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Seasonal effects on ovarian responsiveness to exogenous gonadotrophins and successful artificial insemination in the snow leopard (*Uncia uncia*)

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Abstract. Ovaries of the seasonally-breeding snow leopard (*Uncia uncia*) were examined to determine whether they were responsive to exogenous gonadotrophins throughout the year. The potential of laparoscopic artificial insemination (AI) also was assessed for producing offspring. During the non-breeding, pre-breeding, breeding and post-breeding seasons, females ($n = 20$) were treated with a standardized, dual-hormone regimen given intramuscularly (600 I.U. of equine chorionic gonadotrophin followed 80–84 h later with 300 I.U. of human chorionic gonadotrophin (hCG)). Laparoscopy was performed 45–50 h after administration of hCG, and all ovarian structures were described. Females with fresh corpora lutea (CL) were inseminated, and anovulatory females were subjected to follicular aspiration to examine oocyte quality. Snow leopards responded to exogenous gonadotrophins throughout the year. Mean number of total ovarian structures (distinct follicles mature in appearance plus CL) did not differ ($P \geq 0{\cdot}05$) with season, but the proportion of CL : total ovarian structures was greater ($P < 0{\cdot}01$) for the breeding season compared with all other seasons. The proportion of females ovulating was greater ($P<0{\cdot}05$) during the breeding and post-breeding seasons than during the pre-breeding and non-breeding seasons respectively. No Grade-1 quality oocytes were recovered from follicles of anovulatory females. Serum concentrations of oestradiol-17β appeared elevated in all females, and neither oestradiol-17β concentrations nor progesterone concentrations differed ($P \geq 0{\cdot}05$) among seasons. Of 15 females artificially inseminated, the only one that was inseminated in the non-breeding season became pregnant and delivered a single cub. This is the first successful pregnancy resulting from AI in this endangered species.

Extra keywords: felid, seasonality, ovulation induction, laparoscopy, ovary.

Introduction

Intensive animal management strategies, including zoo breeding programmes that partially rely on assisted reproduction, are being implemented to help conserve rare wildlife species. Artificial insemination (AI), *in vitro* fertilization (IVF) and embryo transfer (ET) in conjunction with genome resource banking (cryopreserved repositories of germ plasm) could play a role in sustaining adequate genetic diversity in populations of endangered wildlife (Wildt *et al*. 1992). Progress in developing assisted reproduction for non-domestic felid species has been encouraging. In recent years, offspring have been produced following laparoscopic AI in the cheetah (*Acinonyx jubatus*) (Howard *et al*. 1992*a*), clouded leopard (*Neofelis nebulosa*) (Howard *et al*. 1996), tiger (*Panthera tigris*) (Donoghue *et al*. 1993), leopard cat (*Prionailurus bengalensis*) (Wildt *et al*. 1992), ocelot (*Leopardus pardalis*) (Swanson *et al*. 1996*a*), and puma (*Puma concolor*) (Barone *et al*. 1994). Although the snow leopard (*Uncia uncia*) does reproduce well in captivity, the use of reproductive tools for helping to genetically manage the species, one of the 'great cats' in danger of extinction, has been endorsed by the Snow Leopard Species Survival Plan (Wildt and Mellen 1994). However, an assisted reproduction regimen that works well for one species can fail in another because of naturally-occurring physiological specificities, even among closely-related species within the same family and genus (Wildt *et al*.

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1992). Therefore, substantial basic research is needed for each species before making practical attempts to implement AI or IVF and ET.

We already have identified reproductive traits unique to the snow leopard. For example, sperm from this species are highly sensitive to conventional felid culture media and conditions (Roth *et al*. 1994, 1996). Furthermore, unlike most felid species, the male snow leopard demonstrates distinct seasonal variation in reproductive traits, producing more sperm and higher peripheral concentrations of testosterone in winter (December–February) compared with summer and autumn (June–November) (Johnston *et al*. 1994). In nature, the snow leopard inhabits the high mountain ranges of China, Mongolia and Russia. Throughout the USA, Canada and Europe, snow leopards typically mate in winter and give birth in the spring after a 90–100-day gestation (Blomqvist 1990; Wharton 1991). One long-term study of two adult females indicated that sexual behaviour occurs in December–April in the presence or absence of a male conspecific (Schmidt *et al*. 1993). Although this seasonal reproductive pattern is widely acknowledged, there has been no large-scale, systematic study to validate a seasonal anoestrus in the snow leopard, and virtually nothing is known about the physiological mechanisms driving reproductive seasonality. This basic information is of fundamental and comparative interest and can be used to gradually mold assisted reproduction techniques into practical management tools.

In the present study, ovarian responsiveness of the snow leopard to an exogenous gonadotrophin treatment administered during specific months throughout the year was evaluated. There are ~230 snow leopards in all of North America and Canada, and almost all individuals are maintained in accredited zoos (Wharton 1991). Therefore, a 'mobile laboratory approach' (Donoghue *et al*. 1990) was used to collect data on a group of females large enough to allow statistical analyses. The specific objectives were: *(1)* to determine if conventional exogenous gonadotrophins could be used to consistently induce follicular development and ovulation throughout the year during different reproductively-active or -inactive periods; and *(2)* to begin to assess the potential of assisted reproduction, specifically laparoscopic AI, as a tool for producing offspring.

Materials and Methods

Animals

Adult female snow leopards (age, 2–14 years; bodyweight, 26–46 kg; $n = 20$) were studied. All were born in captivity and maintained at one of the following institutions: Bronx Zoo/Wildlife Conservation Society (Bronx, NY, USA); Buffalo Zoological Gardens (Buffalo, NY, USA); Cheyenne Mountain Zoological Park (Colorado Springs, CO, USA); Henry Doorly Zoo (Omaha, NE, USA); Houston Zoological Gardens (Houston, TX, USA); Metro Washington Park Zoo (Portland, OR, USA); Nashville Zoo (Nashville, TN, USA); Oklahoma City Zoological Park (Oklahoma City, OK, USA); San Antonio Zoological Gardens and Aquarium (San Antonio, TX, USA); Seneca Park Zoo (Rochester, NY, USA); and Thrigby Hall Wildlife Garden (Norfolk, UK). Despite the institutional variation, all snow leopards at the time of examination were maintained between 29° N and 51° N latitude. Snow leopard sperm donors were maintained on-site at the same institutions housing the females in the study. Males were 4–14 years old, born in captivity, and maintained as singletons for at least two weeks before electroejaculation. All snow leopards were exposed to natural fluctuations in daylength and were in visual and olfactory proximity. Every institution maintained ≥2 females and ≥1 male. Animals were given a standardized meat diet that was supplemented with vitamins and minerals.

