Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season

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The domestic cat experiences circannual variations in ovarian activity and intrafollicular oocyte quality. One result is poor nuclear and cytoplasmic maturation during in vitro maturation (IVM) conducted during the annual non-breeding season (July through November). In an attempt to overcome this seasonal phenomenon immature oocytes were collected from July through November and cultured in a conventional IVM medium (IVM1) or in IVM1 supplemented with different FSH concentrations and antioxidant (ascorbic acid or cysteine). Nuclear status of oocytes was assessed after IVM or IVF. Embryo stage and blastocyst quality were evaluated after 7 days of in vitro culture. Although the addition of antioxidant alone had no effect, the presence of 10 µg FSH ml⁻¹ improved nuclear maturation (75.4 ± 4.1% versus 48.7 ± 8.8% in IVM1; P < 0.05) and fertilization success (47.9 ± 3.2% versus 35.0 ± 5.1% in IVM1; P < 0.05). Furthermore, developmental competence of fertilized oocytes was enhanced (P < 0.05) only in the presence of ascorbic acid (30.6 ± 6.7%) or cysteine (33.6 ± 5.1%) compared with IVM1 (8.1 ± 8.8%). Consequently, blastocyst yield (17% of total oocytes cultured) was highest when oocytes were matured in medium containing higher FSH concentration and antioxidants. The results of this study demonstrate that meiotic and developmental competences are inherent to the immature cat oocyte collected during the non-breeding season. However, appropriate mechanisms (perhaps seasonal variation in FSH receptors or lack of antioxidant capacity of the cumulus–oocyte complex) are inadequate during this period of gonadal quiescence. Regardless, this compromised oocyte function during the non-breeding season can be overridden by altering in vitro culture conditions to include supplemental FSH and antioxidant.

Introduction

In domestic cats, in vitro maturation (IVM) studies of intraovarian oocytes have been conducted since 1989 (Johnston et al., 1989). The success of various IVM conditions has been based on nuclear maturation and successful embryo development after IVF (Johnston et al., 1989; Schramm and Bavister, 1995; Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Karja et al., 2002a), intracytoplasmic sperm injection (Gomez et al., 2000; Bogliolo et al., 2001) and nuclear transfer (Skrzyszowska et al., 2002; Gomez et al., 2003; Kitiyanant et al., 2003), including the production of live offspring after embryo transfer (Pope et al., 1997, 2001). In addition, it is well established that the use of strict selection criteria for freshly collected oocytes assists in ensuring higher rates of subsequent IVM and IVF success (Wolfe and Wildt, 1996; Wood and Wildt, 1997a). It is clear and unsurprising that there are vast differences in the quality of oocytes recovered from ovarian tissue (Wood and Wildt, 1997a), which no doubt explains some of the variation in success of IVM across laboratories. Under optimal culture conditions and using grade I oocytes, 59–80% of immature oocytes, on average, achieve nuclear maturation (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Gomez et al., 2000; Bogliolo et al., 2001; Otoi et al., 2001). When inseminated with conspecific spermatozoa, 45–50% of cultured oocytes are fertilized and develop in vitro to the blastocyst stage (Wolfe and Wildt, 1996; Wood and Wildt, 1997a; Spindler and Wildt, 1999; Freistedt et al., 2001; Karja et al., 2002a).

Spindler and Wildt (1999) first reported the marked effect of season on IVM success in cat oocytes. When oocytes were recovered from cat ovaries during the traditional non-breeding season in North America (from July through November), the incidence of nuclear maturation was approximately 20% and no embryos developed to the blastocyst stage after fertilization. Other studies have confirmed this effect associated with quiescent ovarian
activity and reduced follicular growth, including studies in other geographic regions and at times of the year that differ from the North American breeding season (Freistedt et al., 2001). FSH is known to be an important supplement for IVM success when using oocytes collected during the breeding season (Johnston et al., 1989; Schramm and Bavister, 1995; Wood et al., 1995). For this reason, Spindler and Wildt (1999) speculated that either lower circulating FSH concentrations or fewer FSH receptors may be contributing to reduced IVM efficiency during the non-breeding season, possibly by adversely affecting oocyte quality. It remains unclear whether the poor nuclear maturation observed during the seasonal quiescent period of the cat is linked to a poor meiotic competence, to a failure to meet a threshold concentration of FSH to induce nuclear maturation, or to both factors.

