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Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen–thawed spermatozoa in ocelots (*Felis pardalis*)

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Adult female ocelots (*Felis pardalis*) were treated with one of four dosages of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) (100 iu eCG/75 iu hCG, $n = 3$; 200 iu eCG/150 iu hCG, $n = 4$; 400 iu eCG/150 iu hCG, $n = 5$; 500 iu eCG/225 iu hCG, $n = 5$); hCG was administered 80 h after eCG. Ovaries of each animal were evaluated by laparoscopy 39–43 h after hCG, and blood was collected for progesterone and oestradiol analysis. With progressive increases in gonadotrophin dosage, female ocelots produced more ($P < 0.05$) unovulated follicles (≥ 2 mm in diameter), ranging from 1.3 ± 0.7 (mean ± SEM) follicles per female at the lowest dosage to 8.8 ± 2.8 follicles per female at the highest dosage. Similarly, ocelots produced more ($P < 0.05$) corpora lutea with increasing gonadotrophin dosages, with mean values ranging from $0\text{–}5.0 \pm 1.2$ corpora lutea. However, across treatment groups, a similar proportion ($P > 0.05$) of females ovulated in response to each dosage. At laparoscopy, serum concentrations of oestradiol (overall mean, 330.2 ± 62.2 pg ml^{-1}) and serum concentrations of progesterone (overall mean, 18.5 ± 6.4 ng ml^{-1}) in ovulating females did not differ ($P > 0.05$) across treatment groups. Ten ovulating ocelots were laparoscopically inseminated with fresh ($4.7 \pm 0.2 \times 10^6$; $n = 2$ females) or frozen–thawed ($10.7 \pm 1.8 \times 10^6$; $n = 8$ females), motile spermatozoa. One female treated with 500 iu eCG/225 iu hCG and inseminated with 7.5×10^6 motile, frozen–thawed spermatozoa conceived and gave birth to a healthy male kitten after a gestation of 78 days. We conclude that ocelots are relatively insensitive to exogenous gonadotrophins, requiring much higher dosages (on a per body mass basis) to elicit an appropriate ovarian response than do any other felid species studied to date. Nonetheless, the gonadotrophin-treated female can become pregnant and carry offspring to term after laparoscopic intrauterine insemination with frozen–thawed spermatozoa.

Introduction

The ocelot (*Felis pardalis*) is one of seven small felid species (ocelot; margay, *Felis wiedi*; tigrina, *Felis tigrina*; Geoffroy's cat, *Felis geoffroyi*; pampas cat, *Felis colocolo*; kodkod, *Felis guigna*; Andean mountain cat; *Felis jacobita*) classified on the basis of morphological traits and molecular genetics in the ocelot lineage (Collier and O'Brien, 1985; Slattery *et al.*, 1994). This monophyletic lineage diverged from a common felid ancestor about 12 million years ago (Collier and O'Brien, 1985) and is distinguished by a shared karyotype comprising fewer chromosomes (diploid, $n = 36$) than other cat species (diploid, $n = 38$) (Wurster-Hill and Centerwall, 1982). On the basis of Mace/Lande criteria (Mace and Lande, 1991), all species within the ocelot lineage are threatened with extinction throughout their natural ranges, primarily due to habitat loss and fragmentation and continued poaching pressures (Tewes and Everett, 1986; Tewes and Schmidly, 1987; IUCN, 1995).

Long-term survival of ocelots and other Latin American felids will depend upon both *in situ* and *ex situ* conservation actions, including habitat preservation as well as captive propagation of individuals selected for sustaining genetic diversity. Conservation management programmes for these species theoretically may be enhanced by assisted reproductive technology, including cryostorage of spermatozoa combined with artificial insemination (AI), to facilitate genetic exchange among zoo-maintained individuals and between captive and wild populations (Ballou, 1992; Wildt, 1992, 1994; Wildt *et al.*, 1993a). However, the benefits of assisted reproduction for managing genetic diversity can be realized only when effective protocols are in place for ovulation induction and freezing, thawing and deposition of spermatozoa *in utero*.