Reproductive seasonality in female snow leopards

Birthdate data were summarized from the North American Snow Leopard Studbook (Wharton 1991) to determine when parturition normally occurs in naturally-mated females (Fig. 1). Although offspring were produced from March to September, >95% of births (250/260) occurred from April to July. These data, coupled with a known gestation of 90–100 days (Blomqvist 1990; Wharton 1991), were used to identify January to April as the months of most successful matings (Fig. 1). These months were designated as the 'breeding' season, and the two months before (November and December) and after (May and June) were arbitrarily categorized as the 'pre-breeding' season and the 'post-breeding' season respectively; the period from July to October was designated as the 'non-breeding' season.

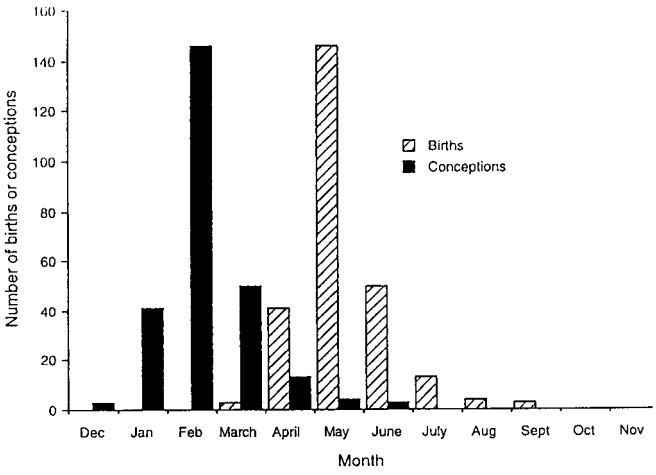

Fig. 1. Number of zoo-managed snow leopard conceptions and births per month based on data recovered from the North American Snow Leopard Studbook (Wharton 1991). Conception data were extrapolated from birthdate data using a known gestation length of 90–100 days.

Semen collection and processing

Semen was collected for AI by inducing a surgical plane of anaesthesia with tiletamine HCl and zolazepam HCl (5 mg kg^{-1} bodyweight) (Telazol; Fort Dodge Laboratories, IA, USA) or ketamine HCl (7·5–8·0 mg kg^{-1} bodyweight) (Ketaset; Fort Dodge Laboratories) and xylazine (0·7–0·8 mg kg^{-1} bodyweight) (Rompun; Mobay, Shawnee, KS, USA). A standardized electroejaculation protocol was followed (Howard *et al*. 1986) using a rectal probe (2·3 cm in diameter) with three longitudinal electrodes. Most animals

received three series of electrical stimuli (80 stimuli in total; range, 2–5 V) over a 30-min interval. In some cases, animals received additional stimuli and/or a fourth series of stimuli to optimize sperm recovery. Ejaculates were evaluated and processed according to previous procedures (Roth *et al*. 1994, 1996). Briefly, total fluid volume, percentage sperm motility (0–100%), and forward progressive motility (scale, 0–5: 0, no movement; 5, rapid forward progression) (Howard *et al*. 1986) were determined. Semen was diluted with an equal volume of pre-warmed (37°C) phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (FCS), 0·1 mM pyruvate, 100 U penicillin mL^{-1}, and 100 U streptomycin mL^{-1} (Roth *et al*. 1994). Semen from all series exhibiting good sperm motility (≥70%) was combined, centrifuged (150*g*, 10 min), the supernatant discarded, and the sperm pellet was resuspended in ∼215 μL of PBS. An aliquot (5 μL) was evaluated for percentage sperm motility and forward progressive motility, and 5 μL were used to determine sperm concentration using a haemocytometer. Samples were maintained at 25°C (protected from light) in ambient air, and percentage sperm motility was evaluated again immediately before AI. A total of $3·0–68·0\times10^6$ motile spermatozoa were inseminated *in utero* as described below.

Semen cryopreservation

Cryopreserved semen prepared according to previous protocols (Howard *et al*. 1986; Swanson *et al*. 1996*a*) served as a source of sperm for AI when fresh samples were unavailable. In brief, after centrifugation, sperm pellets were resuspended in cryoprotectant diluent, consisting of 11% (w/v) lactose, 20% (v/v) egg yolk, 4% (v/v) glycerol, to a final concentration of $\sim100\times10^6$ motile sperm mL^{-1}, cooled at 4°C for 30 min and frozen by pelleting on dry ice. After 3 min on dry ice, pellets were plunged into liquid nitrogen and stored there until thawed for AI.

Sperm pellets were thawed rapidly by immersing each pellet in 100 μL PBS (37°C) in a glass tube (12×75 mm) that was agitated for 30 s in a waterbath (37°C). Thawed samples were combined and centrifuged (150*g*; 10 min), and supernatants were discarded to remove cryoprotectant. The resultant sperm pellet was resuspended in ∼215 μL PBS, evaluated for sperm percentage motility and concentration and immediately used for AI.

Gonadotrophin treatment and laparoscopic evaluation

Preliminary trials were conducted to determine appropriate dosages of exogenous gonadotrophin for inducing ovarian activity. Initial dosages tested were those successful for inducing ovarian activity in cheetahs and pumas, felid species with bodyweights similar to that of snow leopards (Howard *et al*. 1992*a*; Barone *et al*. 1994). Two snow leopards treated intramuscularly in April with 200 I.U. of equine chorionic gonadotrophin (eCG) (Sigma, St Louis, MO, USA) followed with 100 I.U. of human chorionic gonadotrophin (hCG) (Sigma) 80–84 h later had minimal ovarian responses, as diagnosed by laparoscopy (see below). In contrast, 4 females treated with 800 I.U. eCG and 400 I.U. hCG had >10 total follicles (≥3 mm in diameter) on each set of combined ovaries at 24 h after administration of hCG. Based on these preliminary results, female snow leopards in the present study were treated with 600 I.U. eCG followed 80–84 h later by 300 I.U. hCG administered intramuscularly by means of a blow dart or direct syringe injection. Over the two-year study period, 4 females were treated with this exact regimen in 3 of 4 seasons and always with at least 6 months between treatments. The remaining 16 animals were treated once.

To assess ovarian response, laparoscopy was performed 45–50 h after administration of hCG. This timing was chosen based on the fact that big cats ovulate 41–50 h after hCG (Howard *et al*. 1992*a*; Barone *et al*. 1994) and pre-ovulatory anaesthesia inhibits ovulation (Howard *et al*. 1992*b*). Each animal was induced into a surgical plane of anaesthesia with tiletamine HCl and zolazepam HCl (4·5–7·0 mg kg^{-1} bodyweight) or ketamine HCl (5·0–10·0 mg kg^{-1} bodyweight) and xylazine (0·7–2·5 mg kg^{-1} bodyweight). After intubation, a surgical plane of anaesthesia was maintained by delivering a mixture of 1–2% isoflurane gas with oxygen. Laparoscopy was performed using a 10-mm laparoscope inserted 2–4 cm cranial to the umbilicus. Both ovaries were examined thoroughly, and the number and size of follicles and corpora lutea (CL) were recorded. Most females with fresh CL were inseminated by inserting an 18G indwelling catheter (Sovereign Medical, Ireland) percutaneously into the cranial tip of each uterine horn and depositing washed sperm (∼100 μL $horn^{-1}$) directly into the uterine lumen (Howard *et al*. 1992*a*, 1992*b*). In cases where ovaries contained large follicles (≥3 mm in diameter), mature in appearance, but no CL, follicles from one ovary were aspirated to examine oocyte quality and maturational status (based on gross morphology). Laparoscopic follicle aspiration was conducted according to previous methods (Goodrowe *et al*. 1988), and follicular contents were collected into a tube containing PBS with 5% FCS. Oocytes were immediately recovered and assessed for quality grade based on previously reported criteria (Donoghue *et al*. 1990). In brief, Grade-1 oocytes were dark and uniform in shape with an expanded, light-coloured cumulus mass; Grade-2 oocytes were those with ≥2 layers of compact cumulus cells; Grade-3 oocytes had few or no cumulus cells and/or were pale or abnormal in shape; and Grade-4 oocytes included dark or semi-dark oocytes that were uniform in shape and that had expanded, but dark, slightly aggregating cumulus cells that were granular in appearance.

