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Approaches and efficacy of artificial insemination in felids and mustelids

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1. Introduction

Two of the most diverse and species-rich families in the order Carnivora (containing ~280 species) include Felidae (cats) and Mustelidae (commonly called weasels). According to a current taxonomic classification system, the family Felidae consists of 41 felid species, most of which are listed as threatened or endangered with extinction [1]. The family Mustelidae consists of 59 species (including weasels, ferrets, minks, badgers, otters, wolverines), with the North American black-footed ferret (*Mustela nigripes*) being the most endangered [1]. *Ex situ* breeding programs exist for many of these rare carnivores, and one of the highest priorities is the retention of existing gene diversity to ensure species integrity, health and reproduction. Tools associated with 'assisted reproduction' offer multiple advantages for species propagated under the auspices of an organized genetic management plan (e.g., the Species Survival Plan or SSP©; Table 1) [2,3]. Because cooperating institutions (usually zoos) breed animals on the basis of the genetic value of individuals and computerized calculations of kinship (i.e., interrelatedness) [4], the general protocol commonly involves physically moving animals between geographically different zoos for breeding. The logistical complexities, expense and animal stress associated with such transport is substantial. Thus, a primary advantage of assisted breeding is potential simplicity – that is, directly moving germplasm between locations rather than whole, living and stress-sensitive animals. Individuals paired by computer-matchmaking also can be behaviorally incompatible and fail to copulate upon introduction. Additionally, there are some species that generally are reproductively inefficient under *ex situ* conditions (e.g., cheetah, *Acinonyx jubatus*; clouded leopard, *Neofelis nebulosa*), likely related to a suboptimal captive environment. Lastly, one of the most important reasons for adapting assisted breeding to wildlife is to deal with the challenges associated with small population management, especially ensuring that every genetically valuable individual reproduces to pass its genes to the next generation. An excellent example is the black-footed ferret, which barely dodged extinction by declining to a total of 18 individuals of which only seven were 'founders' (i.e., genetically distinct). In such cases, it is essential that every animal reproduces. The advantage of assisted breeding is bolstered even further by an ability to cryopreserve germ plasm, thereby allowing genetic material to be rederived and

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Table 1
Benefits of assisted reproduction techniques and genome resource banks for population management.

Improve reproductive efficiency in breeding programs
Ensure reproduction in genetically valuable individuals
Combat behavior incompatibility between individuals
Enhance founder representation in small populations
Provide insurance from catastrophes (natural disasters; disease epidemics)
Allow exhange of genetic material between populations
Between *ex situ* populations
Avoid risk and expense of shipping animals for breeding
From *in situ* to *ex situ* populations
Restore genetic vigor to *ex situ* captive populations
Animals remain in the wild, protecting habitat
From *ex situ* to *in situ* populations or between *in situ*
Restore genetic vigor to fragmented wild populations
Eliminate reintroduction or translocation of animals
Avoid potential disease transmission

infused into managed collections years after the death of the original donor.

Of the many assisted breeding tools that have been developed for humans and livestock, artificial insemination (AI; manually depositing sperm into a female) has been recognized as most useful for addressing these highest priorities for *ex situ* wildlife collections [5]. Although the potential of AI for enhancing wildlife breeding programs was proposed more than 30 years ago, progress has been slow for all studied taxa, largely due to the need to first understand the fundamental biology of individual species [6]. This certainly has been true for the Carnivora, comprised of families of diverse species with reproductive mechanisms that differ almost completely from humans and domesticated and laboratory animals. Since the 1970s, our laboratory has focused substantial effort on studying felid and mustelid species for the ultimate purpose of improving managed care. Initial activities emphasized applying cattle and human AI techniques directly to wildlife. When such a naïve, overly simplistic approach failed [3,7], we re-designed strategies to develop a thorough knowledge base for each targeted species of interest. Once the reproductive physiology of each species was better understood (generally over the course of years), then positive AI-related results emerged [8–10].

We also have evaluated extensively the applications of cryotechnology in the context of population genetic management as well as helping to conserve entire species (Table 1) [11,12]. In addition to *in situ* (wild) and *ex situ* (captive) populations, repositories of frozen germ plasm (also known as a genome resource bank; GRB) serve as the third important genetic reservoir for ensuring species integrity and survival [12]. However, the first step to developing a sperm GRB useful for AI has been basic research, especially to distinguish the remarkable species-specific differences in a spermatozoon's ability to withstand cryoprotectant exposure, osmotic perturbations, cooling, intracellular ice formation, and then return to ambient temperature [5]. Once this level of cryosensitivity has been determined, it has been possible to systematically develop protocols that permit at least some felid and mustelid sperm to retain post-thaw viability. Furthermore, it has been occasionally feasible to produce wild carnivore offspring from cryopreserved spermatozoa combined with AI (see below).

Indeed, there are examples of the long-term benefits of frozen sperm repositories for protecting gene diversity. For example, in the small black-footed ferret population, genetic variation gradually has decreased over time in the *ex situ* program without an influx of new genes from non-existent wild-born founders. Fortunately, semen samples cryopreserved from black-footed ferrets in the 1990s recently have been used to produce offspring via AI from long-dead donors, illustrating the value of these frozen repositories for re-infusing genetic vigor. These cryo-tactics also have application in underdeveloped range countries, where biodiversity is especially rich [11]. For example, after conducting the prerequisite basic research [13], we have helped establish a sperm GRB for cheetahs in Namibia, a repository that represents genetic diversity of the remaining 3000 free-living cheetahs in that country [14]. Meanwhile, subsets of semen samples from the GRB are exported to support the North American Cheetah SSP [14]. This strategy ensures that cheetahs are not extracted from nature to be moved to zoos. Rather their surplus germ plasm is collected, stored and then used, with the living animals left in the wild where their very presence protects native habitat. Furthermore, it may be possible in the future for these frozen sperm samples to contribute to *in situ* populations. For example, it is theoretically possible to infuse this genetic material into small, wild populations through a process of short-term animal capture, ovulation induction, AI and then reintroduction of pregnant females.

Thus, the potential of AI for wild carnivore management and conservation is enormous. Although the challenges remain substantial, there has been significant progress, which is remarkable given the few researchers in this field. This review discusses key factors, tools and lessons learned from 30 years of

experience developing AI for wild felids and the black-footed ferret.

2. Fecal hormone monitoring

One of the most valuable tools to applying AI successfully to felids and mustelids is not directly related to collecting, processing, storing or depositing spermatozoa. Rather it is noninvasive fecal hormone metabolite monitoring for understanding basic endocrine profiles, mostly in females, but also in males. Hormonal metabolite measures in excreted feces has become a powerful tool for studying carnivore reproduction and advancing assisted breeding [15–17], largely because it is a non-disruptive means of measuring gonadal and adrenal function. Almost all steroid hormones in felids are excreted in feces, rather than urine [15,18]. Validation studies have proven that metabolite profiles generated from serial fecal sample collections reflect physiological hormone fluctuations in peripheral circulation [17,19]. Fecal assessments provide an accurate 'pooled' value of hormonal activity as metabolites are excreted over hours, thereby avoiding the 'noise' and dynamism observed from traditional sequential blood sampling studies. Much of what is currently known about onset of puberty, seasonality, duration of estrus, length of the estrous cycle, time and type (induced versus spontaneous) of ovulation, pregnancy and parturition prediction in felids and mustelids is the result of fecal hormone metabolite monitoring (see reviews [16,17,19]).

Once normative endocrine traits are determined for a given species, such data can have applied management value. For example, fecal hormone monitoring has been useful for discovering that excessive artificial lighting associated with an annual holiday event ('Festival of Lights') altered seasonality and adversely impacted the reproductive cycle of the Pallas' cat (*Otocolobus manul*) [20]. In another case, it was discovered that housing female cheetahs together suppressed normal ovarian cyclicity, a disruption that was mitigated by separating animals into individual enclosures [21]. It also has been possible to monitor adrenal corticoid excretion in feces, with one study in the clouded leopard demonstrating how improved environmental conditions (more vertical cage height, isolation from other larger cats and maintenance off public-display) reduce both physiological and behavioral indices of stress [22].