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For laparoscopic AI in felids, females typically are treated with a sequential combination of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) to induce ovarian follicular development and ovulation, respectively (Howard *et al.*, 1992a). Previous studies from our laboratory of tigers (*Panthera tigris*; Donoghue *et al.*, 1993), cheetahs (*Acinonyx jubatus*; Howard *et al.*, 1992b), pumas (*Felis concolor*; Barone *et al.*, 1994), clouded leopards (*Neofelis nebulosa*; Howard *et al.*, 1993) and domestic cats (*Felis catus*; Howard *et al.*, 1992a) have demonstrated a remarkable species-specific variability in ovarian sensitivity to these exogenous gonadotrophins. Because of the evolutionary divergence of the ocelot lineage relative to other cat species and associated genetic differences, we anticipated additional species-specific challenges in successfully adapting these gonadotrophin treatment and AI protocols. Therefore, our specific objectives were to: (1) examine the ovarian responsiveness of ocelots to several combination dosages of exogenous gonadotrophins (specifically eCG and hCG), and (2) determine the feasibility of laparoscopic AI and the functional competence of frozen–thawed ocelot spermatozoa.

Materials and Methods

Animals

Adult (age range, 2–11 years) male ($n = 2$) and female ($n = 7$), captive-born ocelots housed at five zoological parks (Caldwell Zoo, Tyler, TX; Cheyenne Mountain Zoological Park, Colorado Springs, CO; Dallas Zoo, Dallas, TX; Greenville Zoo, Greenville, SC; Woodland Park Zoological Gardens, Seattle, WA) were studied. Both males and three of the females were proven breeders. All animals were exposed to natural lighting, with variable periods of artificial illumination, in either indoor enclosures (containing windows or skylights) or indoor/outdoor enclosures. All were fed a commercial diet of ground horse meat, enriched with vitamins and minerals (Nebraska Brand Feline or Canine Diets, Central Nebraska Packing, Inc., North Platte, NE; ZuPreem Feline Diet, Premium Nutritional Products, Topeka, KS). At most zoos, diets were supplemented occasionally with whole prey (chicks, rodents, fish). Zoos with both male and female ocelots maintained the genders separately for the duration of the study period (September 1992 through September 1994).

Semen collection and cryopreservation

Male ocelots were anaesthetized with tiletamine HCl/zolazepam HCl (Telazol, Fort Dodge Laboratories Inc., Fort Dodge, IA; 4–7 mg kg^{-1} body mass; i.m.), and semen was collected via a standardized electroejaculation protocol (Wildt *et al.*, 1983; Howard *et al.*, 1986). Recovered spermatozoa were evaluated immediately for percentage motility and rate of forward progressive movement (scale 0–5, with 0 = nonmotile and 5 = rapid linear forward progression) (Wildt *et al.*, 1983; Howard *et al.*, 1986). An aliquot (5–10 μl) was fixed in 0.3% (v/v) glutaraldehyde and later evaluated for sperm morphology (200 spermatozoa per ejaculate) using phase-contrast microscopy (× 1000) to identify specific malformations (Howard *et al.*, 1986). Sperm concentration ($\times 10^6$ ml^{-1}) was determined with 5 μl raw semen, using a red blood cell determination kit/haemocytometer method (Howard *et al.*, 1986), and semen pH was assessed using an indicator strip (EM Science, Gibbstown, NJ). Remaining raw semen was diluted (1:1 or 1:2) in warm (37°C) Ham's F10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% (v/v) fetal calf serum (Irvine Scientific), 0.011 mg pyruvate ml^{-1} (Sigma Chemical Company, St Louis, MO), 100 U penicillin ml^{-1} (Sigma) and 100 μg streptomycin ml^{-1} (Sigma) and then centrifuged at 200 ***g*** for 10 min to remove seminal fluid. The supernatant was discarded, and the sperm pellet was resuspended in Ham's F10 medium (150–200 μl; 22°C) for immediate use (in the case of fresh AI) or processed for cryopreservation.

