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Rescue and Maturation In Vitro of Follicular Oocytes Collected from Nondomestic Felid Species[1]

L. A. JOHNSTON,[3,4,5] A. M. DONOGHUE,[4] S. J. O'BRIEN,[5] and D. E. WILDT[2,4]

National Zoological Park,[4] Smithsonian Institution, Washington, District of Columbia 20008
Laboratory of Viral Carcinogenesis,[5] National Cancer Institute
Frederick, Maryland 21701

ABSTRACT

The potential for rescuing immature oocytes from the ovaries of females of rare felid species which die or undergo medical ovariohysterectomy was evaluated. Ovaries were recovered from 13 species representing 35 individuals in good-to-poor health. Although the majority of females were 10 yr of age or older and in fair-to-poor health, a total of 846 oocytes were recovered of which 608 (71.9%) were classified as fair-to-excellent quality. One hundred of these oocytes were used for initial maturation classification and as parthogenetic controls. Overall, of the 508 fair-to-excellent quality oocytes placed in culture, 164 (32.3%) matured to metaphase II in vitro. For species in which 3 or more individuals yielded oocytes, mean oocyte maturation rates were as follows: 36.2%, tiger; 27.9% leopard; and 8.3%, cheetah. In vitro insemination of oocytes resulted in fertilization (2 polar bodies, 2 pronuclei, or cleavage) rates of 9.1% to 28.6% (leopard) using homologous fresh spermatozoa and 4.0% (lion) to 40.0% (puma) using homologous frozen-thawed spermatozoa. Inseminations using heterologous (domestic cat) spermatozoa also resulted in fertilized oocytes in the tiger, leopard, snow leopard, puma, serval, and Geoffroy's cat (range in fertilization rate, 5.0% for leopard to 46.2% for puma). Cleaved embryos resulted from the insemination of leopard oocytes with homologous sperm (n = 1 embryo) and puma oocytes with domestic cat sperm (n = 3 embryos). These results demonstrate that immature ovarian oocytes from rare felid species can be stimulated to mature in vitro despite an excision-to-culture interval as long as 36 h. These oocytes are capable of fertilization in vitro, although fertilization rates do not approach those achieved in parallel studies in domestic cats. Our observations of successful cross-species sperm-oocyte interaction in vitro (including the production of cleaved embryos) confirms that the oocytes of certain felid species have not developed mechanisms for excluding penetration or fertilization by heterologous felid spermatozoa.

INTRODUCTION

The domestic cat is the only one of 37 felid species not considered endangered or threatened with extinction [1]. For most of these species, the decline of natural populations can be attributed to poaching and human destruction of native ecosystems. As populations decrease, insularization eventually results in subpopulation extinctions caused by detrimental genetic and demographic effects [2]. Long-term survival of many of these species depends on captive breeding and sophisticated management plans that require transporting animals or gametes between isolates to provide artificial corridors for genetic exchange. Reproductive biotechniques including in vitro maturation, in vitro fertilization (IVF), and embryo cryopreservation/transfer could play an important role in managing captive and natural populations as well as in sustaining both genetic and global biodiversity [3, 4].

In vitro maturation of oocytes followed by IVF and embryo transfer have resulted in the birth of live offspring in a variety of laboratory and domestic mammals [5–8]. The ability to mature and then fertilize oocytes in vitro would provide one approach for rescuing genetic material from individuals of rare Felidae species which die or undergo an ovariohysterectomy for medical purposes (i.e., uterine hyperplasia, endometriosis, pyometra) or for population control (contraceptive) purposes. Using the domestic cat as an experimental model, we have initiated in vitro oocyte maturation and fertilization studies [9]. Currently, under optimal conditions, 50–60% of domestic cat follicular oocytes mature in vitro. After insemination and coincubation with homologous sperm, approximately 35% of these oocytes cleave to the 2-cell stage of development.

Based on these results, a cooperative network was established with various zoological institutions throughout North America. The purpose was to obtain ovaries from individual felids that died abruptly or were subjected to ovariohysterectomy or euthanasia. The objectives of this study were 1) to determine the feasibility of recovering viable oocytes from ovaries shipped long distance when the excision-to-culture interval was at least 10 h; and 2) to evaluate the ability of these oocytes to mature and fertilize in vitro using fresh or thawed spermatozoa. As part of a long-term effort to carry out comparative studies of sperm-oocyte interaction in felids [9–18], we also used this opportunity to test the hypotheses that felid oocytes have no mechanism for preventing binding and penetration by sperm from another felid species.