As FSH receptors are located on cumulus cells, the induction of nuclear maturation must be mediated by these cells, which coincidentally also play a key role in cytoplasmic maturation (Shimada et al., 2002; Tanghe et al., 2002; Webb et al., 2002). Cytoplasmic maturation is generally considered to be the ability of an oocyte to support monospermic fertilization, pronuclear formation and early embryo development (Mermillod et al., 1999; Tanghe et al., 2002). A major cytoplasmic factor regulating sperm decondensation and chromatin remodelling is the reduced form of glutathione (GSH) (de Matos et al., 1997). As a result of an increase in oocyte GSH content, the cumulus cells maintain the redox state while simultaneously protecting the oocyte against oxidative stress (de Matos et al., 1997; Sutovsky and Schatten, 1997). Furthermore, high GSH content in the oocyte is also required for supporting successful embryo development in cattle (de Matos et al., 1997; de Matos and Furnus, 2000). It is also known that supplementing pig oocytes during IVM with cysteine, a precursor of GSH synthesis, enhances cytoplasmic maturation (Yoshida et al., 1993; Abeydeera et al., 1999). Likewise, in rats and mice another extracellular antioxidant, ascorbic acid, protects cumulus cells against apoptosis and improves maturation (Behrman et al., 1996; Takami et al., 1999; Eppig et al., 2000). Acquisition of oocyte competence may be lost because of oxidative stress during IVM. However, the impact of antioxidants on IVM during the non-breeding season in carnivore species, including the domestic cat, is unknown.

The aim of the present study was to determine whether the poor nuclear and cytoplasmic maturation observed during the non-breeding season in the cat is related to decreased sensitivity to FSH and increased oxidative stress. This possibility was examined by characterizing the influence of supplemental FSH (at different concentrations) and antioxidants (ascorbic acid or cysteine) on in vitro nuclear and cytoplasmic maturation of intraovarian cat oocytes recovered during the non-breeding season.

Materials and Methods

Oocyte collection and IVM

Ovaries from the adult domestic cat (Felis catus) were collected during the non-breeding season (July to November) from local veterinary clinics and transported in PBS at 4°C to the laboratory within 6 h of ovariectomy. Immature oocytes were then recovered by slicing the ovaries with a scalpel blade in Hepes-buffered minimum essential medium (H-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 1.0 mmol pyruvate l−1, 2.0 mmol L-glutamine l−1, 100.0 iu penicillin ml−1, 100.0 mg streptomycin ml−1 and 4.0 mg BSA ml−1 (Sigma Chemical Co, St Louis, MO). Only grade I immature oocytes (with homogeneous dark cytoplasm, surrounded by several layers of compacted cumulus) (Wood and Wildt, 1997a) were used. These oocytes were selected and pooled before they were cultured in different IVM media for 32 h in 50 µl microdrops (10 oocytes per microdrop) under mineral oil (38.5°C in air with 5% CO2).

IVM control medium (designated IVM1) was used in our laboratory (Wolfe and Wildt, 1996; Spindler and Wildt, 1999) and comprised MEM (Gibco) supplemented with 1.0 mmol L-glutamine l−1, 1.0 mmol pyruvate l−1, 100.0 iu penicillin ml−1, 100.0 mg streptomycin ml−1, 4.0 mg BSA ml−1, 1.0 µg FSH ml−1 (1.64 iu ml−1; NIDDK-ovine FSH-18; National Hormone and Pituitary Program, Rockville, MD), 1.0 µg LH ml−1 (1.06 iu ml−1; NIDDK-OLH-25; National Hormone and Pituitary Program) and 1.0 µg oestradiol ml−1 (Sigma).

In Expt 1, oocytes were cultured in IVM1 supplemented with different FSH concentrations: 1 (control), 5, 10 or 15 µg ml−1. After 32 h of culture, oocytes were fixed in ethanol overnight at room temperature and stained with 10 µg Hoechst 33342 ml−1 (Sigma) (Comizzoli et al., 2001). The meiotic stage of the oocytes was assessed by epifluorescence microscopy (Olympus BX 41, Olympus Corporation, Melville, NY) (Johnston et al., 1989; Wood et al., 1995; Wolfe and Wildt, 1996; Spindler and Wildt, 1999).