For cryopreservation, the sperm pellet was extended in 200–500 μl of cryoprotectant diluent (11% (w/v) lactose, 20% (v/v) egg yolk, 4% (v/v) glycerol; 22°C) (Platz *et al.*, 1978) to a concentration of $140–160 \times 10^6$ motile spermatozoa ml^{-1}, cooled in a refrigerator (5°C) for 30 min and frozen by pelleting onto dry ice (Platz *et al.*, 1978; Howard *et al.*, 1986). The freezing process involved pipetting 30 μl drops of diluted semen into indentations made on a dry ice block, waiting for 3 min and then inverting the block to deposit the resulting pellets into liquid nitrogen. Frozen sperm pellets were placed into labelled screw-top cryovials (2 ml; Vangard Cryos, Sumitomo Bakelite Co., Ltd, Japan) and stored immersed in a liquid nitrogen refrigerator for 6–18 months, until needed.

Stimulation of ovaries and induction of ovulation

Behaviourally anoestrous females ($n = 7$) were treated i.m. with eCG (PMSG, Sigma) and hCG (Sigma) at one of four gonadotrophin dosages (100 iu eCG/75 iu hCG, $n = 3$; 200 iu eCG/150 iu hCG, $n = 4$; 400 iu eCG/150 iu hCG, $n = 5$; 500 iu eCG/225 iu hCG, $n = 5$). Initial gonadotrophin dosages were chosen on the basis of extensive experience with other felids. Hormone injections were delivered by blow-pipe or CO_2-propelled dart pistol, and hCG was administered 80 h after eCG. Most females received multiple gonadotrophin treatments (one female on four occasions; three females on three occasions; one female on two occasions; two females on a single occasion), but at different dosages and with at least 6 months between successive treatments. Females received treatments primarily during two seasons, spring (March; $n = 6$) or autumn (September through December; $n = 10$). One ocelot was treated in the summer (July).

Laparoscopy and artificial insemination

For laparoscopy, anaesthesia was induced with either ketamine HCl (Vetalar, Parke-Davis, Detroit, MI; 3–10 mg kg^{-1}, i.m.) or tiletamine HCl/zolazepam HCl (3–5 mg kg^{-1}, i.m.). Females were intubated and maintained on gas anaesthesia (1–2% isoflurane or halothane) for the duration of the procedure. Blood samples were collected via jugular venepuncture immediately after a surgical plane of anaesthesia was achieved and before laparoscopy, and serum was harvested after centrifugation (at 1200 ***g*** for 10 min) and stored (− 80°C) for later hormone analysis. All aspects of each ovary were evaluated

Table 1. Semen characteristics of freshly collected ocelot electroejaculates

	Male number			
Parameter	1	1	2	1
Ejaculate volume (ml)	2.2	2.0	0.4	1.3
Total number of spermatozoa per ejaculate ($\times 10^6$)	473	65	25	23
Percentage sperm motility	90	85	90	80
Sperm forward progressive status[a]	4.5	4.0	4.0	3.5
Percentage normal sperm forms	50	77	16[b]	76
Processing method	Frozen	Frozen	Fresh	Fresh
Number of females inseminated	5	3[c]	1	1

[a] Scale of 0 (no movement) to 5 (rapid, forward movement).
[b] Probable osmotic shock due to urine contamination.
[c] One pregnancy produced.

laparoscopically 39–43 h after hCG to determine the number of unovulated follicles (≥ 2 mm) and corpora lutea using previously described morphological criteria (Wildt *et al.*, 1977).

Females with recent ovulation sites were inseminated *in utero* (Howard *et al.*, 1992a) using fresh or frozen–thawed spermatozoa as follows. Spermatozoa were deposited directly into the lumen of each uterine horn (90–100 µl per horn) using a 20-gauge, polypropylene intravenous catheter (Sovereign, Sherwood Medical, St Louis, MO) passed transabdominally and then into each horn. The catheter stylette was replaced with polyethylene tubing (PE 10; Intramedic, Clay Adams, Parsippany, NJ) containing the diluted spermatozoa, attached to a 30-gauge needle on a 1-ml syringe (Howard *et al.*, 1992a; Barone *et al.*, 1994). Air (0.2 ml) in the syringe allowed the spermatozoa to be expelled from the tubing into the horn. Fresh and frozen–thawed spermatozoa were inseminated within 1–2 h of collection or 30–40 min of thawing, respectively.