In the context of assisted breeding studies, fecal hormone monitoring has been instrumental in assessing ovarian responses to ovulation induction and AI protocols. Valuable studies have assessed the impact of exogenous gonadotropins on ovarian activity, including the ability of equine chorionic gonadotropin (eCG) to induce folliculogenesis and human chorionic gonadotropin (hCG) to induce ovulation. It especially has been important to document species-specific differences in ovarian response to eCG and hCG (see below) [9,10]. Analysis of fecal hormones also has allowed characterizing which felid species experience induced (i.e., oocyte release only after copulation or hormonal stimulation) or spontaneous (corpora lutea [CL] formation in the absence of a male and copulation) ovulation. Interestingly, the cheetah [23], tiger (*Panthera tigris*) [17,24], tigrina (*Leopardus tigrinus)* [25] and ocelot (*Leopardus pardalis*) [25] appear to be exclusively induced ovulators, similar to the puma (*Puma concolor*) [26], Siberian tiger (*Panthera tigris altaica*) [27] and snow leopard (*Uncia uncia*) [28] assessed in earlier studies using blood analyses. In contrast, spontaneous ovulation during natural estrous cycles has been reported in the clouded leopard [29], lion (*Panthera leo*) [30], leopard (*Panthera pardus*) [31]; margay (*Leopardus wiedii*) [25], Pallas' cat [20] and fishing cat (*Prionailurus viverrinus*) [32,33].

Hormonal monitoring also has been essential for titrating eCG/hCG dosages to avoid ovarian hyperfollicular stimulation and excessive circulating estrogen production (e.g., cheetah and tiger) [23,24,34]. In most cases, the confidence in a specific eCG/hCG regimen can be determined by parallel (or historic) profiling of gonadal endocrine patterns in naturally estrual and/or copulating conspecifics. If the hormonal metabolite patterns are congruent, then there is general assurance in the efficacy and safety of the gonadotropic therapy.

3. Incidence of teratospermia

A biological factor particularly important for developing successful AI in felids is the phenomenon of teratospermia (production of $<60\%$ morphologically normal spermatozoa/ejaculate) (Fig. 1) [3,35,36]. Our laboratory has revealed that 20 of the 23 felid species (or their subspecies) evaluated consistently experienced this condition (range, 4–58% normal sperm; Fig. 2) [36]. Interestingly, only three species (domestic cat, leopard cat, Siberian tiger) ejaculate $>60\%$ structurally normal spermatozoa per ejaculate (Fig. 2) [36].

Although the specific etiology of teratospermia remains to be determined, there is evidence that this condition is directly related to reduced genetic diversity. For example, felid species or populations lacking genetic variability tend to produce more malformed spermatozoa than more genetically diverse counterparts

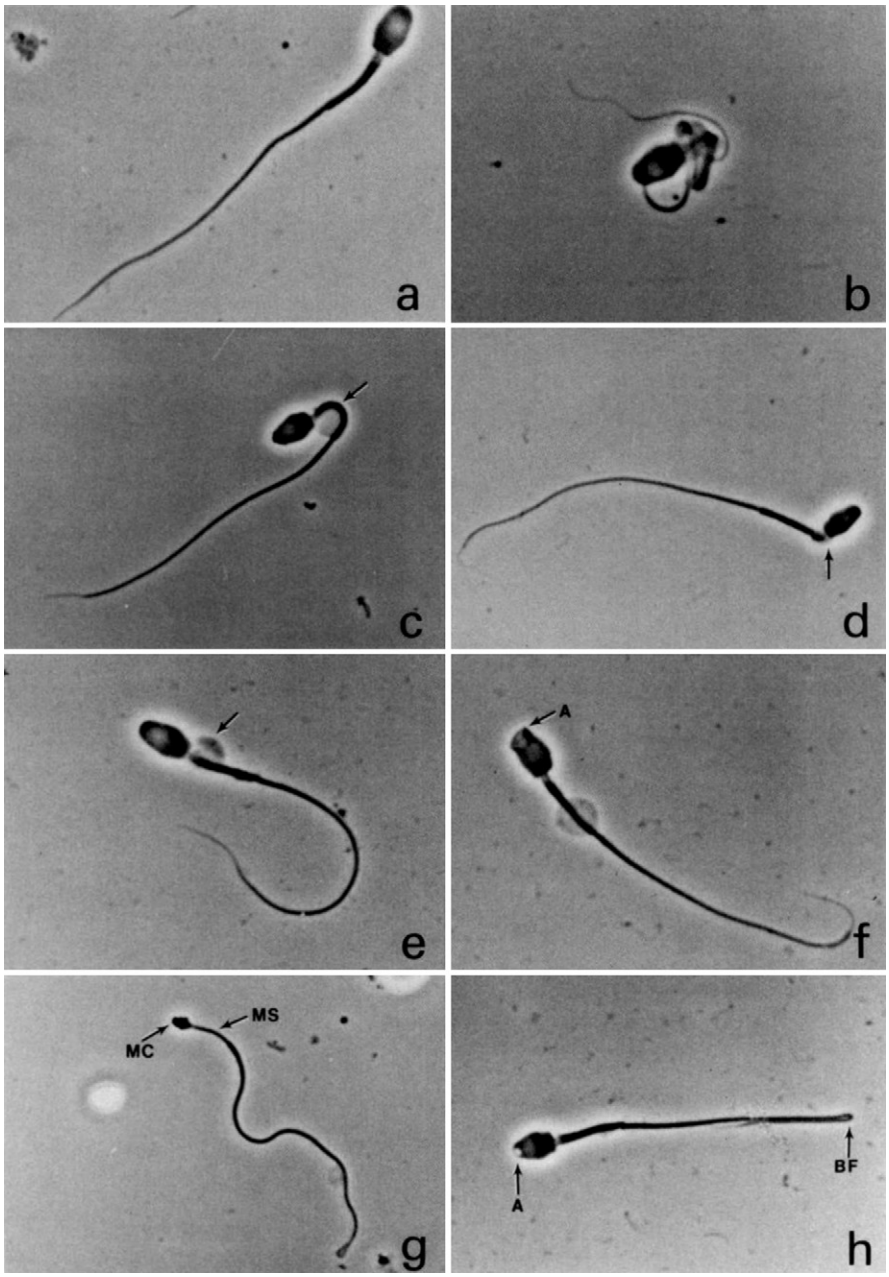

Fig. 1. Phase-contrast photomicrographs of normal and abnormally shaped spermatozoa commonly observed in felid electroejaculates. (a) normal; (b) coiled flagellum; (c) bent midpiece with cytoplasmic droplet; (d) bent neck; (e) proximal cytoplasmic droplet; (f) acrosomal (A) defect and distal cytoplasmic droplet; (g) microcephalic (MC) defect and missing mitochondrial sheath (MS); (h) bent flagellum (BF) and acrosomal defect (A). *Source*: Ref. [2].

[37,38]. The genetically homozygous Florida panther produces extraordinarily high proportions (>90%) of pleiomorphic sperm [39,40]. The cheetah and clouded leopard (two other species well documented to have low levels of gene diversity) also routinely ejaculate >70% malformed spermatozoa [36,37,41]. A more direct link between heterozygosity and sperm integrity in felids has been shown from prospective inbreeding studies in the domestic cat, whereby one generation of sib-to-sib matings increases pleiomorphic sperm production in offspring [42].