The results of Expt 1 were used to design the composition of the IVM media used in Expts 2 and 3. For these studies, oocytes were cultured in IVM1 supplemented with either 10.0 µg FSH ml−1 (FSH+), 0.5 mmol ascorbic acid l−1 (AS; Sigma), 0.13 mmol cysteine l−1 (CYS; Sigma), 10.0 µg FSH ml−1 + 0.5 mmol ascorbic acid l−1 (FSH+/AS) or 10.0 µg FSH ml−1 + 0.13 mmol cysteine l−1 (FSH+/CYS). The dosage of the two antioxidants was based on previous studies in pig (Abeydeera et al., 1999) and mouse oocytes (Eppig et al., 2000).

In vitro fertilization and development

IVF was performed in Expts 2 and 3 using a standard protocol originally developed in our laboratory for oocytes matured in vivo (Johnston et al., 1991a) and later
for oocytes matured in vitro (Wood et al., 1995). The most recent description of the protocol has been provided by Spindler and Wildt (1999). Briefly, frozen–thawed motile spermatozoa from a single sperm donor were selected by swim-up processing (Goodrowe et al., 1988) in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 25 mmol Hepes l⁻¹, 1 mmol pyruvate l⁻¹, 2 mmol glucose l⁻¹, 100 iu penicillin ml⁻¹, 100 mg streptomycin ml⁻¹ and 5% (v/v) fetal calf serum (complete Ham's medium with Hepes). Oocytes were inseminated with 5 × 10⁵ motile spermatozoa ml⁻¹ in 50 µl microdrops of complete Ham's medium without Hepes under equilibrated mineral oil at 38.5 °C in air with 5% CO₂. Some of the oocytes (n=12 per IVM condition) were incubated without spermatozoa to assess the incidence of parthenogenetic activation. At 16 h after insemination, cumulus cells were removed by vortexing at maximum setting (Vortex-Genie, Scientific Industries, Bohemia, NY) for 2 min. For Expt 2, presumptive zygotes were fixed and stained as described by Comizzoli et al. (2001) to assess pronuclear formation. Pronucleus morphology was recorded according to its size and localization within the cytoplasm (Hewitson et al., 2000). For Expt 3, presumptive zygotes were cultured in vitro for 7 days in complete Ham's F-10 (38.5 °C in air with 5% CO₂). In vitro development was observed on day 6 and again on day 7 (day 0 corresponding to the day after the in vitro insemination). Embryos were then fixed and stained as described by Comizzoli et al. (2001) to determine the developmental stage and number of blastomeres.

**Experimental design and statistical analysis**

Grade I immature oocytes from different ovaries were pooled before random and equal distribution across different IVM conditions. In Expt 1, incidence of nuclear maturation was defined as the number of oocytes at the telophase I or the metaphase II stage relative to the total number of oocytes cultured in vitro. Oocytes that arrested at the germinal vesicle breakdown (GVBD) stage or that only progressed to the metaphase I stage were considered immature (Wolfe and Wildt, 1996; Spindler and Wildt, 1999). Oocytes with fragmented cytoplasm or without chromatin were considered degenerated. In Expt 2, oocytes were considered fertilized when two pronuclei were present in the cytoplasm. The number of unfertilized oocytes in metaphase II was also assessed in this experiment to determine the incidence of nuclear maturation at the time of in vitro insemination. In Expt 3, the percentage of cleaved embryos and blastocyst yield were calculated relative to the total number of oocytes cultured in vitro. Developmental competence was defined as the number of blastocysts produced relative to the total number of cleaved embryos. All experiments were replicated three or four times on different days with different batches of oocytes. Average values were expressed as mean ± SD. Percentage data were transformed using arcsin transformation before analysis. Comparison between IVM condition and among replicates were analysed by ANOVA and Tukey's multiple comparison testing (SigmaStat, SPSS, Chicago, IL).

**Results**

**Ovarian activity and oocyte recovery during the non-breeding season**

Of the 414 ovaries collected from July through November for this study, none contained corpora lutea or corpora haemorrhagica. Furthermore, all antral follicles observed were < 1 mm in diameter. The mean number of grade I oocytes recovered per ovary was 2.7 ± 0.7.