When cryopreserved spermatozoa were used, each frozen pellet was thawed by rapid immersion in warm (37°C) Ham's F10 medium (100 µl) contained in a 12 mm × 75 mm glass test tube (Curtin Matheson Scientific Inc., Jessup, MD) that was gently agitated for 30 s in a water bath at 37°C. Thawed spermatozoa were centrifuged at 200 **g** for 10 min and the supernatant aspirated and discarded to remove cryoprotectant. The resulting sperm pellet was resuspended in Ham's F10 medium (150–200 µl; 37°C) and evaluated immediately for percentage motility and rate of progressive movement. An aliquot (5 µl) of thawed, washed spermatozoa was used to determine sperm concentration ($\times 10^6$ ml^{-1}) (Howard *et al.*, 1986). Acrosomal status was assessed by mixing a sperm aliquot (5 µl) with 10–20 µl of a rose bengal/fast green stain (Pope *et al.*, 1991), maintaining it at 22°C for 2 min and spreading it onto glass slides. The percentages of intact, partially intact and nonintact acrosomes were determined by evaluating 200 spermatozoa per ejaculate using bright-field microscopy ($\times$ 400) (Pope *et al.*, 1991).

Analysis of serum hormones

Thawed serum samples were assessed for oestradiol and progesterone concentrations using solid-phase ^{125}I radioimmunoassay kits (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA). Binding inhibition curves of serially diluted ocelot serum were parallel to the oestradiol and progesterone standard curves. Net recovery of oestradiol and progesterone added to ocelot serum was 109% ($y = 0.98x + 19.8$; $r = 0.99$) and 111% ($y = 1.06x + 0.14$; $r = 0.99$), respectively. All samples were evaluated simultaneously in a single radioimmunoassay for each hormone. Assay sensitivities (based on 90% of maximum binding) for oestradiol and progesterone were 5.0 pg ml^{-1} and 0.05 ng ml^{-1}, respectively. The intra-assay coefficients of variation were < 10% for both assays.

Statistical analyses

For each gonadotrophin dosage, mean (± SEM) values for number of corpora lutea, unovulated follicles and serum oestradiol and progesterone concentrations were determined. Differences in mean values with increasing dosages were evaluated using regression analysis (Steel and Torrie, 1980). For individual females receiving multiple gonadotrophin treatments, means for total number of ovarian structures were determined and compared using regression analysis. Furthermore, because ovarian responses to the second and third successive treatments were immunologically relevant (Swanson *et al.*, 1995a), mean numbers of follicles, corpora lutea and total ovarian structures for these treatments were compared using a Student's *t* test (Steel and Torrie, 1980). Differences in the proportion of ovulating females at each gonadotrophin dosage were evaluated using Fisher's exact test (Steel and Torrie, 1980). For fresh and frozen–thawed sperm samples, mean values were calculated for sperm motility traits (percentage motility, rate of forward progressive movement) and compared using a Student's *t* test (Steel and Torrie, 1980).

Results

Ejaculates containing spermatozoa were obtained from both males ($n = 4$ total ejaculates) (Table 1). One ejaculate was contaminated by urine (based on an acidic pH) and contained > 80% morphologically abnormal spermatozoa (primarily