Increased homozygosity appears to increase the incidence of sperm head and acrosomal abnormalities in some felids, including the Florida panther and clouded

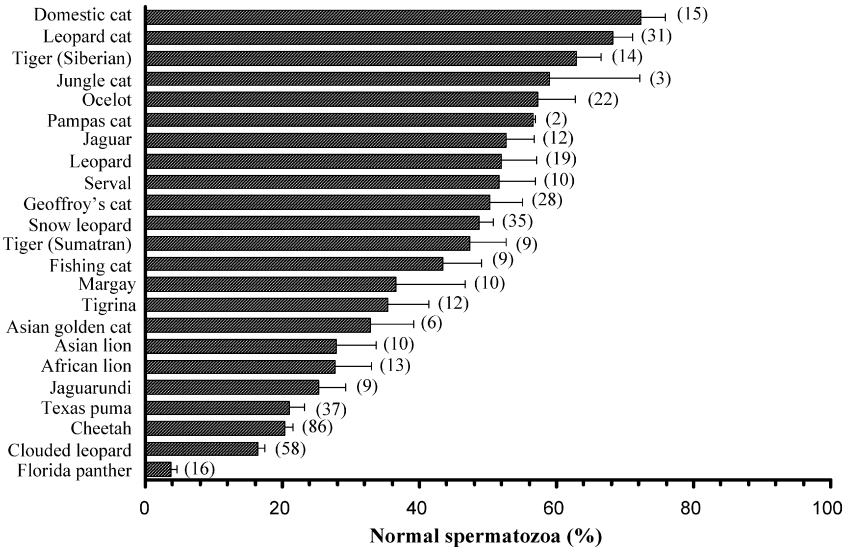

Fig. 2. Proportion of total structurally normal spermatozoa in 23 species (or subspecies) in the Felidae family. Values are means ± SEM. Numbers in parentheses indicate number of males evaluated. *Source*: Ref. [36].

leopard (Fig. 1F–H). Transmission and electron microscopy reveal large vacuoles, a knobbed acrosome and abnormal acrosomal matrix in such animals [36]. These types of sperm anomalies now are known to originate within the testes, which is indicative of dysfunctional spermatogenesis and spermiogenesis [43]. In the case of the Florida panther, the extreme teratospermia has occurred in parallel with an increased incidence of cryptorchidism and prevalence of heart anomalies (atrial septal defect) and hypersensitivity to disease pathogens [39].

Such observations reinforce the importance of retaining genetic diversity of felid populations (in captivity as well as the wild) and for stimulating interest in understanding the functional impact of malformed sperm in the ejaculate. We have demonstrated in our laboratory that even structurally normal spermatozoa from teratospermic ejaculates are compromised in fertilization ability [36,44,45]. Sperm from such ejaculates are less able to undergo capacitation and the acrosome reaction [46,47], penetrate the zona-pellucida [44], fertilize conspecific oocytes [44], and survive cryopreservation [48].

Because of its prevalence in felids and ability to reduce fertilization, teratospermia becomes a key factor to developing effective AI protocols. Even so, the specific influence of significant numbers of malformed and dysfunctional sperm on AI efficiency still is unknown. Conducting systematic, prospective *in vivo* investigations is logistically difficult (due to the rarity of study specimens) and expensive. The few attempts to produce pregnancies in the naturally mating Florida panther held in captivity met with failure [49]. In contrast, it is recognized that a cheetah can become pregnant after a single mating, despite the male ejaculating mostly malformed spermatozoa [34]. Furthermore, AI-derived pregnancies also have resulted in this species from inseminating comparative low sperm density inseminates comprised of >70% abnormally shaped cells (see below) [34,50]. Thus, the threshold for an adverse effect of teratospermia on conception is relatively high or perhaps the cheetah is comparatively reproductively 'efficient', requiring few total spermatozoa (even in the face of many malformed cells) to achieve successful fertilization. This perhaps is one of the primary reasons why AI has been relatively effective in this species (see below). Regardless, it is clear that the structurally defective cells do not participate in fertilization. Thus, AI protocols for felids must ensure that at least some (preferably optimal numbers) of the morphologically normal spermatozoa are available to maximize conception potential.

Teratospermia is not unique to felids and, interestingly, an important condition in the black-footed ferret. Detailed comparative studies have been conducted involving this rare species and two related counterparts (domestic ferret, *Mustela putorius furo*; Siberian [steppe] polecat, *Mustela eversmanii*). Both of the latter have higher genetic diversity than the former, which was derived from a small founder base [51]. Black-footed ferrets routinely ejaculate ∼79%

malformed sperm compared to the domestic ferret (~33%) and Siberian polecat (~25%) [52–55].

4. AI in felids

Procedural efficiency and effectiveness of AI in felids is related to having significant baseline information on: (1) site of insemination; (2) time of insemination; and (3) ovulation induction.

4.1. Site of insemination

In the 1970s, domestic cat kittens were born from females that were artificially inseminated vaginally (while fully conscious) with fresh sperm during a natural estrus (Table 2) [56]. Subsequently, anesthetized queens in natural estrus or a gonadotropin-induced estrus were vaginally inseminated with frozen–thawed spermatozoa which resulted in live kittens (Table 2) [57]. Despite an incidence of pregnancy success of only ~11% in that study, this AI approach was applied to cheetahs, tigers and clouded leopards (with an occasional transcervical insemination), but no pregnancies resulted, including after inseminations of 23 cheetahs (Table 2), 11 tigers, and seven clouded leopards [2,58,59].

The first successful AI in a wild felid occurred in 1981 at the Zoological Society of London with a puma cub produced by *in utero* deposition of sperm at laparotomy [60,61]. Shortly thereafter, a Persian leopard (*Panthera pardus saxicolor*) was produced at the Cincinnati Zoo and Botanical Garden after a transcervical AI [62]. Our laboratory focused research on why the intravaginal/intracervical approach was so inefficient. It soon was discovered that sperm transport through the reproductive tract was poor in anesthetized felids, probably due to reduced uterine horn contractility caused by anesthesia [59]. To circumvent this challenge, a laparoscopic intrauterine insemination technique was developed to ensure that sperm were deposited near to the site of fertilization (oviduct). This transabdominal approach involved inserting a laparoscope through a cannula device (and a 3 cm skin/muscle opening) in the abdominal wall, followed by catheterizing the uterine lumen and depositing sperm in the cranial aspect of the uterine horn (Fig. 3) [63]. Laparoscopy also has been used extensively for characterizing felid reproductive tract anatomy and ovarian events, including documenting follicular development, presence of fresh (corpora hemorrhagica, CH) and mature corpora lutea (CL) and the effectiveness of various gonadotropin therapies [9,10]. This laparoscopic AI technique was utilized initially in the domestic cat and then successfully extrapolated to the cheetah (Table 2) [34,63].

A major advantage of intrauterine AI is the need for fewer spermatozoa than required during a vaginal insemination (Table 2) [64]. Tanaka et al. [65] reported a high incidence of pregnancy success (~78%) in naturally estrual domestic cats vaginally inseminated with 80×10^6 fresh sperm. It was recognized that two semen collection procedures may be required to achieve this high cellular density for a single insemination [65]. In contrast, Tsutsui et al. [66,67] achieved a conception rate of 80% in the cat by intrauterine insemination (by laparotomy) but using only 8×10^6 fresh sperm into a single uterine horn. This relationship between insemination site and number of sperm required for conception also has been observed in the dog [68]. In humans, similar findings have been attributed to the filtering role

Table 2
Influence of site of sperm deposition and number of motile sperm inseminated on pregnancy success after vaginal/cervical (non-surgical) versus intrauterine (laparoscopy or laparotomy) artificial insemination (AI) in various species.

Species	Gonadotropins	Sperm deposition	Sperm type	No. motile sperm inseminated	No. pregnancies (%)	Reference
Domestic cat	Natural estrus/hCG	Vaginal	Fresh	$5–50 \times 10^6$	14/26 (53.8%)	[56]
Domestic cat	Natural estrus and pFSH/hCG	Vaginal	Frozen	$50–100 \times 10^6$	6/56 (10.7%)	[57]
Domestic cat	eCG/hCG	Intrauterine (laparoscopy)	Fresh	$2–19 \times 10^6$	9/18 (50.0%)	[63]
Domestic cat	Natural estrus/hCG	Vaginal	Fresh	80×10^6	7/9 (77.8%)	[65]
Domestic cat	Natural estrus/hCG	Intrauterine (laparotomy)	Fresh	8×10^6	8/10 (80.0%)	[66]
Domestic cat	Natural estrus/hCG	Intrauterine (laparotomy)	Frozen	50×10^6	8/14 (57.1%)	[70]
Cheetah	pFSH/hCG	Transcervical	Fresh/frozen	30×10^6	0/23 (0%)	[2]
Cheetah	eCG/hCG	Intrauterine (laparoscopy)	Fresh	$4–21 \times 10^6$	6/13 (46.2%)	[34]
European ferret	Natural estrus/hCG	Vaginal	Fresh	$4–19 \times 10^6$	0/10 (0%)	[106]
European ferret	Natural estrus/hCG	Intrauterine (laparoscopy)	Fresh	$2–10 \times 10^6$	17/24 (70.8%)	[106]
European ferret	Natural estrus/hCG	Intrauterine (laparoscopy)	Frozen	$2–6 \times 10^6$	7/10 (70.0%)	[107]