**Effects of FSH on oocyte nuclear maturation**

In this experiment designed to assess the impact of FSH concentration, an examination of oocytes before fixation revealed no apparent gross influence of gonadotrophin concentration on expansion of the surrounding cumulus mass. However, in the presence of 10 or 15 µg FSH ml⁻¹, the percentage of oocytes in telophase I or metaphase II was significantly higher (P < 0.05), and the percentage of oocytes undergoing GVBD and reaching metaphase I was significantly lower (P < 0.05) compared with oocytes in the presence of 1 or 5 µg FSH ml⁻¹ (Fig. 1). The incidence of oocyte degeneration was not significantly different among treatments. As nuclear maturation was significantly enhanced in the presence of...
10 μg FSH ml⁻¹ and was not further increased by 15 μg FSH ml⁻¹, subsequent experiments were carried out in the presence of 10 μg FSH ml⁻¹.

Effect of higher FSH concentration in the presence or absence of antioxidant on oocyte nuclear maturation and fertilization

Expt 2 replicated the findings of Expt 1 in that nuclear maturation was positively influenced (P < 0.05) by the higher FSH concentration (FSH+ versus IVM1; Fig. 2). This improved maturation was also noted in the presence of 10 μg FSH ml⁻¹ combined with ascorbic acid (FSH+/AS) or cysteine (FSH+/CYS) (Fig. 2). As might be expected, the percentages of oocytes in GVBD and at metaphase II were significantly lower (P < 0.05) after IVM in the presence of higher FSH concentration and antioxidant (FSH+/AS, CYS, FSH+/CYS; Fig. 4). There was no enhancement effect by the presence of either ascorbic acid or cysteine, and neither antioxidant alone promoted cleavage. The percentage of blastocysts relative to the total number of oocytes was significantly higher (P < 0.05) after IVM in the presence of the antioxidants and with or without the higher FSH concentration (AS, CYS, 60.0 ± 5.2%; FSH+/AS, 63.5 ± 3.9%; FSH+/CYS, 62.8 ± 5.9%) compared with IVM1 only (32.5 ± 2.1%) or the higher concentration of FSH in the absence of antioxidant (FSH+, 32.5 ± 3.8%). For the last two treatments, pronuclei were generally smaller and more distantly spaced at 16 h after insemination (Fig. 3b).

Effect of higher FSH concentration in the presence or absence of antioxidant on embryo cleavage, developmental competence and blastocyst yield

In Expt 3, the same proportion of fertilized oocytes progressed to the first cleavage stage regardless of the IVM treatment. Compared with the IVM1 control, the percentage of cleaved embryos was higher (P < 0.05) when IVM was performed in the presence of 10 μg FSH ml⁻¹ with or without the antioxidants (FSH+, FSH+/AS, FSH+/CYS; Fig. 4). There was no enhancement effect by the presence of either ascorbic acid or cysteine, and neither antioxidant alone promoted cleavage. The percentage of blastocysts relative to the number of cleaved embryos (developmental competence) was always significantly higher (P < 0.05) after IVM in the presence of the antioxidants and with or without the higher FSH concentration (AS, CYS, FSH+/AS, FSH+/CYS; Fig. 4). The presence of higher FSH concentration and antioxidant during oocyte IVM (FSH+/AS, FSH+/CYS) increased (P < 0.05) blastocyst yield (number of blastocysts relative to the total number of oocytes) compared with all other IVM treatments (that is, IVM1, FSH+, AS, CYS; Fig. 4).

The developmental stages of embryos across treatments are shown (Fig. 5). More embryos (P < 0.05) developed beyond the 2- to 8-cell stage after IVM in all supplemented media compared with IVM1. Embryo development was mainly arrested at the 8–16-cell stage (P < 0.05; Figs 5, 6b) when the original
oocytes were cultured in IVM1 or supplemented with higher FSH alone. In contrast, the presence of the antioxidant with or without the higher FSH concentration enhanced (P < 0.05; Fig. 5) the proportion of resulting morulae and blastocysts (Fig. 6c,e). On day 6 of culture in vitro, blastocyst formation was observed only in the four treatments containing antioxidant. The morphological benefits of supplemental ascorbic acid or cysteine are shown (Fig. 6). In addition to more uniform blastomeres and the absence of pycnotic nuclei at different developmental stages, the mean number of cells for blastocysts was higher (P < 0.05) after IVM in the presence of antioxidant (88.4 ± 10.3 cells, combined average for AS, CYS, FSH+/AS, FSH+/CYS) compared with the absence of antioxidant (59.6 ± 8.8 cells, combined average for IVM1 and FSH+).