Radioimmunoassays for serum concentrations of oestradiol-17β and progesterone

Immediately after anaesthesia and before laparoscopy, a 10-mL blood sample was collected from each female, and the serum was harvested and stored at −80°C. As an indicator of ovarian function, concentrations of oestradiol-17β and progesterone were measured in unextracted serum using solid-phase ^{125}I radioimmunoassay (RIA) kits (Coat-a-Count, Diagnostic Products, Los Angeles, CA, USA). This assay has been standardized and tested thoroughly for felid serum (Swanson *et al*. 1995*a*). All serum samples were evaluated simultaneously in a single RIA for each respective hormone. Assay sensitivities (based on 90% of maximum binding) for oestradiol-17β and progesterone were 5 pg mL^{-1} and 0·05 ng mL^{-1} respectively. The intra-assay coefficients of variation were <10% for both assays.

Statistical analysis

Means±s.e.m. were calculated. Total ovarian structures (number of follicles plus number of CL) were compared by ANOVA, and least significant difference comparisons were used to determine seasonal variations. The proportion of CL : total ovarian structures and the proportion of females ovulating were compared among seasons by χ^2 analysis. Correlation coefficients were calculated across all seasons between follicle number, CL number, total ovarian structures, and serum concentrations of oestradiol-17β and progesterone. Correlation coefficients also were determined between the two independent variables, animal age and bodyweight, and the number of CL and total ovarian structures, as well as concentrations of oestradiol-17β and progesterone. Among-season differences in ovarian and endocrine responses of the four females treated three times with exogenous gonadotrophins were determined using a paired Student's *t*-test.

Results

Ovarian responses to exogenous gonadotrophins

Twenty-eight individual gonadotrophin treatments followed by laparoscopic assessments were made in the 20 female snow leopards. A minimum of 6 females was treated during each of the non-breeding ($n = 6$), pre-breeding ($n = 6$), breeding ($n = 9$), and post-breeding ($n = 7$) seasons. The proportion of females ovulating was greater ($P < 0{\cdot}05$) during the breeding and post-breeding seasons than during the non-breeding season (Table 1). Mean number of total ovarian structures (follicles + CL) observed at laparoscopy did not differ ($P \geq 0{\cdot}05$) among seasons. However, the CL : total ovarian structures ratio was different, with the greatest proportion ($P < 0{\cdot}01$) of CL observed during the breeding season (52/98) and the smallest proportion ($P < 0{\cdot}01$) observed during the non-breeding and pre-breeding seasons (9/119 and 6/123 respectively). Furthermore, there were more ($P < 0{\cdot}01$) CL and fewer follicles during the breeding and post-breeding seasons than during the non-breeding and pre-breeding seasons. The ovaries of some females during the breeding season ($n = 2$) and post-breeding season ($n = 1$) contained CL that were older in appearance (abundant, highly organized, white luteal tissue with no recent haemorrhaging; see Plate I*a*).

Repeated responses to exogenous gonadotrophins

In the four females treated with gonadotrophins three times, Treatment 1 was administered during the post-breeding season, Treatment 2 was given 7 months later (pre-breeding season), and Treatment 3 occurred 14 months after Treatment 2 (breeding season). All of these females had ovarian follicular and/or luteal activity at all three times (Table 2). More ($P<0{\cdot}05$) follicles and fewer ($P < 0{\cdot}05$) CL were produced after Treatment 2 compared with Treatment 1, but mean follicle numbers and CL numbers after Treatment 3 did not differ ($P \geq 0{\cdot}05$) from those following Treatment 1 or Treatment 2. Mean numbers of total ovarian structures and concentrations of oestradiol-17β and progesterone were comparable ($P \geq 0{\cdot}05$) among treatments.

Serum concentrations of oestradiol-17β and progesterone

Based on a single blood sample collected from each animal at laparoscopy, serum concentrations of oestradiol did not differ ($P \geq 0{\cdot}05$) among the non-breeding (203±40 pg mL^{-1}), pre-breeding (291±53 pg mL^{-1}), breeding (226±39 pg mL^{-1}), and post-breeding (228±33 pg mL^{-1}) seasons. Serum concentrations of oestradiol were <100 pg mL^{-1} in only two females, one of which exhibited no follicular or luteal activity and the other contained only fluid-filled, cystic follicles.

Similarly, serum concentrations of progesterone did not differ ($P \geq 0{\cdot}05$) among seasons, ranging from 0·6±0·2 ng mL^{-1} in the non-breeding season to 4·0±2·9 ng mL^{-1} in the post-breeding season. Only three snow leopards had circulating concentrations of progesterone >3·0 ng mL^{-1} (4·9, 9·4 and 21·2 ng mL^{-1}), and the ovaries of all of these females contained CL that appeared older and well developed (see Plate I*a*).

Effect of age and bodyweight

There was an inverse relationship between female age and total ovarian structures ($r = -0{\cdot}39$; $P < 0{\cdot}05$) and a positive correlation between follicle number and serum oestradiol-17β ($r = 0{\cdot}39$; $P < 0{\cdot}05$). However, there was no correlation between serum progesterone and CL number ($r = -0{\cdot}08$; $P \geq 0{\cdot}05$). Furthermore, there was no significant relationship ($P \geq 0{\cdot}05$) between bodyweight and follicle number ($r = -0{\cdot}25$) and CL number ($r = -0{\cdot}42$).

Oocyte aspiration and evaluation

Ovarian follicles were aspirated from six snow leopards. In the non-breeding season, 34 oocytes were obtained from four females and 12 of these were classified as Grade-3, 13 as Grade-2, and 9 as Grade-4 oocytes (see Fig. 2). In the pre-breeding season, 11 oocytes were recovered from two females and 4 were classified as Grade-2, 5 as Grade-3, and 2 as Grade-4 oocytes. No oocyte from any female met the Grade-1 criteria.