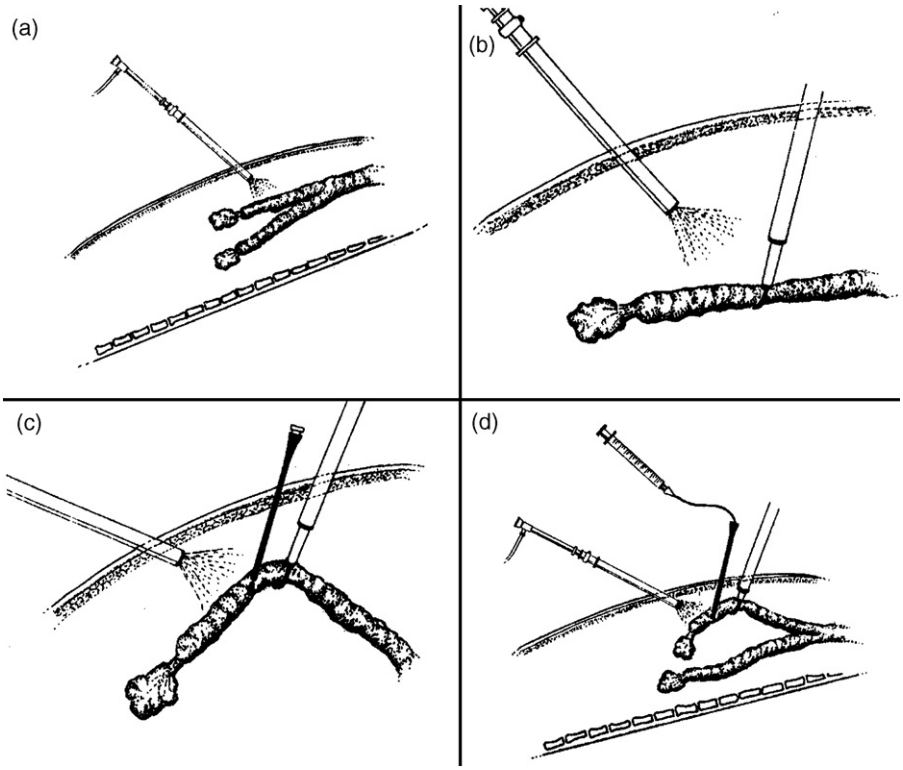

Fig. 3. Laparoscopic intrauterine insemination in felids. (a) The laparoscope is used to identify the reproductive tract; (b) accessory forceps are used to grasp the uterine horn; (c) the horn is elevated, and a catheter punctured through ventral abdominal wall is inserted into the uterine lumen; and (d) the stylette is withdrawn from the catheter and tubing containing spermatozoa is guided through the catheter into the uterine lumen for sperm deposition. *Source*: Ref. [63].

of the cervix and its secretions on limiting sperm reaching the site of fertilization [69]. These observations are important in the context of wild felids that routinely produce comparatively low sperm densities (often with high accompanying pleiomorphisms). Therefore, it is not surprising that early vaginal and intracervical inseminations failed, and the more prudent approach is to ensure that sperm are deposited as near as possible to the oviduct. This is especially important in cases of using valuable thawed sperm that may well be further compromised as a result of cryopreservation/thawing [48]. Due to reduced post-thaw motility and acrosomal integrity, more frozen–thawed sperm may be required for intrauterine insemination [70].

4.2. Time of insemination

Timing the insemination also influences AI success in felids. Logically, it would appear that insemination should occur just before ovulation, so that spermatozoa are present in the oviduct upon arrival of ova. However, studies from our laboratory revealed that the use of anesthesia prior to ovulation (to allow animal handling) compromises ovum release and subsequent pregnancy establishment in felids. When domestic cats are treated with eCG/hCG and anesthetized with ketamine hydrochloride, acepromazine and gaseous halothane immediately before ovulation, only a few females ovulate, and pregnancy success after AI is low (14%; Table 3) [63]. Conversely, when the interval from hCG administration-to-anesthesia is increased to 6–14 h after the anticipated time of ovulation, all cats release ova, and 50% of these become pregnant and deliver live offspring (Table 3) [63]. Tsutsui et al. [66] found conflicting results. These investigators evaluated conception rates in domestic cats that were inseminated *in utero* via laparotomy either before or after ovulation. Queens were induced to ovulate with hCG on Days 2–4 of a natural estrus, then anesthetized with ketamine, acepromazine and halothane gas before AI. Ten of 18 (55.6%) cats that were anesthetized before ovulation conceived, which was higher than the five of 24 (20.8%) cats inseminated post-ovulation (Table 3). Perhaps the reason for the difference between the two laboratories was ‘type’ of estrus. Cats in the Tsutsui investigation were in natural estrus, whereas follicular development

Table 3
Influence of time of insemination conducted pre-ovulation versus post-ovulation on pregnancy success following intrauterine artificial insemination (AI) in felids.

	Gonadotropins		No. pregnancies (%)		
	eCG (IU)	hCG (IU)	Pre-ovulation	Post-ovulation	Reference
Domestic cat	100	75	2/14 (14.3%)	9/18 (50.0%)	[63]
Domestic cat	Natural estrus	100 × 2 or 250	10/18 (55.6%)	5/24 (20.8%)	[66]
Cheetah	200	100	0/3 (0%)	6/13 (46.2%)	[34,50]

in the queens in our study was stimulated using eCG. These two conditions may create different levels of follicular sensitivity to ovum release and responsiveness to the cascade of events associated with anesthetic drug use.

Nonetheless and most importantly, we also observed compromised ovulation in wild felids due to use of anesthesia. Three of four preovulatory tigers treated with gonadotropins followed by anesthesia and laparoscopy at 39–42 h after hCG failed to ovulate (based on laparoscopic observations) [71]. Similar results for cheetahs are depicted in Table 3. Three females failed to ovulate when anesthesia and insemination were conducted before ovulation, whereas pregnancies were established in females anesthetized and inseminated after ovulation (Table 3) [34,50].

As a result of such findings and experiences, gonadotropin-stimulated felids in our laboratory always are artificially inseminated after ovulation has commenced. This practice obviously requires having detailed information on time of ovulation which is species-specific. Examples of variance in onset of ovulation after eCG/hCG treatment are illustrated in Table 4 and range from 25 to 30 h (post-hCG) in the domestic cat and leopard cat (*Prionailurus bengalensis*) to 39–46 h for the tiger [9,10].

4.3. Ovulation induction and sensitivity to exogenous gonadotropins

Most wild felids in *ex situ* collections do not reliably exhibit overt signs of estrus, or behavioral indices of sexual receptivity are inconsistent or too difficult (or dangerous) to identify. Periods of estrus also can be determined by fecal hormonal monitoring (see above), although such analyses generally are retrospective because of the laboratory time required to assay samples for hormone content. Thus, the most effective means for scheduling an AI is to stimulate ovarian activity using exogenous gonadotropins. This process benefits from the tendency of many felids to be 'induced' (rather than 'spontaneous') ovulators (as discussed above). Thus, ovulation can be controlled and will not normally occur in the absence of administrating an ovulation-inducing hormone, such as hCG [72–74], luteinizing hormone (LH) [10] or gonadotropin releasing hormone (GnRH) [73]. In contrast, spontaneously ovulating felids (like the clouded leopard) will frequently ovulate and produce CL and an endocrine milieu that can interfere with provoking a new crop of pre-ovulatory ovarian follicles, thereby complicating the AI process (see below) [10].

Substantial data on ovulation induction therapies and success are available in felids, in part, because of significant early data on the domestic cat (as a model for wild felids) [2,72] and the use of laparoscopy. For three decades, laparoscopic viewing of felid ovaries has allowed documenting follicular development and ovulation in non-gonadotropin-treated cycles [75], as well as the timing and impacts after various hormonal therapies [9,72,76]. This has included tracking and photographing the fate of immature and mature follicles, fresh (CH) and mature (CL) ovulation sites over time [63].