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[2]Correspondence: David E. Wildt, National Zoological Park, Smithsonian Institution, Washington, DC 20008.
[3]Current address: Omaha's Henry Doorly Zoo, 3701 South 10th St., Omaha, NE 68107.

MATERIALS AND METHODS

Ovary Collection and Oocyte Recovery/Maturation

Ovaries representing 13 felid species were opportunistically obtained from 35 females during a 2.5-yr period. On the basis of discussions with the veterinary staff of each institution, the health of each individual was classified subjectively as 1) good (i.e., female was healthy and ovaries were recovered at elective ovariohysterectomy, or female was healthy immediately before an accidental death); 2) fair (i.e., for health purposes a medical ovariohysterectomy was mandatory); or 3) poor (i.e., female either died naturally or was euthanized because of declining health). In cases of ovariohysterectomy or euthanasia, ovaries were removed immediately and stored in cold (4°C) PBS supplemented with 10% serum and antibiotics (0.05 mg/ml penicillin and streptomycin). In instances of natural death, ovaries generally were recovered 6–12 h later and placed immediately in the same PBS medium. Because ovaries were transported from many different sites of origin (ranging from 60–4 200 km from our laboratory), the interval from ovarian removal to oocyte recovery varied from 1–48 h. In most cases, ovarian material was shipped by air express and in all instances maintained at 4°C by packing in insulated transport boxes containing ice packs.

For oocyte recovery, ovaries were placed into Eagle's Minimum Essential Medium (MEM) (water, Irvine Scientific, Irvine, CA; chemicals, Sigma Chemical Co., St. Louis, MO) [6] supplemented with 3 mg/ml BSA (Irvine Scientific) [9]. Oocytes were liberated from follicles (~0.5–3.0 mm in size) by puncturing each ovary repeatedly with a 22-gauge needle. Oocytes were classified according to the exact criteria described previously for the domestic cat [9]: 1) excellent (i.e., medium to darkly pigmented and completely surrounded by cumulus cells and a tight corona radiata); 2) good/fair (i.e., lightly pigmented or mottled with partial cumulus and corona radiata); or 3) degenerate (i.e., oocyte abnormal in shape, pale and lacking cumulus cells or a corona radiata). Degenerate oocytes were discarded, and all others were washed three times in Eagle's MEM before they were placed into culture (the optimal culture system developed previously for the domestic cat [9] was used). In brief, oocytes were placed in 2.5 ml MEM supplemented with 3 mg/ml BSA and 1 μg/ml each of ovine FSH (NIADDK-oFSH-17 AFP-6446C) and ovine LH (NIADDK-oLH-25 AFP-5551B). Oocytes were cultured at 38°C in a 5% CO_2, 5% O_2, 90% N_2 gas atmosphere.

The fate of all individual fair-to-excellent oocytes is shown in Table 1. As described in *Results,* some oocytes were used only to assess the stage of maturation at the time of insemination; the remainder were used to determine maturation and fertilization 30 h post-insemination. Stages of the meiotic cycle were used as indices of normal oocyte maturation. Pictorial depictions of oocyte staging criteria are provided in a related, recent publication from this laboratory [9]. In brief, this process involved fixing the oocytes for 3–5 days in methanol/glacial acetic acid (3:1), air-drying on slides, and staining with 2% Giemsa [19]. Oocytes were classified as mature when chromosomes were in either telophase I or metaphase II. Telophase I is characterized by the appearance of 2 equally spread groups of chromosomes and metaphase II by one of these groups spread and the other clustered and comprising the polar body. Oocytes that became fertilized after insemination (as demonstrated by the presence of 2 pronuclei) also were classified as mature.

Semen Collection and IVF

Oocytes from 15 individuals representing 8 species (tiger, lion, leopard, snow leopard, puma, Geoffroy's cat, leopard cat, serval) were inseminated using homologous fresh or thawed spermatozoa or thawed domestic cat spermatozoa. Domestic cat sperm were used for two reasons. First, there was no available conspecific sperm donor for some species. Second, we were interested in assessing the ability of heterologous gametes to interact in vitro.

Fresh spermatozoa were collected from a leopard and a leopard cat using a standardized electroejaculation procedure [20, 21]. When males were unavailable to provide a fresh ejaculate, frozen-thawed spermatozoa were used for insemination. In these cases, conspecific or domestic cat semen samples from the National Zoological Park's Genetic Resource Bank were used. These ejaculates had been collected 3 mo to 5 yr earlier by electroejaculation and frozen in egg-yolk cryodiluent PDV-62 [22] using the "pelleting" method [23]. For each insemination, two pellets were removed from liquid nitrogen storage and thawed rapidly in 250 μl of Ham's F10 medium (Irvine Scientific) [13, 14] maintained at 37°C. For both fresh and thawed ejaculates, spermatozoal percent motility and progressive status (range, 0–5: 0 = no forward sperm movement; 5 = rapid, linear, forward movement [20]) were assessed objectively. Samples with at least a 45% sperm motility and 2.75 sperm progressive status rating were used for IVF attempts. A sperm motility index (SMI), based on progressive status and motility, was calculated for both fresh and thawed samples [16].