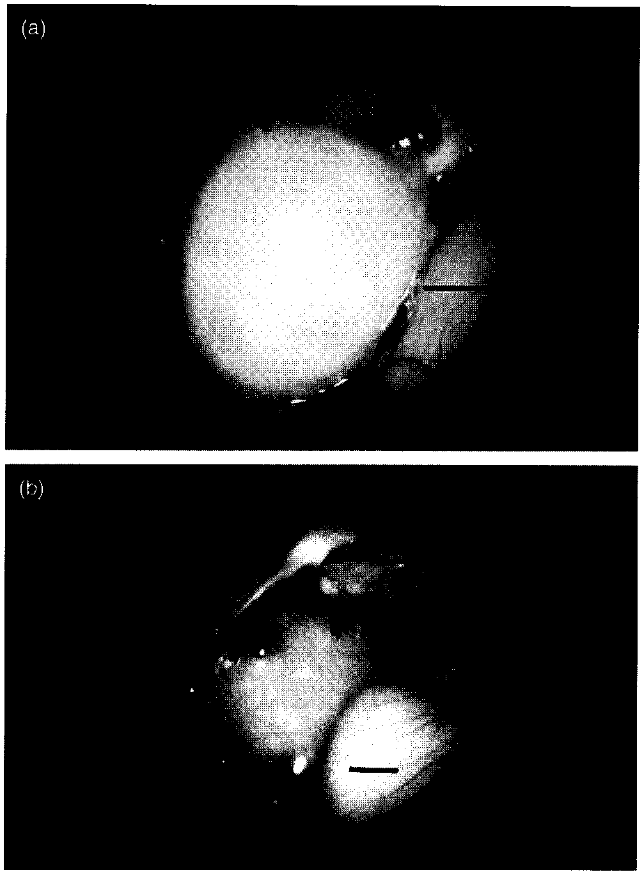

Fig. 1. Laparoscopic observations of ovarian responses after exogenous gonadotrophin treatment of ocelots. (a) Minimal ovarian activity resulting from a low gonadotrophin dosage (100 iu eCG/75 iu hCG); and (b) moderate ovarian activity, including several recent ovulations (arrows), resulting from a high gonadotrophin dose (500 iu eCG/225 iu hCG). Scale bars represent 5 mm.

bent and coiled flagella), considerably more than the other three electroejaculates (mean, 32 ± 9% abnormal forms). Two ejaculates were used immediately for AI, and two were cryopreserved for later use.

Female ocelots treated with exogenous gonadotrophins produced more ($P < 0.05$) unovulated follicles (≥ 2 mm in diameter) and more ($P < 0.05$) corpora lutea (Fig. 1) with increasing dosages (Table 2). However, with the exception of the lowest dosage, most females ovulated in response to treatment and the proportion of ovulatory females did not differ ($P > 0·05$) (Table 2). Serum oestradiol concentrations (overall mean, 330.2 ± 62.2 pg ml^{-1}) did not differ ($P > 0.05$) among females treated with increasing gonadotrophin dosages, and serum progesterone concentrations in ovulating females (overall mean, 18.5 ± 6.4 ng ml^{-1}) were similar ($P > 0.05$) across treatment groups (Table 2). For nonovulating females, progesterone concentrations were low (mean, 1.5 ± 0.4 ng ml^{-1}; range, 0.5–2.9 ng ml^{-1}) within each group (data not shown). Ocelots ($n = 4$) receiving three or more eCG/hCG treatments, at progressively higher dosages, did not ($P > 0.05$) experience decreased ovarian responsiveness (Table 3). Notably, these females had similar ($P > 0.05$) mean numbers of follicles (5.0 ± 0.8 versus 6.3 ± 1.5), corpora lutea (4.0 ± 1.8 versus 3.8 ± 1.3) and total ovarian structures (9.0 ± 2.3 versus 10.0 ± 2.7) after receiving 400 iu eCG/150 iu hCG (as their second or third treatment) and 500 iu eCG/225 iu hCG (as their third or fourth treatment), respectively.

Of eleven ovulating females, ten were inseminated with fresh ($n = 2$ females) or frozen–thawed ($n = 8$ females) spermatozoa (Table 4). After thawing and processing, sperm percentage motility and rate of progressive movement ranged from 40–60% and 3.0–4.0, respectively, at the time of insemination and were not different ($P > 0.05$) from that of freshly ejaculated, processed inseminates. However, a high percentage (range 53–69%) of thawed spermatozoa had nonintact acrosomes (Fig. 2) compared with fresh, unfrozen spermatozoa (< 10%). To compensate, more total motile spermatozoa were used for AI with frozen–thawed (range, $6.5–20.7 \times 10^6$) compared with freshly collected (range, $4.5–4.8 \times 10^6$) inseminates. One nulliparous female treated with a high gonadotrophin dosage (500 iu eCG/225 iu hCG) and inseminated with frozen–thawed spermatozoa (7.5×10^6 motile spermatozoa; 47% intact acrosomes) conceived and gave birth to a healthy male kitten after a gestation of 78 days.