Initial studies in our laboratory focused on using serial injections of follicle stimulating hormone (porcine derived; pFSH for 5 d) and hCG (for 1 or 2 d) prior to the planned AI [57,72]. Although this protocol resulted in the production of domestic cat kittens after AI with fresh [72] or thawed [57] spermatozoa, ovarian hyperstimulation (excessive development of follicles) was not uncommon. We also encountered logistical challenges to giving multiple (daily) FSH injections to wild felids (i.e., increased animal anxiety). And, although this hormone treatment sometimes resulted in normal-appearing follicles and ovulation sites, occasional abnormal ovarian responses occurred [2,59,76,77]. Most importantly, none of the cheetahs, leopards, lions or tigers given this gonadotropin became pregnant post-AI. This was incentive for subsequent studies that examined the longer-acting eCG that required only a single injection and has been relatively effective in diverse felid species (Table 4) [9,10,63,74]. Nonetheless, exogenous gonadotropins

Table 4
Successful ovulation induction and laparoscopic intrauterine artificial insemination (AI) conducted post-ovulation in various felids.

Species	Average body weight (kg)	Gonadotropin dosages		Time of ovulation post-hCG (h)	Type of sperm	No. pregnancies (%)	Reference
		eCG (IU)	hCG (IU)				
Domestic cat	2	100	75	25–30	Fresh	9/18 (50.0%)	[63]
Leopard cat	2	100	75	25–30	Fresh and frozen	2/2 (100.0%)	[7,86]
Tigrina	2	200	150	30–36	Fresh	1/4 (25.0%)	[85,87]
Ocelot	9	400	200	30–36	Fresh	1/4 (25.0%)	[85]
Ocelot	9	500	225	30–36	Frozen	1/4 (25.0%)	[88]
Clouded leopard	15	100	75	37–40	Fresh	1/20 (5.0%)	[34,58]
Snow leopard	30	600	300	~40	Fresh	1/15 (6.7%)	[97]
Cheetah	35	200	100	40–42	Fresh	6/13 (46.2%)	[34]
Cheetah	35	200	100	40–42	Frozen	3/11 (27.2%)	[83,84]
Puma	35	200	100	33–40	Fresh	1/8 (12.5%)	[98]
Tiger	250	1000	750	39–46	Fresh	1/10 (10.0%)	[71,99]

can disturb endocrine profiles causing an aberrant follicular, oviductal or uterine environment and disruptions in oocyte maturation, embryo development, or implantation [78–80]. Safety also can be an issue with eCG and hCG as these are large, foreign glycoproteins that persist for days in circulation and can induce gonadotropin-neutralizing antibodies after subsequent eCG/hCG treatments [74,81,82]. Too frequent administration results in refractoriness, which can be avoided by giving eCG or hCG treatments no more than once every 6–12 mo [81,82].

Currently, the most common means of stimulating ovum release from ovarian follicles prior to AI is to give two im injections, the first eCG, followed by hCG 80–84 h later. There are rather remarkable differences among felid species in ovarian response to gonadotropin dosage. Certain species (including the cheetah) demonstrate an extreme level of sensitivity to eCG and hCG, with higher dosages causing hyper-elevated fecal estradiol and progestogen concentrations [17]. As dosages are lowered (for example, to 200 IU eCG and 100 IU hCG in the cheetah), fecal hormones return to concentrations observed during and after natural estrus and mating (Fig. 4) [23]. Laparoscopic assessments also have revealed that gonadotropin dosage influences CL morphology [34]. In the cheetah, high eCG/hCG dosages (i.e., 400 IU/250 IU) result in abnormally small CL (2–4 mm diameter; Fig. 5A), whereas lower concentrations (200 IU eCG/100 IU hCG) produce larger (5–8 mm diameter), more structurally normal CL (Fig. 5B) [34]. It also is now known that there is a threshold eCG dose effect; for example, 100 IU in the cheetah produces ovarian follicles that are incapable of ovulating, even in the presence of high hCG [34]. Such titration studies have led to developing an effective gonadotropin protocol for the cheetah that has resulted in 11 pregnancies and 20 cubs after intrauterine AI (Table 4) [34,50,83,84].

Certain felid species appear to be insensitive to gonadotropins, requiring increased dosages to achieve a similar ovulation induction response. For example, the tigrina requires twice the dosages of eCG (200 IU) and hCG (150 IU) than the domestic cat and leopard cat (100 IU eCG/75 IU hCG), despite being the same weight (Table 4) [7,85–87]. The ocelot, which is about one-fourth the body mass of a cheetah, requires

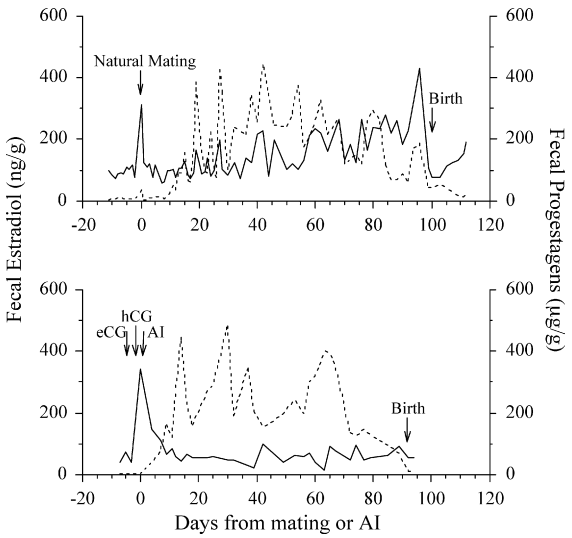

Fig. 4. Mean (± SEM) fecal estradiol (solid line) and progestogen (dashed line) metabolite profiles in pregnant cheetahs demonstrating similar hormone concentrations following a natural estrus and natural mating compared to a gonadotropin-induced cycle and laparoscopic intrauterine insemination. A low dose of equine chorionic gonadotropin (200 IU) was used to induce estrus, followed 80 h later by human chorionic gonadotropin (100 IU) to induce ovulation. This ovulation induction and AI protocol has resulted in 11 cheetah pregnancies and 20 cubs. *Source*: Ref. [23].

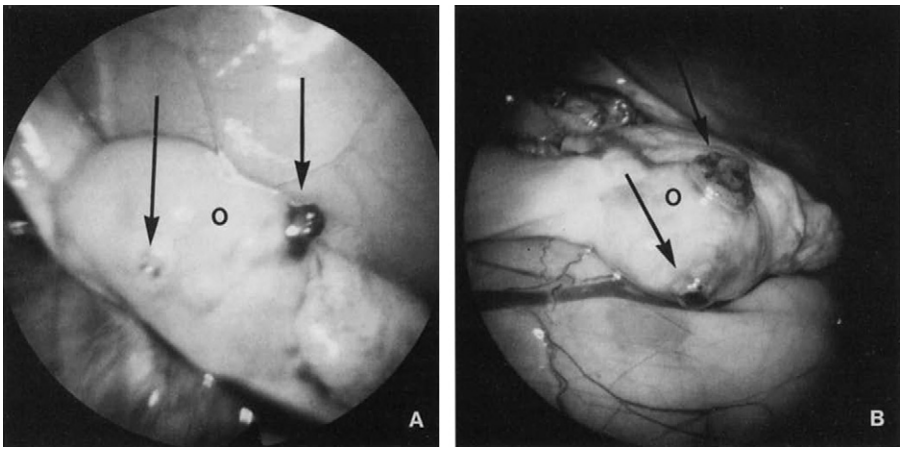

Fig. 5. Cheetah ovaries (O), each containing one of two types of fresh corpora lutea (CL) after gonadotropin stimulation. (A) The small CL (arrows) were 2–4 mm in diameter and observed following high doses (400 IU eCG/250 IU hCG) of gonadotropins. (B) The large CL (arrows) were 5–8 mm in diameter and observed when lower gonadotropin doses (200 IU eCG/100 IU hCG) were used. *Source*: Ref. [34].

400–500 IU of eCG (twice the cheetah dosage) to mimic similar ovarian activity (Table 4) [34,85,88].

4.4. Ovarian control prior to AI

The ability to suppress gonadal activity prior to ovarian stimulation has improved AI success in domesticated livestock [89]. Generally, an inactive ovary is considered to be more likely to provide a uniform response to exogenous gonadotropins. Therefore, we have speculated that AI efficiency in cats could be enhanced (especially in those species that tend to spontaneously ovulate) by artificially creating a quiescent ovary prior to gonadotropin administration. This may be especially important for the clouded leopard, where up to 43% of females spontaneously release ova in the absence of mating (based on fecal steroid data) [29]. In the presence of mature, functional CL (Fig. 6), even the ideal eCG/hCG dose fails to elicit normal follicle development and ovulation (Fig. 7) [29,34].