Discussion

The present study provides important insight into two potential causes of compromised IVM/IVF capacity in intraovarian cat oocytes collected during the traditional non-breeding season. First, it was discovered that the...
conventional IVM medium for this species benefited from a higher FSH concentration that, in turn, increased both nuclear maturation and fertilization success. As there was no difference in the results from using 10 or 15 μg FSH ml⁻¹, there appeared to be a threshold effect after which there was no further advantage of more gonadotrophin in the culture medium. Second, supplementing the IVM medium with the antioxidants ascorbic acid or cysteine had a particularly beneficial impact on advanced embryo development in vitro, especially from the 8–16 cell to the blastocyst stage. Both of these findings were significant in helping to speculate about arrested or dysfunctional mechanisms in oocytes during intervals of reproductive acyclicity in this species. Finally, the results of the present study demonstrate that simple supplements to conventional IVM conditions circumvented the maturational and developmental compromise that, in turn, resulted in healthy embryo numbers near those reported in studies conducted during the breeding season (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Freistedt et al., 2001; Karja et al., 2002a).

More specifically, the percentages of cat oocytes reaching metaphase II in the control group of the present study were comparable using the same IVM medium in our laboratory and 1 μg FSH ml⁻¹ (previous study, breeding season) (Spindler and Wildt, 1999) versus 10 μg FSH ml⁻¹ (the present study, non-breeding season). The findings of the present study were similar to percent-ages of oocytes reaching metaphase II or undergoing degeneration after IVM using oocytes collected during the breeding season by other laboratories (Pope et al., 1998; Gomez et al., 2000; Bogliolo et al., 2001; Otoi et al., 2001; Karja et al., 2002a). Culturing in the presence of higher concentrations of FSH also promoted meiotic resumption from both the GVBD stage and the metaphase I stage. Although increased FSH concentration in the culture medium has been shown to enhance nuclear maturation in bovine oocytes (Izadyar et al., 1998; Choi et al., 2001), the present study was the first to report that supplementation with high concentrations of gonadotrophins enhanced oocyte function in vitro during a seasonally related interval of compromised gamete function. It was apparent that immature cat oocytes recovered during the non-breeding season had full meiotic competence, but required supplemental FSH to advance the maturation process. Although circannual FSH patterns have not been studied in the cat, it is well known that GnRH and FSH secretion patterns decrease in other seasonally breeding mammals (Lehman et al., 1997). In addition, treating the squirrel monkey (Yeoman et al., 1994) or ewe (Stenbak et al., 2001) (the whole animal) with FSH during seasonal periods of ovarian inactivity is known to improve oocyte nuclear maturation and developmental competence. Particularly interesting from the present study is that FSH priming in cats can be delayed until the oocytes are placed in culture. The mechanism by which higher FSH exerts an effect in culture remains unknown, although a plausible explanation could be a circumvention of a naturally depressed number of functional FSH receptors. Supplementing FSH may largely allow targeting all available receptors to facilitate communication between the somatic cells and the oocyte itself (Eppig et al., 2000; Tanghe et al., 2002; Webb et al., 2002). Concentrations of FSH are difficult to compare among IVM studies in the domestic cat from 0.02 iu bovine FSH ml⁻¹ (Freistedt et al., 2001) to 1.0 μg FSH ml⁻¹ (Schramm and Bavister, 1995) because of different purity and origin of the hormones. However, the highly purified FSH used in the present study did not contain any contaminants that might have influenced the nuclear maturation and the cumulus expansion.

Although the higher FSH concentration promoted fertilization success similar to that measured during the breeding season (Spindler and Wildt, 1999; Freistedt et al., 2001; Karja et al., 2002a), the addition of only ascorbic acid or cysteine to the IVM medium failed to enhance either nuclear maturation or fertilization. The latter observation is consistent with previous reports for pig (Yoshida et al., 1993; Tatemoto et al., 2001), rat (Takami et al., 1999) and goat oocytes (Mayor et al., 2001). However, these antioxidants profoundly influenced the morphology of cat pronuclei at 16 h after insemination, indicative of a more rapid and improved chromatin remodelling which was probably due to enhanced cytoplasmic maturation (Yoshida et al., 1993; Collas, 1998; Tatemoto et al., 2001).