Discussion

This study represents the first documentation of reproductive characteristics and the initial attempt to apply assisted reproduction to a species from the ocelot lineage of cats. In contrast to many other felid species that routinely ejaculate > 50% malformed cells (Howard, 1993; Swanson *et al.*, 1995b), freshly ejaculated sperm quality in the ocelot was excellent, and a high proportion of cells was morphologically normal. Furthermore, ocelot spermatozoa exhibited good forward motility and a comparatively high incidence of intact acrosomal membranes. The excellent semen quality of the ocelot should be a distinct advantage in the routine use of assisted reproduction for management/conservation purposes.

The female ocelot, rather than the male, proved a greater challenge for developing the AI protocol, being relatively insensitive to the exogenous gonadotrophins, eCG and hCG, and requiring markedly higher dosages (on a per body mass basis) than other felid species to elicit an appropriate ovarian response. Ocelots required approximately twice the gonadotrophin dosage (per kg body mass) of domestic cats (Howard *et al.*, 1992a) and three to ten times the dosage reported for other nondomestic felid species to achieve comparable ovarian responses (i.e., 3–7 ovulations, few unovulated, residual follicles) (Howard *et al.*, 1992b, 1993; Donoghue *et al.*, 1993; Barone *et al.*, 1994). For example, female cheetahs and pumas, with average body masses of about 35 kg (or about four times the mass of the ocelot) required only 200 iu eCG and 100 iu hCG to induce similar ovarian responses (Howard *et al.*, 1992b; Barone *et al.*, 1994). The underlying cause for the insensitivity of ocelots is unknown, but perhaps is related to evolutionary divergence and genetic differences of the ocelot lineage. Because radiation of species within the ocelot lineage has been relatively recent (within the past 2–5 million years) (Slattery *et al.*, 1994), this characteristic may be conserved among species, a possibility being explored in parallel studies of the margay and tigrina.

Table 2. Ovarian responses and serum hormone concentrations in ocelots treated with low or high gonadotrophin dosages

Gonadotrophin dosage	Number of ocelots	Ovarian response[a]			Serum hormones[a]	
		Number of ocelots ovulating	Number of follicles[b]	Number of corpora lutea[b]	Oestradiol (pg ml^{-1})	Progesterone[c] (ng ml^{-1})
100 iu eCG/75 iu hCG	3	0	1.3 ± 0.7	0	150.1 ± 112.6	NA
200 iu eCG/150 iu hCG	4	3	4.8 ± 2.6	1.0 ± 0.4	213.7 ± 51.5	11.5 ± 5.4
400 iu eCG/150 iu hCG	5	4	4.6 ± 0.7	4.0 ± 1.4	448.9 ± 164.0	33.4 ± 15.5
500 iu eCG/225 iu hCG	5	4	8.8 ± 2.8	3.4 ± 1.1	412.7 ± 87.2	8.9 ± 1.4

[a] Means ± SEM.
[b] Number of follicles and corpora lutea increased ($P < 0.05$) with increasing gonadotrophin dosages.
[c] For ovulating females only.

Table 3. Ovarian responses of ocelots ($n = 4$) receiving three or more successive treatments with exogenous gonadotrophins at progressively higher dosages

Gonadotrophin dosage	Total number of ovarian structures (number of follicles/number of corpora lutea)				
	Female number				
	1	2	3	4	Mean ± SEM[a]
100 iu eCG/75 iu hCG	2 (2/0)	NA	0 (0/0)	2 (2/0)	1.3 ± 0.7
200 iu eCG/150 iu hCG	0 (0/0)	12 (11/1)	NA	NA	6.0 ± 6.0
400 iu eCG/150 iu hCG	5 (5/0)	14 (7/7)	12 (5/7)	5 (3/2)	9.0 ± 2.3
500 iu eCG/225 iu hCG	3 (3/0)	10 (5/5)	16 (10/6)	11 (7/4)	10.0 ± 2.7

[a] Number of ovarian structures increased ($P < 0.05$) with increasing gonadotrophin dosages.