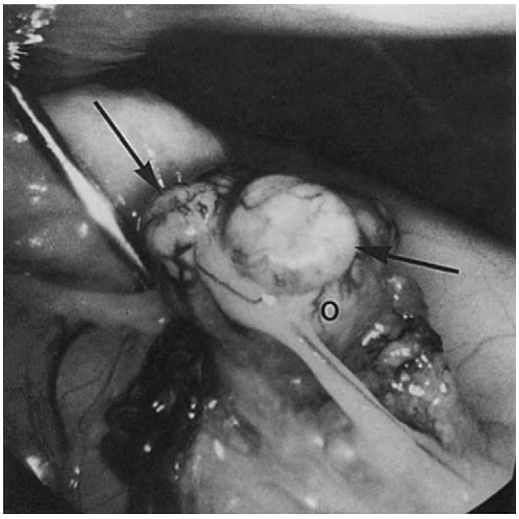

Fig. 6. Clouded leopard ovary (O) containing mature, aged corpora lutea (CL; arrows) in a spontaneously ovulating female, a finding that interferes with ovarian response following exogenous gonadotropin treatments. Aged CL were spherical structures (10–12 mm in diameter, 8–10 mm in height) with extensive surface vascularization. *Source*: Ref. [34].

Our contemporary studies are exploring means of applying short-term, reversible control on ovarian activity hoping to elicit a more consistent response to eCG/hCG treatment. Inducing lutelolysis (a typical means of regulating the estrous cycle in other mammals; [89]) is ineffective in felids. The CL is nonresponsive to prostaglandins (even at high dosages) [90] and is refractory to dopamine agonists up to 40 d post-ovulation [91]. Therefore, we reasoned that follicular inhibition would be a more reasonable approach for eliciting synchrony. Five hormone options have been investigated in felids: (1) GnRH agonist, leuprolide acetate (Lupron®); clouded leopard; (2) GnRH antagonist, antide; domestic cat; (3) melatonin; domestic cat; (4) progestin, levonorgestrel (Norplant®; implant); domestic cat, fishing cat and clouded leopard; and (5) progestin, altrenogest (Regu-Mate®; oral); domestic cat, fishing cat and clouded leopard. Although all suppressed ovarian activity in the species listed [92–96], only the progestin implant (levonorgestrel) and the oral progestin (altrenogest) have successfully improved subsequent ovarian response to exogenous gonadotropins [95,96].

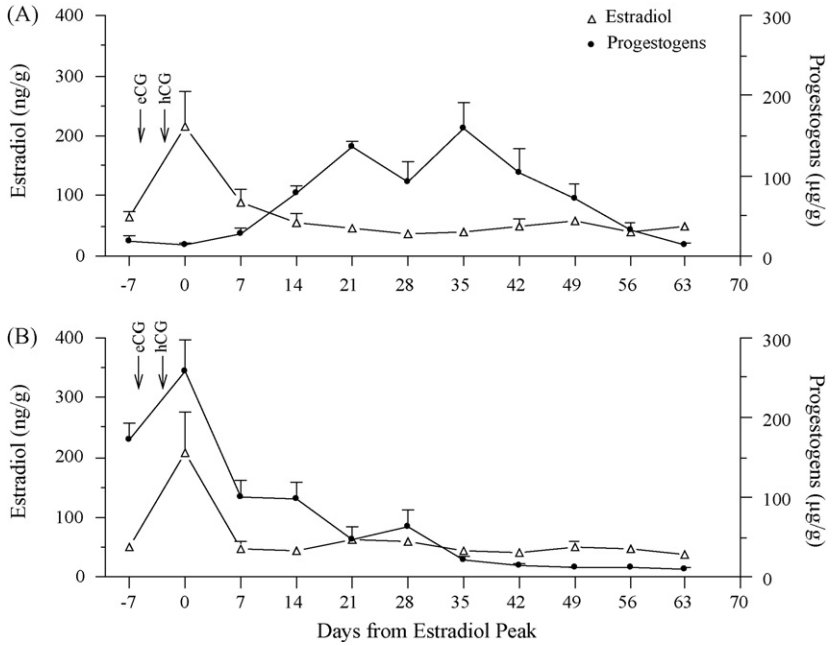

Fig. 7. Mean (±SEM) fecal estradiol (open triangle) and progestogen (closed circle) metabolite profiles in clouded leopards demonstrating two different responses to ovulation induction. (A) Normal ovarian response to eCG/hCG treatment ($n = 3$) comprised of a provoked increase in estradiol followed by a sustained rise in progesterone as a result of ovulation. (B) Failed ovulation because of initially elevated fecal progestogen concentrations due to the presence of mature ovarian CL as a result of spontaneous ovulation (without copulation) occurring prior to eCG/hCG treatment ($n = 3$). The result was only an abbreviated progesterone response to eCG/hCG stimulation. All data were aligned to the estradiol peak. *Source*: Ref. [29].

More specifically, pre-treatment with the levonorgestrel implant before eCG/hCG has resulted in consistent CL numbers and quality in the domestic cat and fishing cat [33,96]. Tendencies for spontaneous ovulation have been eliminated, and endocrine profiles during follicular and luteal phases have been normal (Fig. 8). However, levonorgestrel has reduced ovarian responsiveness to gonadotropin stimulation in the

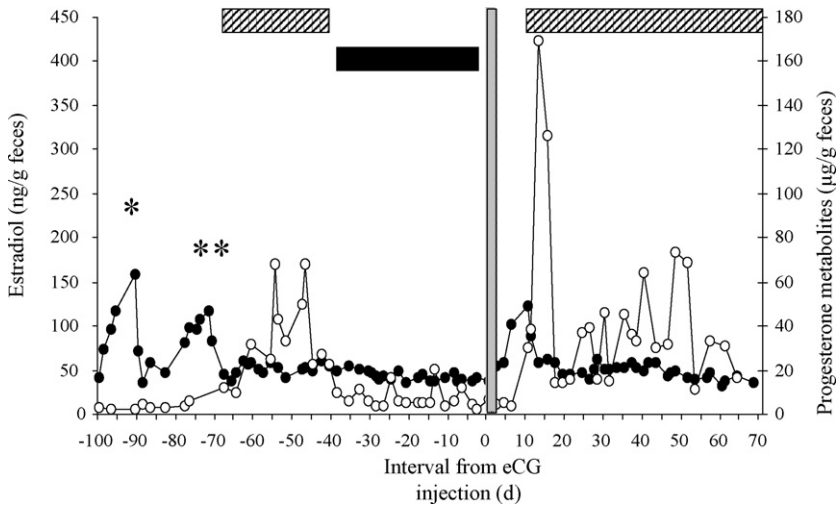

Fig. 8. Prevention of ovulation by the progestin levonorgestrel in a domestic cat prone to ovulating spontaneously followed by ovulation induction with eCG and hCG. Estradiol (closed circles) and progesterone metabolite (open circles) profile demonstrating a spontaneous ovulation event (hatched horizontal bars) prior to, but not during, inhibition treatment with levonorgestrel implant, followed by an induced follicular phase and ovulation. Asterisks indicate estradiol peaks associated with a follicular phase. Black horizontal bar represents the 37-d implant period. Vertical bar represents the day of eCG injection. Horizontal bar following eCG represents an ovulation induced by hCG. *Source*: Ref. [96].

clouded leopard. Only three of eight (37.5%) clouded leopards treated with levonorgestrel before eCG/hCG ovulated, and all females had multiple (6–26) unovulated ovarian follicles [10].