Ejaculate traits

Good quality ejaculates were collected throughout the year. Seminal volume and total number of sperm ejaculated varied among individuals, ranging from 1·4–5·0 mL and 22–185×10^6 sperm respectively. Percentage sperm motility was high (range, 70–90%), and neither it nor the percentage of morphologically-normal sperm cells (range, 49–67%) differed with season ($P \geq 0{\cdot}05$).

Artificial insemination (AI)

Females with fresh CL at laparoscopy (15/17) were artificially inseminated (Table 3). All of these females but one were inseminated with fresh spermatozoa collected by electroejaculation 1–5 h earlier (mean, 20·8±4·6×10^6 motile spermatozoa; range, 3–68×10^6; percentage morphologically normal, 49·6±3·2%). The aim was to inseminate ≥20×10^6 motile spermatozoa, however, ejaculate quality and/or percentage sperm motility at the time of AI did not always permit attaining this goal. The remaining female was inseminated with 6·7×10^6 motile, frozen–thawed spermatozoa because no male was available for electroejaculation. No females inseminated during the pre-breeding ($n = 2$), breeding ($n = 6$), or post-breeding

Table 1. Seasonal impact on ovarian responsiveness to exogenous gonadotrophins in the snow leopard

Individual females were administered 600 I.U. of equine chorionic gonadotrophin and 300 I.U. of human chorionic gonadotrophin intramuscularly. Values are mean±s.e.m. for total number of females treated within each season. Within columns, values with different superscripts differ (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$)

Season	No. females ovulating/ no. females treated*	No. fresh CL[A]	No. follicles ≥ 3 mm in diameter**
Non-breeding (July–October)	1/6 (16·7%)[a]	1·5±1·5	18·3±5·0[a]
Pre-breeding (November–December)	2/6 (33·3%)[ab]	1·0±0·8	19·5±3·6[a]
Breeding (January–April)	7/9 (77·8%)[bc]	5·8±2·1	5·1±2·2[b]
Post-breeding (May–June)	7/7 (100%)[c]	5·0±1·2	8·4±3·3[b]

[A]CL, corpora lutea.

Table 2. Ovarian and endocrine responses in four snow leopards following repeated treatment with exogenous gonadotrophins

Individual females were administered 600 I.U. of equine chorionic gonadotrophin and 300 I.U. of human chorionic gonadotrophin intramuscularly. Treatment 1 was administered during the post-breeding season, Treatment 2 was given 7 months later during the pre-breeding season, and Treatment 3 occurred during the breeding season, 14 months after Treatment 2. Within columns, mean values with different superscripts differ ($P < 0{\cdot}05$)

Animal	No. follicles ≥3 mm in diameter	No. fresh CL[A]	Serum concentrations of: oestradiol-17β (pg mL^{-1})	progesterone (ng mL^{-1})
Treatment 1 (post-breeding season)				
No. 35	10	10	113	0·5
No. 37	3	8	259	0·7
No. 39	1	4	183	1·6
No. 41	3	6	163	2·0
mean±s.e.m.	4·3±2·0[a]	7·0±1·3[a]	179±30	1·2±0·3
Treatment 2 (pre-breeding season)				
No. 35	25	1	230	1·2
No. 37	6	5	141	0·3
No. 39	15	0	252	1·6
No. 41	16	0	472	0·3
mean±s.e.m.	15·5±3·9[b]	1·5±1·2[b]	274±70	0·9±0·3
Treatment 3 (breeding season)				
No. 35	0	3	139	0·7
No. 37	3	4	184	1·2
No. 39	6	16	440	1·1
No. 41	1	16	216	1·5
mean±s.e.m.	2·5±1·3[ab]	9·8±3·6[ab]	245±67	1·1±0·2

[A]CL, corpora lutea.

Table 3. Results of artificial insemination in the snow leopard

Values are mean±s.e.m.

Season	No. females inseminated	No. follicles ≥3 mm in diameter	No. fresh CL[A]	Total motile sperm inseminated ($\times 10^6$)[B]	No. pregnancies	No. offspring
Non-breeding	1	15	9	9	1	1
Pre-breeding	2	15·5±9·5	3·0±2·0	8·0±0·0 (8–8)	0	0
Breeding	6	6·2±3·1	7·2±2·9	26·3±8·6 (10–68)	0	0
Post-breeding	6	7·2±3·6	5·7±1·2	19·2±6·1 (3–42)	0	0

[A]CL, corpora lutea.
[B]Values in parentheses represent range ($\times 10^6$).