Table 4. Inseminate characteristics of fresh and frozen–thawed ocelot spermatozoa used for laparoscopic artificial insemination (AI)

Gonadotrophin dosage	Inseminate status	Number of AIs	Number of motile spermatozoa ($\times 10^6$)	Acrosome intact (%)	Number pregnant
100 iu eCG/75 iu hCG	Fresh	0	NA	NA	NA
	Thawed	0	NA	NA	NA
200 iu eCG/150 iu hCG	Fresh	0	NA	NA	NA
	Thawed	2	10.2 ± 3.7[a]	31	0
400 iu eCG/150 iu hCG	Fresh	1	4.5	ND[b]	0
	Thawed	3	7.8 ± 0.6[a]	31	0
500 iu eCG/225 iu hCG	Fresh	1	4.8	98	0
	Thawed	3	13.9 ± 3.8[a]	47	1

[a] Mean ± SEM.
[b] Not determined.

Our investigation required that two potentially confounding variables, reproductive seasonality and immunological response to exogenous gonadotrophins, be controlled. Definitive information on seasonality is limited, but anecdotal evidence indicates that ocelots reproduce throughout the year in both tropical and temperate regions (Eaton, 1977; Mondolfi, 1982). Any seasonal influences were balanced by administering gonadotrophin treatments primarily in the spring and autumn, with a fairly equal distribution across low and high dosage groups. Because most ocelots were treated on multiple occasions with exogenous gonadotrophins, we could indirectly assess potential concerns that anti-gonadotrophin immunoglobulin formation may affect ovarian responsiveness over time. Domestic cats treated multiple times with eCG/hCG at intervals of 7 weeks have progressively decreased ovarian responsiveness owing to the formation of eCG and hCG neutralizing immunoglobulins (Swanson *et al.*, 1995a). Typically, a substantial reduction in number of ovarian follicles and percentage of mature oocytes coincides with the third successive eCG/hCG treatment. However, subsequent studies

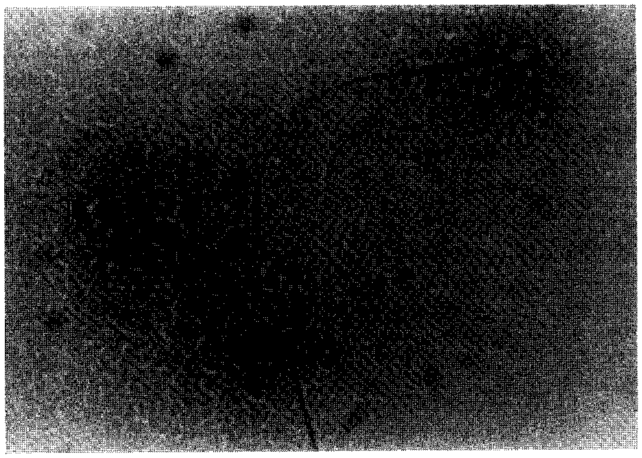

Fig. 2. Frozen–thawed ocelot spermatozoa stained with a rose bengal/fast green acrosome stain. Spermatozoa with an intact acrosome (black arrow) stained dark blue over the head region, whereas spermatozoa with a nonintact acrosome (open arrow) stained pale pink. Scale bar represents 5 μm.

indicated that intervals of 4–5 months between successive treatments preclude the development of immunologically mediated ovarian refractoriness (Swanson *et al.*, in press). In the present study, ocelots were allowed a minimum of 6 months between treatments and received a maximum of four treatments. Even after multiple treatments, ocelots remained responsive to eCG and hCG, producing comparable numbers of follicles and corpora lutea at the two highest dosages. This finding suggests that immunological interference with gonadotrophin-induced ovarian activity in ocelots could be avoided by using prolonged successive treatment intervals.

This study demonstrated that ocelots can become pregnant and carry offspring to term after exogenous gonadotrophin treatment and laparoscopic intrauterine insemination. This birth represents the sixth nondomestic felid species (cheetah, Howard *et al.*, 1992b; clouded leopard, Howard *et al.*, 1993; tiger, Donoghue *et al.*, 1993; leopard cat, *Felis bengalensis*, Wildt *et al.*, 1993a; puma, Barone *et al.*, 1994) produced by laparoscopic AI technology, but only the second (the other being the leopard cat; Wildt *et al.*, 1993a) produced after AI with cryopreserved spermatozoa. Despite fairly substantial acrosome damage, frozen–thawed ocelot spermatozoa appeared functionally competent. With the sperm pelleting cryopreservation technique, about 50–60% of ocelot spermatozoa had acrosome damage after thawing, similar to values reported for frozen–thawed ferret spermatozoa (Howard *et al.*, 1991) and slightly less than the approximately 60–80% acrosome damage reported for frozen–thawed domestic cat spermatozoa (Hay and Goodrowe, 1993; Wood *et al.*, 1993). Extensive acrosomal damage may be one reason for lower conception rates (about 11%) in domestic cats after vaginal artificial insemination with frozen–thawed (Platz *et al.*, 1978) compared with fresh (50–75% conception rate; Sojka *et al.*, 1970) spermatozoa. To compensate for cryopreservation-associated injury to ocelot spermatozoa, we usually doubled the number of motile spermatozoa per inseminate for frozen–thawed compared with fresh samples.