The major disadvantage of the levonorgestrel implant is the need for two anesthetic procedures, to insert and then remove the hormone-releasing device. Therefore, after determining in early studies that progestin administration was effective for short-term ovarian suppression, all subsequent studies have focused on the oral altrenogest option. These studies have revealed that females given this progestin have excellent ovarian responses post-gonadotropin treatment. Interestingly, one study in the domestic cat determined that altrenogest treatment increased ovarian sensitivity subsequent to exogenous gonadotropins. Lower doses of eCG (50 IU) and hCG (38 IU) produced the same number of follicles and subsequent ovulations (Fig. 9) as the traditional, higher dose (100 IU eCG/75 IU hCG) treatment [95]. However, priming with the progestin altrenogest in the clouded leopard appeared to interfere with ovulation following gonadotropin stimulation, similar to the ovarian response observed with the progestin levonorgestrel [10]. Recent findings have suggested that ovarian down-regulation with these steroid hormones in the clouded leopard appears to decrease ovarian sensitivity and increase the required subsequent dose of both eCG and hCG (Pelican and Howard, unpublished data, 2008). Clouded leopards actually have required significantly higher eCG (300 IU) and hCG (225 IU) after altrenogest, indicating that the ovary has become more resilient to exogenous gonadotropins. This new protocol (altrenogest priming and high dosages of eCG/hCG) recently has been used for AI in clouded leopards with excellent ovarian responses (13–17 CL and only 3–4 unovulated follicles/female); pregnancy results are pending (Pelican and Howard, unpublished data, 2008). Therefore, although spontaneous ovulation is species-dependent, it appears possible to circumvent this confounding problem that complicates AI effectiveness in some felids, by short-term suppression with an oral progestin.

4.5. Integrating results and summary of AI successes in felids

Once species-specific requirements were determined among felids, then eCG/hCG ovulation induction protocols emerged that have allowed successful AI with fresh sperm in the domestic cat and eight wild felid species, including the leopard cat [7,86], tigrina [85,87], ocelot [85,88], clouded leopard [34,59]; snow leopard [97], cheetah [34,50], puma [98], and tiger [71,99] (Table 4).

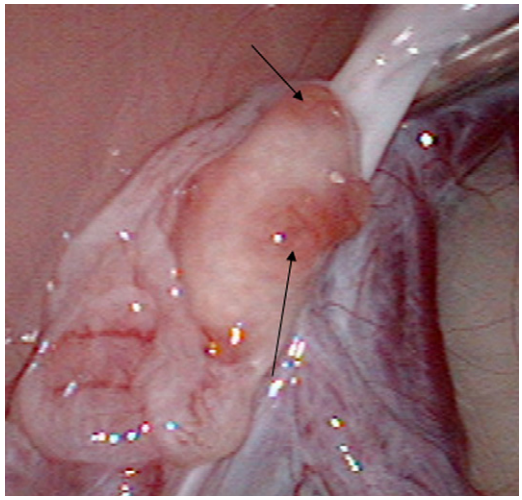

Fig. 9. Domestic cat ovary containing corpora lutea (CL; arrows) after inhibition treatment with oral progestin (altrenogest) for 38 d, followed by ovulation induction with eCG and hCG.

Pregnancies also have resulted following intrauterine insemination with frozen–thawed spermatozoa in the leopard cat, ocelot and cheetah (Table 4). Three cheetah litters have been produced using sperm collected from wild-caught males in Namibia Africa, then cryopreserved and imported into the United States for use in the Cheetah SSP Program [12,83,84]. Of the 11 cheetahs inseminated with frozen–thawed Namibian sperm, conception success was influenced by number of motile sperm inseminated. No pregnancies resulted in five females inseminated with fewer than 4×10^6 motile sperm/AI, whereas three of six (50%) females inseminated with 6–16 $\times$ 10^6 motile sperm/AI became pregnant and produced six cubs (1–3 cubs/litter). Overall, these findings among felid species continue to emphasize the fundamental differences in reproductive mechanisms among related species in the same family.

5. AI in ferrets

5.1. Recovery of the endangered black-footed ferret

Once considered extinct until a remnant population was discovered in Wyoming in 1981, the last remaining 18 black-footed ferrets were captured in 1985–1987 to save the species through captive breeding. Fortunately, intensive management resulted in production of the first offspring *ex situ* in 1987. Given the species' critical status, the U.S. Fish & Wildlife Service developed the

'Black-Footed Ferret Recovery Plan' in 1988 that emphasized a host of species preservation tactics beyond natural breeding (including developing assisted reproductive technology) and all of which might contribute to returning the species to nature. Ferret offspring were distributed among six North America zoos to begin cooperative breeding/research under the guidance of a Black-Footed Ferret SSP Program. The *ex situ* breeding goal was to generate and then maintain ~250 genetically healthy ferrets at multiple locations (to avoid a catastrophe that might affect any single facility). These animals would be the source of black-footed ferrets for release to meet a reintroduction target of 1500 ferrets in at least 10 self-sustaining, free-ranging populations by the year 2010.

Since 1987, the breeding program has been highly successful, and >6100 black-footed ferrets have been born. Extensive knowledge has resulted on ferret biology and husbandry that has significantly improved managed care. The ability to produce ferrets in captivity allowed reintroduction to begin in 1991. Ferrets initially were released in Wyoming and later in South Dakota, Montana, Mexico, Colorado, Utah, Arizona, Kansas, and New Mexico. Numerous factors have continually threatened survival of released ferrets, including disease (i.e., canine distemper and sylvatic plague) and rodent poisoning to reduce colonies of prairie dogs (primary prey for the black-footed ferret) near reintroduction sites. Despite these risks, successful reproduction and offspring production has been documented at all release sites. Currently, ~1000 black-footed ferrets survive in nature today in eight states and Mexico. Plans currently are in progress for Canada to initiate the next reintroduction program.

5.2. Developing assisted reproduction via studying ferret 'models'

The potential benefits of reproductive technologies for contributing to black-footed ferret conservation were recognized from the onset of the recovery program. Even at the time of rescuing the last 18 individuals, there was speculation that AI with fresh or frozen spermatozoa could help retain genetic diversity by ensuring reproduction in every valuable individual. Additionally, the concept of a GRB of cryopreserved spermatozoa was recognized as a means of preserving extant genes for future 'infusions' to ensure the long-term well-being of this small population.

The National Zoological Park's Conservation & Research Center was invited to take a lead role in studying ferret reproductive biology and participating in *ex situ* breeding. We began reproductive investigations using the domestic ferret and Siberian polecat as animal models for simply developing an informational database on the intricacies of 'ferret biology' [2]. This approach was deemed appropriate, because early molecular analyses revealed that the common ferret, Siberian polecat, and black-footed ferret were phylogenetically related [51]. Others had determined that all of these species were seasonal, 'long-day' breeders [100–103] with increases in testis size beginning in January and peaking from March through June with declines thereafter [104]. The breeding season for the female had been determined to be restricted to March through June and characterized by changes in vaginal cytology and increased vulvar size. The only other information available was that all ferrets appeared to be induced ovulators, with ovulation commencing ~30 h after a single copulation or hCG or LH injection [105].

With that baseline information, we began extensive studies in common (European) ferrets to develop a consistently effective approach for collecting and analyzing fresh or cryopreserved spermatozoa [106,107]. Ejaculates were collected to address the: (1) temporal spermatogenesis patterns and sperm viability; (2) comparative effectiveness of vaginal versus intrauterine insemination via a laparoscopic approach; (3) influence of sperm number and time of hCG administration on pregnancy success, gestation interval, and number of offspring produced; and (4) influence of cryodiluent, freezing method, and thawing temperature on biological competence of thawed ferret spermatozoa [106,107]. Such basic studies were crucial to developing reliable assisted breeding protocols. For example, vaginal insemination was determined to be ineffective in the domestic ferret as none of 10 females became pregnant (Table 2) [106]. In contrast, transabdominal-intrauterine AI via laparoscopy (Fig. 3) was efficient. Seventeen of 24 ferrets (70.8%) inseminated in this fashion became pregnant and delivered live young (Table 2) [106]. Meanwhile, comparative assessments of 12 cryopreservation methods determined that a combination of an egg-yolk/lactose cryodiluent, the pellet freezing method and a 37 °C thawing temperature was effective for recovering maximal ferret sperm motility and acrosomal integrity. By this approach, seven of 10 females (70.0%) inseminated *in utero* with thawed spermatozoa became pregnant (Table 2) [107]. Overall, reproductive efficiency was high (70.6%) in European ferrets after laparoscopic intrauterine AI with fresh or frozen spermatozoa (Table 5).

Table 5
Comparison of laparoscopic intrauterine artificial insemination (AI) with fresh or frozen–thawed spermatozoa in closely related ferret species.

	Domestic (European) ferret	Siberian polecat	Black-footed ferret
No. females inseminated	34	10	82
No. pregnant females	24	8	49
Incidence of pregnancy success (%)	70.6	80.0	59.8
No. kits born	116	42	139
Mean (± SEM) number of kits/litter	4.8 ± 0.8	5.2 ± 1.0	2.8 ± 0.5

Source: [52,53,106–108].