Regardless of whether fresh or thawed samples were used, spermatozoa were diluted with Ham's F10 medium (supplemented with 5% fetal calf serum; Irvine Scientific) to an insemination concentration of 2×10^5 motile cells/ml. Oocytes from each individual were divided randomly into two groups to be either 1) fixed at 48 h for maturation assessment; or 2) inseminated at 48 h with homologous or heterologous spermatozoa after assessment of oocyte maturation on the basis of an expanded cumulus cell matrix and no visible signs of degeneration [10, 11]. Oocytes used for inseminations were placed in a 100-μl drop of sperm suspension (14 oocytes or fewer per drop) and placed under washed paraffin oil [6]. To determine the incidence of parthogenetic development, control oocytes (n = 1–5 oo-

TABLE 1. In vitro oocyte recovery, maturation, and fertilization of follicular oocytes recovered from 13 species of nondomestic felids.

Species	No.	Age	Health	Transit time[a]	Total oocytes	Oocyte classification			Number matured (%)	Number fertilized (%)
						Excellent	Good/Fair	Degenerate		
Tiger										
	T1	16	Poor	12	33	7	12	14	4/17 (23.5)	—
	T2	9	Poor	10	54	37	14	3	23/47 (48.9)	3/33 (9.1)[b] 1/14 (7.1)[c]
	T3	8	Good	24	66	35	16	15	11/41 (26.8)	4/41 (9.8)[b]
	T4	UNK	Poor	48	14	0	0	14	—	—
	T5	18	Poor	24	0	0	0	0	—	—
	T6	20	Poor	24	0	0	0	0	—	—
	T7	UNK	Poor	24	0	0	0	0	—	—
Lion										
	LN1	12	Good	24	88	18	9	61	3/25 (12.0)	1/25 (4.0)[b]
	LN2	2	Good	24	20	10	2	8	5/10 (50.0)	3/10 (30.0)[b]
	LN3	17	Poor	24	0	0	0	0	—	—
	LN4	14	Poor	24	0	0	0	0	—	—
Leopard										
	LD1	15	Fair	24	22	6	11	5	8/14 (57.1)	4/14 (28.6)[c]
	LD2	12	Fair	36	23	15	8	0	6/21 (28.6)	6/21 (28.6)[d]
	LD3	14	Fair	10	0	0	0	0	—	—
	LD4	12	Poor	12	17	7	5	5	2/11 (18.2)	1/11 (9.1)[d]
	LD5	UNK	Fair	0	0	0	0	0	—	—
	LD6	16	Fair	10	47	24	16	7	7/29 (24.1)	0/16 (0.0)[c]
	LD7	11	Fair	24	54	23	25	6	9/42 (21.4)	1/20 (5.0)[c]
Jaguar										
	J1	12	Poor	24	21	6	9	6	4/12 (33.3)	—
	J2	17	Fair	24	0	0	0	0	—	—
Snow Leopard										
	SNL1	11	Fair	10	32	25	7	0	16/30 (53.3)	5/18 (27.8)[c]
Puma										
	P1	2.5	Good	12	83	42	36	5	15/41 (36.6)	2/5 (40.0)[b] 7/36 (19.4)[c]
	P2	2	Good	24	23	16	7	0	13/23 (56.5)	6/13 (46.2)[c]
	P3	17	Poor	24	0	0	0	0	—	—
Cheetah										
	CH1	15	Poor	24	10	3	0	7	0/3 (0.0)	—
	CH2	16	Poor	24	7	3	3	1	1/6 (16.7)	—
	CH3	13	Poor	24	8	2	1	5	0/3 (0.0)	—
	CH4	7	Poor	48	20	0	0	20	—	—
Clouded Leopard										
	CL1	14	Poor	24	2	2	0	0	0/2 (0.0)	—
Bobcat										
	B1	2	UNK	36	102	0	61	41	8/51 (15.7)	—
Serval										
	S1	10	Good	10	33	16	10	7	15/24 (62.5)	9/24 (37.5)[c]
Geoffroy's Cat										
	GYC1	9	Good	30	45	18	27	0	10/43 (23.3)	10/43 (23.3)[c]
Golden Cat										
	GNC1	15	Poor	24	0	0	0	0	—	—
Leopard Cat										
	LC1	UNK	Poor	1	5	0	0	5	—	—
	LC2	UNK	Poor	1	17	3	11	3	4/13 (30.8)	2/13 (15.4)[d]