Studies of cattle, humans and sheep have demonstrated lower fertilizability of frozen-thawed compared with fresh spermatozoa, presumably owing to decreased motility or acrosomal integrity (Shannon, 1978; Critser *et al.*, 1987; Maxwell *et al.*, 1993). However, in tigers, fresh and frozen–thawed spermatozoa were equally effective in fertilizing conspecific oocytes *in vitro* (Donoghue *et al.*, 1992a). In addition, pregnancy rates of 70% have been reported in ferrets after laparoscopic AI with frozen–thawed spermatozoa, using females in natural oestrus but induced to ovulate with hCG (Howard *et al.*, 1991). Accordingly, we suspect that low pregnancy success after AI in ocelots is more a consequence of suboptimal oocyte quality or inadequate maternal support (associated with the exogenous gonadotrophins) rather than due to poor acrosomal integrity or compromised function in frozen–thawed spermatozoa.

Felid oocyte quality is sensitive to exogenous gonadotrophin dosages (and gonadotrophin interaction) and the timing of the FSH-like and LH-like signals (Goodrowe *et al.*, 1988; Donoghue *et al.*, 1992b). In our domestic cat studies, both variables have been optimized for oocyte fertilizability through comparative studies using *in vitro* fertilization and various combinations of eCG and hCG. This basic information was applied in laparoscopic AI protocols that consistently resulted in multiple ovulations (~ 8 corpora lutea per queen) and high conception rates (50%) with proper timing of insemination (Howard *et al.*, 1992a). However, average litter sizes after AI continue to be small (about two kittens per litter), suggesting either recruitment of a large subpopulation of poor quality oocytes or establishment of an inadequate maternal milieu to support embryo or fetal development. This phenomenon also may be occurring in ocelots.

Dosages of eCG/hCG that were sufficient to induce desired folliculogenic and ovulatory responses in ocelots also produced extremely high (about 400 pg ml^{-1}) serum concentrations of oestradiol. Serum oestradiol was three- to tenfold greater in ocelots than in other felid species treated with eCG/hCG (Donoghue *et al.*, 1990, 1992b; Howard *et al.*, 1992b). Serum concentrations of progesterone in ovulatory females frequently were variable and, in most instances, were higher than would be expected from a recent ovulation (i.e., < 24 h after ovulation). Although there are no comparative serum hormonal data from female ocelots mated under natural conditions, these new observations suggest that hormone secretory patterns associated with exogenous gonadotrophin treatment may be abnormal and adversely affecting AI success in ocelots.

Improving AI conception rates in ocelots and other nondomestic felids may depend upon making additional species-specific adjustments in ovarian stimulation protocols. One approach is suggested by our experiences with cheetahs, a species typically characterized by ovarian acyclicity in females and a high incidence of teratospermia in males (Wildt *et al.*, 1993b). Despite these characteristics (normally associated with low fertility), AI success has been relatively high (nine litters produced to date), possibly because cheetah ovaries are 'downregulated' before exogenous gonadotrophin treatment. In felids normally producing much more cyclic follicular activity, a pharmaceutical approach to ovarian downregulation (such as with GnRH agonists or antagonists) may be useful as an adjunct treatment to reduce individual variability and produce more consistent ovarian responses for planned AI. Although pregnancy success was low in ocelots, the present results demonstrate the potential of assisted reproductive technology for producing offspring, thus reinforcing our earlier assertion that these tools will be useful in the genetic management of endangered cat species. Although encouraging, these findings also indicate the need for additional research to improve efficiency for conservation purposes.

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