Most importantly and in contrast to our findings in felids, we determined that pre-ovulatory anesthesia in ferrets did not compromise ovulation and AI success [106]. High pregnancy rates (67–75%) were achieved in ferrets when anesthesia and insemination were conducted at either 24 h post-hCG (~6 h before anticipated ovulation) or the time of hCG administration (~30 h before ovulation). Interestingly, these ferrets were in natural estrus, similar to the cats in the study conducted by Tsutsui et al. [66] that demonstrated an 80% pregnancy success. These findings further support the concept that eCG-induced estrus might affect follicular sensitivity to the ovulatory process.

Knowledge from investigations of the common ferret then was applied to the Siberian polecat and ultimately the black-footed ferret. Sperm motility was similar between the domesticated ferret and Siberian polecat (~81%), but lower in the black-footed ferret (~51%) [53]. Structurally normal spermatozoa were far less prevalent in the black-footed ferret (~21%) than the common ferret (~67%) and Siberian polecat (~75%), perhaps due to the historically restricted founder base of the former species [53,107,108]. The proportion of sperm with normal, intact acrosomes also was lower in the black-footed ferret (~67%) than the other two species (~92–97%) [53–55,107,108]. Sperm motility and membrane integrity after cryopreservation and thawing also were reduced in the black-footed ferret compared to domestic ferret and Siberian polecat [107,108], again probably related to differences in species heterozygosity. Nevertheless, laparoscopic intrauterine AI, developed in the domestic ferret, was effective in the Siberian polecat (8 of 10 females becoming pregnant; Table 5), a finding that gave the confidence to proceed to AI studies in the rare black-footed ferret.

5.3. Enhanced reproduction using AI in black-footed ferrets

Early experience in the breeding program revealed that some black-footed ferrets consistently failed to reproduce. Analysis of breeding records indicated that most females (>90%) demonstrated a spring estrus on the basis of vaginal cytology changes (markedly increased numbers of superficial, cornified, squamous epithelial cells) using the Papanicoloau (PAP) stain (Fig. 10) [16,109]. Our evaluations of fecal estradiol and progestogen metabolites in pregnant versus pseudo-pregnant females revealed similar profiles (Fig. 10) and no indications of endocrine dysfunction [16].

However, records analysis did indicate a remarkably high proportion (>50%) of prime breeding age males (1–3 years old) that inexplicably failed to sire offspring. In 1995, there were 40 such adult males (54.8% of the breeding age male population) that were exposed to prime age, estrual females and yet did not produce young. Simultaneous lineage evaluations also revealed that one of the original wild-born ferret founders had few offspring in the modern *ex situ* population (only 43 descendants compared to more than 300 progeny from each of the other founders). Thus, it was realized that (1) many males were not breeding naturally and (2) a valuable founder descendant was under-represented. A more detailed assessment of the first challenge revealed a high incidence of sexual incompatibility between designated mates, largely due to improper copulatory positioning, inter-animal aggression and poor testis development [110,111]. These findings collectively justified the need to begin applying AI within the black-footed ferret breeding program. A first step was to assess reproductive traits and breeding behaviour in males with proven versus unproven fertility, followed by the systematic cryobanking of spermatozoa from the most genetically valuable males. These data then could quickly allow the application of AI for improving reproductive efficiency in non-breeders. Success would both help sustain gene diversity as well as increase number of kits for reintroduction.

From 1996 through 2008, 82 females were monitored for natural estrus and were given hCG or LH (to induce ovulation) 5–7 d after maximal vulvar swelling and >90% superficial cornified vaginal cells. Twelve to 20 h later, each female was anesthetized and, under

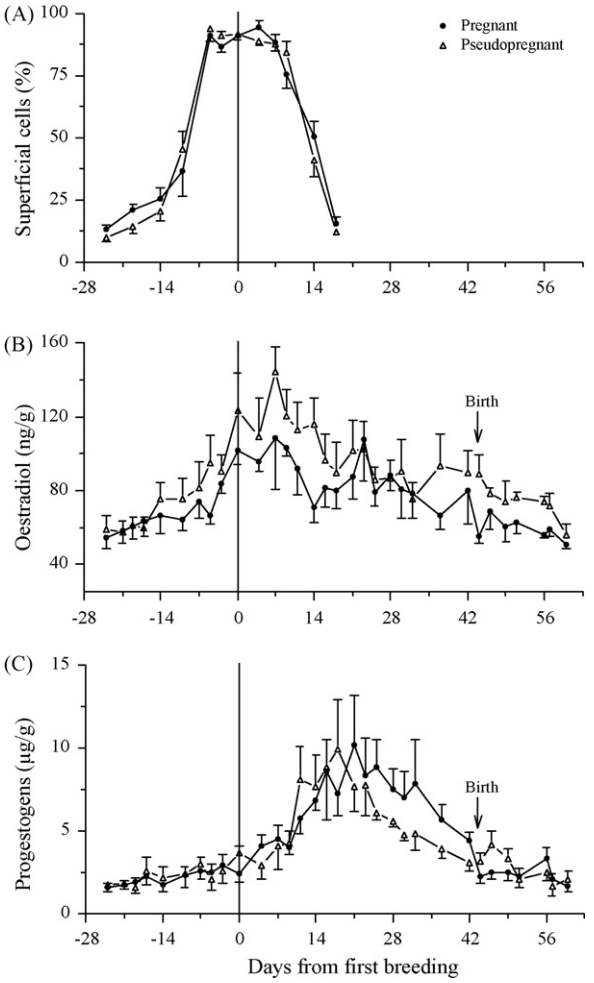

Fig. 10. Mean (± SEM) percent superficial cells in vaginal lavages (A), and fecal estradiol (B) and progestogen (C) metabolite concentrations in pregnant ($n = 7$; closed circle) and pseudopregnant ($n = 9$; open triangle) black-footed ferrets. Day 0 is the time of first mating. *Source*: Ref. [16].

laparoscopic observation, inseminated *in utero*. Overall, 49 of 82 (59.8%) black-footed ferrets inseminated before ovulation with fresh or frozen–thawed semen became pregnant. Furthermore, AI resulted in 139 additional black-footed ferret kits, offspring that never would have been born from natural mating (Table 5) [9,108]. Of these, five kits have been born with frozen/thawed semen, including two valuable kits in 2008 produced in two females inseminated with semen cryobanked 10–11 years previously from sires that had been dead for 8 and 9 years, respectively. This milestone emphasizes the importance of developing a GRB for small populations with limited gene diversity. In this case, the ability to re-derive genetic material from a decade ago from deceased animals illustrates the power of AI in combination with sperm cryopreservation.

6. Conclusions and challenges

The carnivores, many of which are top predators requiring vast amounts of 'wild' space', will continue to face severe risks to surviving in nature. Evitable habitat losses also will increase the likelihood of losing gene diversity that, in turn is known to adversely affect fitness, especially in felids and mustelids. Therefore, these *ex situ* carnivore populations are especially valuable for maintaining the integrity of 'insurance' populations while offering important research opportunities and (at least in the case of the black-footed ferret) animals for return to the wild. Successful managed care programs, however, largely depend on the ability to secure reproduction in every valuable individual while controlling breeding to retain maximum gene diversity. When natural breeding fails, or is logistically impossible or too expensive, then AI can become an attractive alternative.

Throughout this manuscript, we have illustrated the history, experiences, challenges and accomplishments of adapting AI to felids and mustelids. It is clear that the AI success is totally dependent on understanding the idiosyncratic mechanisms for each target species. It is judicious that all efforts to apply assisted reproduction to any wildlife species first focus on basic research that: (1) characterizes the uniqueness of the species; (2) provides an understanding of the optimal way to control ovarian function; and (3) develops methods to collect, process, store and deposit viable sperm at the appropriate place and time in the female's reproductive tract. That information, combined with requisite animal availability and the inter-institutional collaboration associated with cooperative breeding programs, will vastly improve the likelihood of success. Advances also will be facilitated by intensively studying past successes (such as the cheetah and black-footed ferret), as well as failures. Future efforts to improve the efficiency of AI in felids will be focused on improving methods to control and synchronize ovarian activity following gonadotropin stimulation and ovulation induction.

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