As more oocytes exposed to higher FSH concentration underwent successful maturation in culture in the present study, more fertilized and cleaved. As others have already asserted (Schramm and Bavister, 1995; Wood et al., 1995), the benefits of this gonadotrophin are exerted via its impact on nuclear maturation rather than improved fertilization and developmental competence. After 7 days in culture, the proportion of embryos developing beyond the 2- to 8-cell stage was increased markedly in all supplemented media. This finding was not influenced by parthenogenetic activation. Although IVM culture supplementation influenced nuclear and cytoplasmatic maturation, the percentage of blastocysts eventually developing was 10% less than that reported in comparable studies conducted during the breeding season (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Freistedt et al., 2001; Karja et al., 2002a). Nonetheless, blastocyst quality in terms of developmental pace in vitro, total number and uniformity of blastomeres produced was better in the presence, rather than the absence, of either antioxidant. Furthermore, these traits were similar to those reported for IVM/IVF oocytes collected from cats during the breeding season (Spindler and Wildt, 2002; Karja et al., 2002b).

The benefits of adding ascorbic acid to IVM culture conditions have been reported for pigs (Tatemoto et al., 2001). Extracellular cysteine supplementation too has
been shown to enhance embryo development in bovine (de Matos et al., 1997; de Matos and Furnus, 2000) and pig (Yoshida et al., 1993) oocytes. It is possible that the maternal–zygotic transition and the onset of embryonic transcription (which occurs at the 5- to 8-cell stage in cats; Hoffert et al., 1997) was improved in the presence of antioxidant because we observed more embryos developed beyond the 8–16-cell stage when IVM medium contained ascorbic acid or cysteine. Clearly, the presence of these antioxidants promoted developmental competence. As ascorbic acid and cysteine provided similar beneficial effects on early chromatin remodelling (either by protecting cumulus cells via the former or improving GSH synthesis via the latter), it appeared that these early stage, immature oocytes were particularly sensitive to oxidative stress during the non-breeding season. The antioxidant capacity of the oocyte could be decreased during this period because of a depletion of GSH synthesis or an altered metabolism (Sutovsky and Schatten, 1997; Spindler et al., 2000). It is highly likely that the beneficial effect observed in this study was induced by a positive signal, which then was transferred from cumulus cells to the oocyte (Behman et al., 1996) without any interaction with the FSH concentration. Regardless, although supplemental antioxidant seemed to ensure improved chromatin remodelling (as demonstrated by excellent pronuclear morphology after insemination) and maternal–zygotic transition, embryos arrested at the morula–blastocyst transition. This block is not directly related to IVM conditions or the onset of embryonic transcription, but rather to inadequate culture conditions during the early stages of embryo development (Schramm and Bavister, 1995; Hoffert et al., 1997).

The present study has demonstrated that cat follicles during the non-breeding season (most of which are <1 mm in diameter) contain oocytes that already have meiotic and developmental competence. Furthermore, despite an impressive amount of follicular atresia (approximately 65%) occurring at any given time in this species (Wood et al., 1997b), a significant number of viable oocytes are recoverable and can undergo embryogenesis by altering culture conditions. Nuclear and cytoplasmic maturation benefit from supplemental FSH, and developmental competence is improved by the antioxidants ascorbic acid or cysteine. The exact mechanism responsible for this phenomenon is currently under investigation in our laboratory. Specifically, the seasonal changes in FSH receptor concentration and functionality as well as modulation of GSH contents in the cumulus–oocyte complex are being investigated.

Finally, these studies have application to our parallel efforts to manage small populations of endangered felid species. Some basic studies in these species have been carried out in our laboratory (Johnston et al., 1991b) as well as the laboratories of others (Jewgenow et al., 1997). Wild felid species are especially prone to seasonal breeding patterns to ensure the production of young at times of the year most conducive to offspring survival (Wildt et al., 1998). Thus, the findings of the present study will also be used to continue comparative assessments of reproductive mechanisms among species of the Felidae as well as hopefully to improve IVF–IVF results in zoo-housed, valuable specimens that die unexpectedly during seasonal anoestrus.

This study was supported by a fellowship to P. Comizzoli from the Smithsonian Institution Scholarly Studies Program, and the National Institutes of Health to B. S. Pukazhenthi (K01 RR00135). The authors thank M. Cranfield and B. Whitaker (Maryland Line Animal Rescue) and D. Thornburg (Petworth Animal Hospital) for providing domestic cat ovaries. The authors also thank R. E. Spindler for valuable scientific advice.

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Received 20 June 2003.
First decision 5 August 2003.
Revised manuscript received 18 August 2003.
Accepted 27 August 2003.