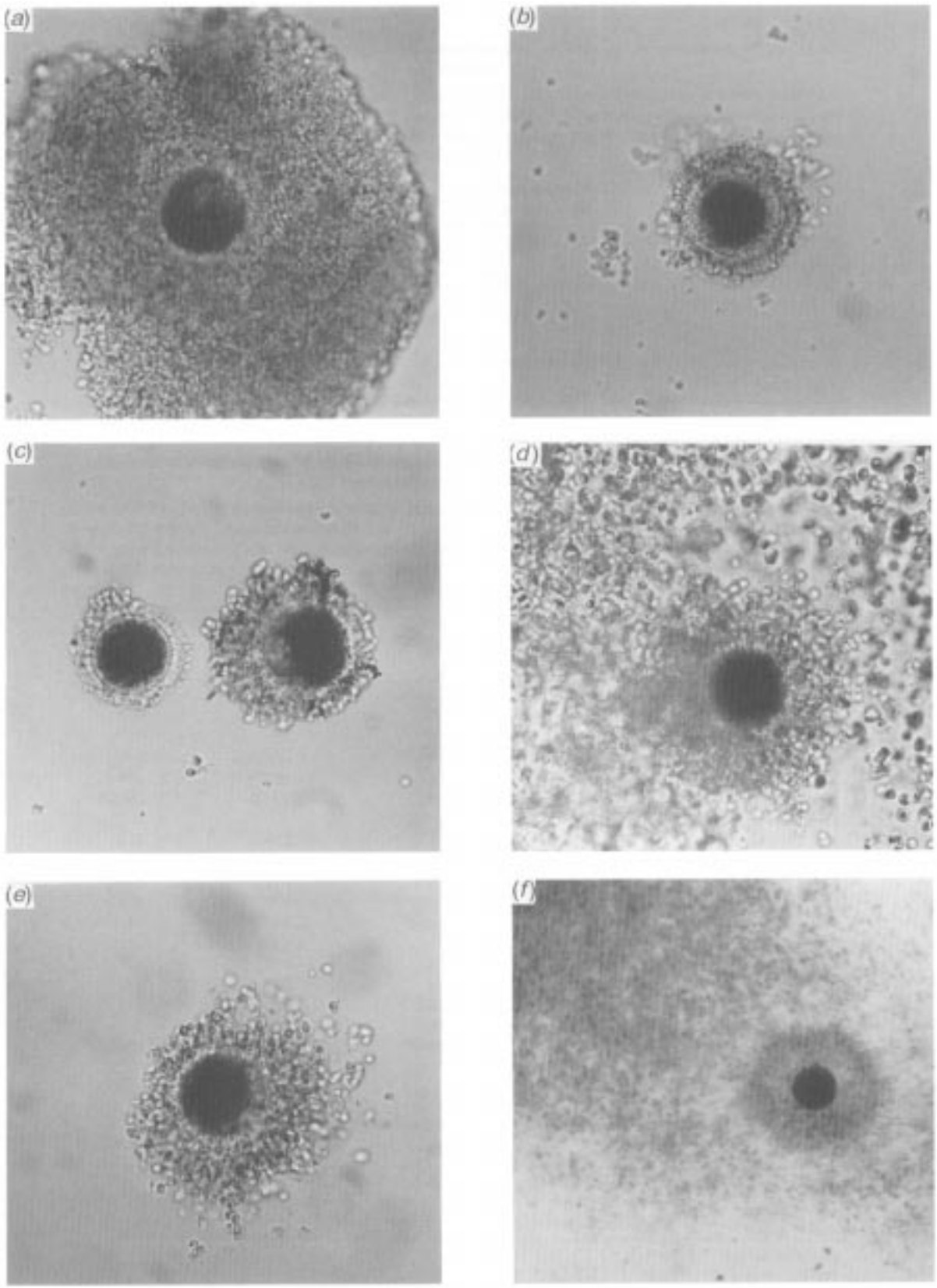

Fig. 2. Oocytes recovered from ovarian follicles of eCG+hCG-treated snow leopards 45–50 h after administration of hCG during the non-breeding season. (*a and b*) Oocytes, immature in appearance (Grade 2), with ≥2 layers of compact cumulus cells. *(c)* Degenerate or poor quality (Grade 3) oocytes with inconsistent colouring of the cytoplasm or very few cumulus cells. *(d)* Oocytes, mature in appearance but aged (Grade 4), with expanded (but dark and granular) cumulus. *(e)* Oocytes, mature in appearance but aged (Grade 4), with cumulus masses that appear dark and granular. *(f)* No oocytes recovered from snow leopard follicles during the pre-breeding and non-breeding seasons were considered mature, Grade-1 oocytes like those from another study shown here.

($n = 6$) seasons became pregnant. However, the only ovulating, inseminated female in the non-breeding season (October) became pregnant and delivered a single cub in January after a 100-day gestation (Day 1, day of AI). This female was inseminated 50 h after administration of hCG with 9×10^6 motile spermatozoa electroejaculated 5 h earlier.

Discussion

Felids generally are regarded as induced ovulators, and a direct relationship between the coitus-induced luteinizing hormone (LH) surge and ovulation success has been measured in the domestic cat (Concannon *et al*. 1980; Wildt *et al*. 1980, 1981; Swanson *et al*. 1995*a*). Follicular development can be stimulated in the domestic cat with exogenous follicle-stimulating hormone (FSH) or eCG (Wildt *et al*. 1978; Howard *et al*. 1986; Goodrowe *et al*. 1988; Pope *et al*. 1993), and ovulation can be induced with gonadotrophin-releasing hormone (GnRH) or hCG (Chakraborty *et al*. 1978; Howard *et al*. 1986; Goodrowe and Wildt 1987). For non-domestic felids, eCG followed by hCG has become the regimen of choice to avoid animal stress associated with multiple injections of FSH. However, an appropriate dosage of eCG and hCG must be determined for each felid species because of unique sensitivities to exogenous gonadotrophins that appear independent of bodyweight (Howard *et al*. 1996; Swanson *et al*. 1996*a*). The bodyweight of female snow leopards (26–45 kg) is equivalent to that of cheetahs or pumas, yet preliminary trials suggested that they are less sensitive to eCG, requiring three times the gonadotrophin dosage found effective for the latter two species (Howard *et al*. 1992*a*; Barone *et al*. 1994). Treating snow leopards with 600 I.U. eCG and 300 I.U. hCG during the breeding and post-breeding seasons induced adequate follicular growth and ovulation. In contrast, an abundance of unovulated follicles developed in the non-breeding and pre-breeding seasons, and perhaps a higher hCG dosage or a different eCG to hCG interval should be tested during these months.

Environmental factors, including food availability, temperature, pheromonal cues and social pressures are known to influence reproduction in mammals. The most common regulator of seasonal reproduction is photoperiod. In the domestic cat and tigers, reduced daylength causes seasonal anoestrus (Scott and Lloyd-Jacob 1959; Seal *et al*. 1985). Other researchers (Johnston *et al*. 1994; Schmidt *et al*. 1993) have presented evidence of reproductive seasonality in both male and female snow leopards, a finding supported by the analysis of historical parturition records. It seems probable that reproductive seasonality in the snow leopard also is mediated by natural photoperiodicity. For some non-felid species (hamsters, ferrets, sheep), increased daylength is associated with reduced GnRH secretion resulting in decreased secretion of FSH, LH, and testosterone (Bronson 1988). Endocrine dynamics of the male snow leopard appear to follow this trend with concentrations of FSH, LH, and testosterone all reaching a nadir in the summer, then increasing as the daylength decreases to reach a winter peak (Johnston *et al*. 1994). The same endogenous gonadotrophin pattern probably also occurs in the female snow leopard. A general theory indicates that FSH induces LH receptor expression, and LH induces androgen secretion by the theca interna (for a review, see Lipner 1988). In the presence of granulosa cells, androgen is converted into oestrogen, and follicular growth ensues. Therefore, with increasing daylength, a gradual photoperiodic-induced decrease in GnRH secretion and, consequently, FSH could lead to reduced LH receptor expression, follicular growth, and the eventual onset of anoestrus in late spring and summer. Interestingly, Schmidt *et al*. (1993) reported increased social and sexual behaviour without mating in paired snow leopards during the early winter. They suggested that follicles may begin to develop in early winter but regress without completely maturing until late in winter when mating is observed. This theory would support the idea of progressive ovarian changes (or priming), perhaps due to photoperiodic-induced increases in pulsatile GnRH and FSH leading to enhanced LH receptor expression, androgen production, and eventual conversion to high oestrogen concentrations culminating in oestrous behaviour in early winter. Although eCG is capable of stimulating follicular development during the pre-breeding and non-breeding seasons, deficient or delayed LH receptor expression during these seasons may be partially responsible for ovarian refractoriness to hCG-induced ovulation.

In the present study, it appeared that the eCG+hCG-induced follicular development during the pre-breeding and non-breeding seasons was, to some extent, normal. Gross examination revealed numerous large follicles that, when aspirated, yielded oocytes of various developmental stages and qualities, including those classified as Grade-4 oocytes (mature-but-aged). The latter categorization was based on cumulus cell expansion and morphology, known to correlate well with nuclear maturation (Dekel and Kraicer 1978; Testart *et al*. 1983). Further evidence that normal follicular development occurred during the non-breeding season was irrefutable, a pregnancy and birth of a cub following exogenous hormone treatment and AI conducted in October.

