since the beginning of the assisted reproduction technologies both in non-domestic felids and felids in captivity. However, these attempts are still not sufficient enough to use this technology for consistent population management in global scale. This fact proves that the true potential of ART in wild felids has not been yet fully discovered and thus needs to be studied further.

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