One of the challenges of this type of research is the limited number and restricted accessibility of animals. Although we were able to include 20 female snow leopards in the present study, it was necessary to use four females on multiple occasions. By treating the

same individuals during three seasons, we had the advantage of conducting a paired comparison (albeit on a small dataset) to examine seasonal differences in ovarian response within animals. This analysis also revealed a greater sensitivity to exogenous gonadotrophins in the breeding and post-breeding seasons than in the pre-breeding season. Because of the overall sample size, we also considered bodyweight and age as confounding factors. However, the only significant correlation was an inverse relationship between age and ovarian structures, a dependent variable that did not differ across seasons.

The production of gonadotrophin-neutralizing immunoglobulins after repeated exogenous gonadotrophin administration is well documented in a variety of species (Jainudeen *et al*. 1966; Greenwald 1970; Bavister *et al*. 1986), including the domestic cat (Swanson *et al*. 1995*b*). In our study, the four snow leopards treated repeatedly with eCG and hCG produced similar numbers of ovarian structures with subsequent treatments, indicating that ovarian responsiveness to eCG did not decrease. Furthermore, the responses of these four females during the breeding season (their third treatment) were no different than those of the additional five females in this season treated for the first time. However, a minimum of six months was maintained between gonadotrophin treatments, thereby probably avoiding anamnestic responses. The kinetics of humoral immune responses following the administration of exogenous gonadotrophins in the domestic cat suggest that a four-month interval between treatments is sufficient to avoid secondary immune responses that neutralize gonadotrophins (Swanson *et al*. 1996*b*). Although it is likely that species-specific immune sensitivities to exogenous gonadotrophins exist, a six-month interval appears sufficient to limit immunoglobulin production in several felids, including the domestic cat (Swanson *et al*. 1996*b*), ocelot (Swanson *et al*. 1996*a*), cheetah (Howard *et al*. 1992*a*) and tiger (Donoghue *et al*. 1990).

By comparison with other felid species (Brown *et al*. 1994), snow leopards produce relatively high basal and peak concentrations of oestradiol-17β. However, serum concentrations of oestradiol-17β were 3–10 times higher than those reported for snow leopards in natural oestrus (Schmidt *et al*. 1993). This possibly was due to ovarian hyperstimulation considering that a relatively high number of follicles was observed at laparoscopy, and there was a positive correlation between follicle number and oestradiol-17β concentrations. Yet, no strong conclusions regarding oestradiol concentrations should be made based on a single serum sample from each animal. Serum concentrations of progesterone increase as early as 24 h after follicle aspiration in the domestic cat (Goodrowe *et al*. 1988; Donoghue *et al*. 1992). In all but three of the snow leopard females, peripheral concentrations of progesterone were relatively low ($<3{\cdot}0$ ng mL^{-1}), confirming that the ovulations we classified as 'fresh' were probably <24 h old at the time of laparoscopy. Three females with higher progesterone concentrations contained CL that were older in appearance. The two females with progesterone concentrations $>3{\cdot}0$ ng mL^{-1} but <10 ng mL^{-1} may have been in the follicular phase when treated with eCG, and the LH-like activity in eCG (Licht *et al*. 1979) could have caused premature ovulation. The third female had a much higher progesterone concentration (>20 ng mL^{-1}), suggesting that CL were present well before gonadotrophin administration. Elevated progesterone concentrations have been reported for $\sim$6 weeks after copulation in females failing to produce offspring, suggesting a non-pregnant luteal phase similar to that in other felids (Schmidt *et al*. 1993). This, in addition to the corpora albicantia observed on ovaries of several females (Plates I*a* and I*b*) that had not been housed with a male support recent claims that many felid species occasionally ovulate spontaneously (Brown *et al*. 1995).

One of the highest priorities in developing assisted reproduction as a practical management tool for felids is the ability to completely control (downregulate and stimulate) ovarian activity. Among non-domestic felids, AI is most successful in the cheetah, a species that inexplicably becomes anoestrus at random times during the year (Wildt *et al*. 1993; Brown *et al*. 1996). A possible reason for this relatively high pregnancy rate is reproductive quiescence at the onset of eCG+hCG treatment, allowing the ovaries to be more consistently responsive to the exogenous gonadotrophins. The snow leopard presents a fascinating comparison and paradox. Our only pregnancy resulting from AI occurred during the non-breeding season, which also is a time when the species is least sensitive to eCG+hCG. The relatively quiescent state of the ovaries before administration of eCG+hCG may have allowed a rebound effect that produced nine fresh ovulations and resulted in a pregnancy. Yet, even this female produced only a single cub, whereas snow leopards typically produce litters of two or three, which could mean that gonadotrophin administration recruits poor or sub-quality follicles and/or oocytes. Multiple CL and small litter sizes after laparoscopic AI are observed consistently in felid species (Howard *et al*. 1992*a*, 1992*b*; Donoghue *et al*. 1993; Barone *et al*. 1994; Swanson *et al*. 1996*a*). This, and pregnancy success after AI in felids, appear unrelated to sperm number or even quality. Litters of cheetahs have been produced using fresh or cryopreserved–thawed spermatozoa, even though $>70\%$ of ejaculated cheetah spermatozoa are malformed. In the present study, the snow leopard conceiving after AI was inseminated with only 9×10^6 motile spermatozoa, whereas other females received up to 68×10^6 motile cells.

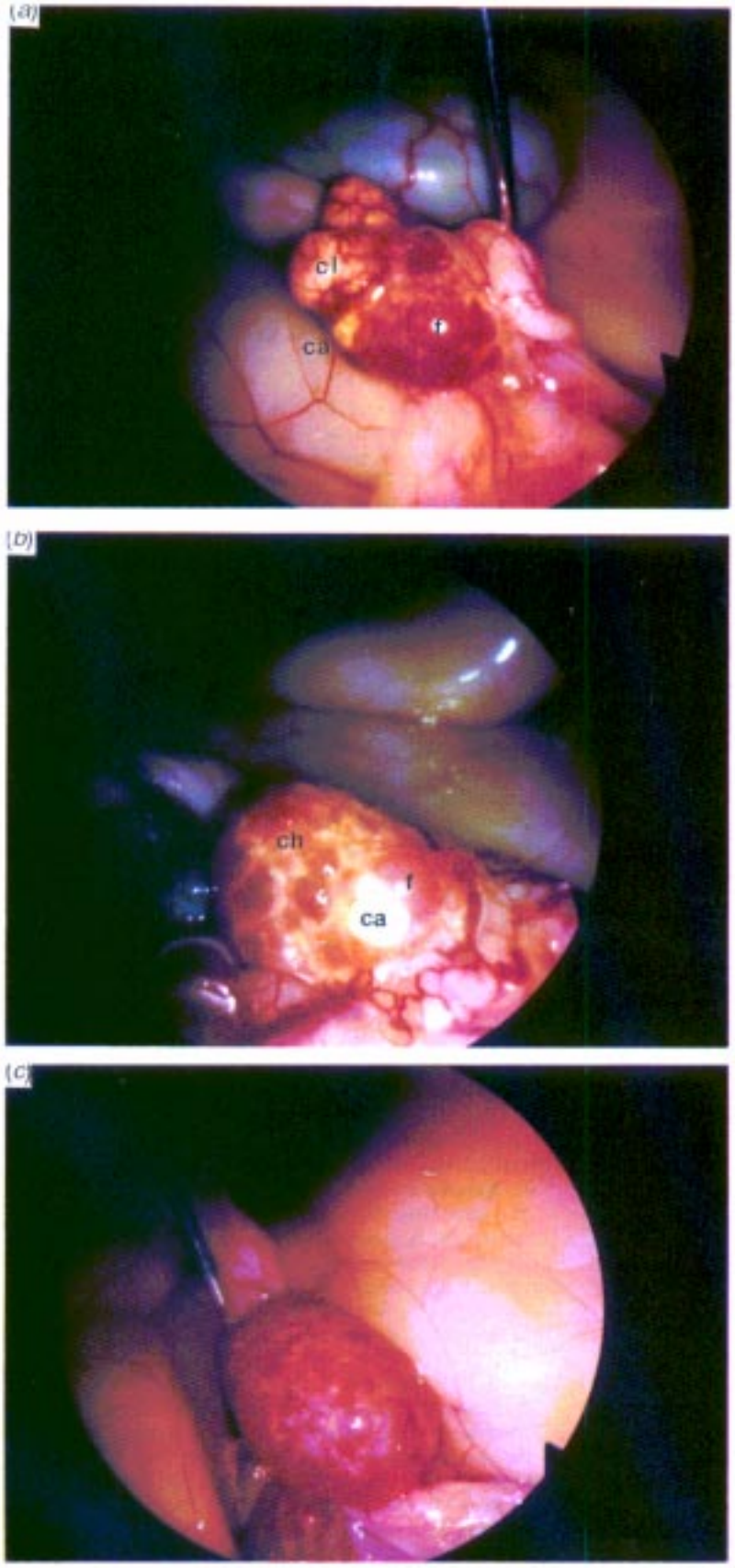

Plate I. Laparoscopic view of snow leopard ovaries following treatment with eCG+hCG 45–50 h after administration of hCG. (*a and b*) During the post-breeding season, ovaries frequently contained unovulated follicles (f) that were mature in appearance, yellow corpora albicantia (ca), fresh corpora haemorrhagica (ch), and, occasionally, well-organized corpora lutea (cl) that were older in appearance. *(c)* During the non-breeding and pre-breeding seasons, most ovaries contained only multiple-raised, vascular follicles and no ch or cl. eCG, equine chorionic gonadotrophin; hCG, human chorionic gonadotrophin.

Although reproductive technologies will be useful for optimizing genetic diversity in zoo-maintained reservoir populations of endangered species, such techniques will become efficient only when the basic biology of a given species is thoroughly understood. The present study has provided an example of how natural effects on reproductive function (seasonality) can influence ovarian sensitivity to exogenous gonadotrophins. We now know that this factor must be considered in designing a consistently effective assisted reproduction strategy for the snow leopard and probably other seasonal felids. Regardless, it was encouraging that treatment with exogenous gonadotrophins combined with AI could be used to override the natural (seasonal-induced) down-regulation of reproductive function to produce a pregnancy and offspring.

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References

Barone, M. A., Wildt, D. E., Byers, A. P., Roelke, M. E., Glass, C. M., and Howard, J. G. (1994). Gonadotrophin dose and timing of anaesthesia for laparoscopic artificial insemination in the puma (*Felis concolor*). *J. Reprod. Fertil.* **101**, 103–8.

Bavister, B. D., Dees, C., and Schultz, R. D. (1986). Refractoriness of rhesus monkeys to repeated ovarian stimulation by exogenous gonadotropins is caused by nonprecipitating antibodies. *Am. J. Reprod. Immunol.* **11**, 11–16.

Blomqvist, L. (Ed.) (1990). 'International Pedigree Book of Snow Leopards: *Panthera uncia*.' pp. 169–253. (Helsinki Zoo: Finland.)

Bronson, F. H. (1988). Seasonal regulation of reproduction in mammals. In 'The Physiology of Reproduction'. (Ed. E. Knobil and J. Neill.) pp. 1831–73. (Raven Press: New York.)

Brown, J. L., Wasser, S. K., Wildt, D. E., and Graham, L. H. (1994). Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol. Reprod.* **51**, 776–86.

Brown, J. L., Wildt, D. E., Graham, L. H., Byers, A. P., Collins, L., Barrett, S., and Howard, J. G. (1995). Natural *versus* chorionic gonadotropin-induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroid analysis. *Biol. Reprod.* **53**, 93–102.

Brown, J. L., Wildt, D. E., Wielebnowski, N., Goodrowe, K. L., Graham, L. H., Wells, S., and Howard, J. G. (1996). Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. *J. Reprod. Fertil.* **106**, 337–46.

Chakraborty, P. K., Wildt, D. E., and Seager, S. W. J. (1978). Serum luteinizing hormone and ovulatory response to luteinizing hormone-releasing hormone in the estrous and anestrous domestic cat. *Lab. Anim. Sci.* **28**, 301–7.

Concannon, P., Hodgson, B., and Lein, D. (1980). Reflex LH release in estrous cats following single and multiple copulations. *Biol. Reprod.* **23**, 111–17.

Dekel, N., and Kraicer, P. F. (1978). Induction *in vitro* of mucification of rat cumulus oophorus by gonadotrophins and adenosine 3′,5′-monophosphate. *Endocrinology* **102**, 1797–802.

Donoghue, A. M., Johnston, L. A., Seal, U. S., Armstrong, D. L., Tilson, R. L., Wolff, P., Petrini, K., Simmons, L. G., Gross, T., and Wildt, D. E. (1990). *In vitro* fertilization and embryo development *in vitro* and *in vivo* in the tiger (*Panthera tigris*). *Biol. Reprod.* **43**, 733–44.

Donoghue, A. M., Johnston, L. A., Brown, J. L., Munson, L., and Wildt, D. E. (1992). Influence of gonadotropin treatment interval on follicular maturation *in vitro* fertilization, circulating steroid concentrations, and subsequent luteal function in the domestic cat. *Biol. Reprod.* **46**, 972–80.

Donoghue, A. M., Johnson, L. A., Armstrong, D. L., Simmons, L. G., and Wildt, D. E. (1993). Birth of a Siberian tiger cub (*Panthera tigris altaicia*) following laparoscopic intrauterine artificial insemination. *J. Zoo Wildl. Med.* **24**, 185–9.

Goodrowe, K. L., and Wildt, D. E. (1987). Ovarian response to human chorionic gonadotropin or gonadotropin releasing hormone in cats in natural or induced estrus. *Theriogenology* **27**, 811–17.

Goodrowe, K. L., Wall, R. J., O' Brien, S. J., Schmidt, P. M., and Wildt, D. E. (1988). Developmental competence of domestic cat follicular oocytes after fertilization *in vitro*. *Biol. Reprod.* **39**, 355–72.

Greenwald, G. S. (1970). Development of ovulatory refractoriness in the rabbit to cyclic injections of human chorionic gonadotropin. *Fertil. Steril.* **21**, 163–8.

Howard, J. G., Bush, M., and Wildt, D. E. (1986). Semen collection, analysis, and cryopreservation in nondomestic mammals. In 'Current Therapy in Theriogenology'. (Ed. D. A. Morrow.) pp. 1047–53. (W. B. Saunders: Philadelphia.)

Howard, J. G., Donoghue, A. M., Barone, M. A., Goodrowe, K. L., Blumer, E., Snodgrass, K., Starnes, D., Tucker, M., Bush, M., and Wildt, D. E. (1992*a*). Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (*Acinonyx jubatus*). *J. Zoo Wildl. Med.* **23**, 288–300.

Howard, J. G., Barone, M. A., Donoghue, A. M., and Wildt, D. E. (1992*b*). The effect of pre-ovulatory anaesthesia on ovulation in laparoscopically inseminated domestic cats. *J. Reprod. Fertil.* **96**, 175–86.

Howard, J. G., Byers, A. P., Brown, J. L., Barrett, S. J., Evans, M. Z., Schwartz, R. J., and Wildt, D. E. (1996). Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (*Neofelis nebulosa*). *Zoo Biol.* **15**, 55–70.

Jainudeen, M. R., Hafez, E. S. E., Gollnick, P. D., and Moustafa, L. A. (1966). Antigonadotropins in the serum of cows following repeated therapeutic pregnant mare serum injections. *Am. J. Vet. Res.* **27**, 669–75.

Johnston, L. A., Armstrong, D. L., and Brown, J. L. (1994). Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*). *J. Reprod. Fertil.* **102**, 229–36.

Licht, P., Bona-Gallo, A., Aggarwal, B. B., Farmer, S. W., Castelino, J. B., and Papkoff, H. (1979). Biological and binding activities of equine pituitary gonadotropins and pregnant mare serum gonadotropin. *J. Endocrinol.* **83**, 311–22.

Lipner, H. (1988). Mechanism of mammalian ovulation. In 'The Physiology of Reproduction'. (Eds. E. Knobil and J. Neill.) pp. 447–88. (Raven Press: New York.)

Pope, C. E., Keller, G. L., and Dresser, B. L. (1993). *In vitro* fertilization in domestic and non-domestic cats including sequences of early nuclear events, development *in vitro*, cryopreservation, and successful intra- and interspecies embryo transfer. *J. Reprod. Fertil.* (Suppl.) **47**, 189–201.

Roth, T. L., Howard, J. G., Donoghue, A. M., Swanson, W. F., and Wildt, D. E. (1994). Function and culture requirements of snow leopard (*Panthera uncia*) spermatozoa *in vitro*. *J. Reprod. Fertil.* **101**, 563–9.

Roth, T. L., Swanson, W. F., and Wildt, D. E. (1996). Snow leopard (*Panthera uncia*) spermatozoa are sensitive to alkaline pH, but motility *in vitro* is not influenced by protein or energy supplements. *J. Androl.* **17**, 558–66.

Schmidt, A. M., Hess, D. L., Schmidt, M. J., and Lewis, C. R. (1993). Serum concentrations of oestradiol and progesterone and frequency of sexual behaviour during the normal oestrous cycles in the snow leopard (*Panthera uncia*). *J. Reprod. Fertil.* **98**, 91–5.

Scott, P. P., and Lloyd-Jacob, M. A. (1959). Reduction in the anoestrus period of laboratory cats by increased illumination. *Nature (Lond.)* **184**, 2022.

Seal, U. S., Plotka, E. D., Smith, J. D., Wright, F. H., Reindl, N. J., Taylor, R. S., and Seal, M. F. (1985). Immunoreactive luteinizing hormone, estradiol, progesterone, testosterone, and androstenedione levels during the breeding season and anestrus in Siberian tigers. *Biol. Reprod.* **32**, 361–8.

Swanson, W. F., Roth, T. L., Brown, J. L., and Wildt, D. E. (1995*a*). Relationship of circulating steroid hormones, luteal luteinizing hormone receptor and progesterone concentration, and embryonic mortality during early embryogenesis in the domestic cat. *Biol. Reprod.* **53**, 1022–9.

Swanson, W. F., Horohov, D. W., and Godke, R. A. (1995*b*). Production of exogenous gonadotrophin-neutralizing immunoglobulins in cats after repeated eCG–hCG treatment and relevance for assisted reproduction in felids. *J. Reprod. Fertil.* **105**, 35–41.

Swanson, W. F., Howard, J. G., Roth, T. L., Brown, J. L., Alvarado, T., Burton, M., Starnes, D., and Wildt, D. E. (1996*a*). Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen–thawed spermatozoa in ocelots (*Felis pardalis*). *J. Reprod. Fertil.* **106**, 87–94.

Swanson, W. F., Roth, T. L., Graham, K., Horohov, D. W., and Godke, R. A. (1996*b*). Kinetics of the humoral immune response to multiple treatments with exogenous gonadotropins and relation to ovarian responsiveness in domestic cats. *Am. J. Vet. Res.* **57**, 302–7.

Testart, J., Frydman, R., DeMouzon, J., Lassalle, B., and Belaisch, J. C. (1983). A study of factors affecting the success of human fertilization *in vitro*. I. Influence of ovarian stimulation upon the number and condition of oocytes collected. *Biol. Reprod.* **28**, 415–24.

Wharton, D. (1991). 'North American Snow Leopard (*Panthera uncia*) Studbook.' (New York Zoological Park: New York.)

Wildt, D. E., and Mellen, J. D. (Eds) (1994). 'Felid Action Plan: Conservation Assessment and Management Plan.' (Metro Washington Park Zoo: Oregon.) 116 pp.

Wildt, D. E., Kinney, G. M., and Seager, S. W. J. (1978). Gonadotropin-induced reproductive cyclicity in the domestic cat. *Lab. Anim. Sci.* **28**, 301–7.

Wildt, D. E., Seager, S. W. J., and Chakraborty, P. K. (1980). Effect of copulatory stimuli on ovulatory and serum luteinizing hormone response in the cat. *Endocrinology* **107**, 1212–17.

Wildt, D. E., Chan, S. Y. W., Seager, S. W. J., and Chakraborty, P. K. (1981). Ovarian activity, circulating hormones, and sexual behavior in the cat. I. Relationships during the coitus-induced luteal phase and the estrous period without mating. *Biol. Reprod.* **25**, 15–28.

Wildt, D. E., Monfort, S. L., Donoghue, A. M., Johnston, L. A., and Howard, J. G. (1992). Embryogenesis in conservation biology — or how to make an endangered species embryo. *Theriogenology* **37**, 161–84.

Wildt, D. E., Brown, J. L., Bush, M., Barone, M. A., Cooper, K. A., Grisham, J., and Howard, J. G. (1993). Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: the benefits of physiological surveys for strategic planning. *Zoo Biol.* **12**, 45–80.

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