[a]From removal of ovaries in situ to oocyte recovery into culture medium.
[b]Inseminated with homologous, thawed sperm.
[c]Inseminated with heterologous domestic cat, thawed sperm.
[d]Inseminated with homologous, fresh sperm.

cytes used for IVF per individual) were placed in Ham's F10 medium without spermatozoa.

After 12 h of culture, oocytes were removed from the fertilization dishes and washed in a 0.2% hyaluronidase solution to remove residual cumulus cells and loosely attached spermatozoa; control oocytes were treated similarly. Oocytes were returned to culture in fresh Ham's F10 medium under oil for an additional 12–18 h and then were assessed for fertilization as defined by the presence of 2 polar bodies, 2 pronuclei, or cleavage to at least the 2-cell stage [10]. The oocyte cytoplasm of all species was darkly pigmented, making it difficult to accurately identify intracellular structures by light microscopy. Therefore, all oocytes not cleaving by 30–36 h of culture were treated with a DNA-specific fluorescent stain, Hoescht 33342 (H342; bizbenzamide; Sigma Chemical Co.) using techniques previ-

ously described for in vivo-matured/in vitro-fertilized domestic cat [10], leopard cat, [11], puma [14], and tiger [15] oocytes. Oocytes cleaving by 30 h post-insemination were placed in fresh Ham's F10 medium, allowed to continue culture, and then were examined at 24-h intervals for the extent of embryo development in vitro. When these embryos failed to advance within any 24-h interval, they were stained with Hoescht 33342, and the blastomeres were counted.

RESULTS

Overall, 846 oocytes were recovered from 25 of the 35 (71.4%) individual donors. Of the 22 individuals in which oocyte recovery/quality permitted culture, oocyte maturation on the basis of chromosomal configuration was detected on 19 (86.4%) occasions (Fig. 1). Figures 2 and 3 depict gross evidence of oocyte maturation on the basis of a loosening and expansion of the cumulus cells. Of the 15 cases in which IVF was attempted, 14 (93.3%) resulted in evidence of fertilization (Fig. 4), including the production of cleaved embryos (Fig. 5) on 3 occasions. None of the oocytes cultured without sperm demonstrated evidence of fertilization. Specific results on the basis of individual species are summarized in Table 1 and as follows:

Tiger (Panthera tigris). These 7 donors ranged from 8–20 yr of age and all (except one) were in poor health (medical problems included mammary carcinoma and renal failure). No oocytes were recovered from the 2 oldest females (T5 and T6), but the ovaries from T5 contained cystic follicles. When the interval between ovariohysterectomy and oocyte collection was 10–24 h (T1, T2, T3), most of the oocytes were fair-to-excellent in quality. When the interval was extended to 48 h (T4), all oocytes were degenerate. A total of 167 oocytes were recovered from 4 females; of the 105 oocytes cultured, 36.2% matured to metaphase II. Seven of 74 oocytes inseminated with thawed tiger spermatozoa (SMI = 79.0) were fertilized as determined by the presence of 2 pronuclei. One oocyte (from T2) inseminated with thawed, domestic cat spermatozoa (SMI = 90.0) also fertilized on this basis.

Lion (Panthera leo). Lion donors were in poor- (LN3, LN4) to good (LN1, LN2) health. A total of 108 oocytes were collected from LN1 and LN2, but none were recovered from LN3 and LN4, even though the gross appearance of these ovaries was normal. Although oocytes were recovered within ~24 h of accidental death in LN1, most (69.3%) were degenerate. Because this animal died in July, the seasonal ambient temperature may have contributed to oocyte degeneration. However, for all fair-to-excellent quality oocytes, maturation rates averaged 22.9%, and insemination with thawed, conspecific spermatozoa (mean SMI = 47.5) resulted in 11.4% of the oocytes fertilized (on the basis of 2 pronuclei present).

Leopard (Panthera pardus). Even though all of these individuals were more than 10 yr of age and in only fair or poor condition, many oocytes were recovered from 5 of 7 donors. Overall, 163 oocytes were collected of which 140 (85.9%) were categorized as fair-to-excellent. The average maturation rate was 27.4%, and the mean IVF rates were 21.9% and 10.0% using fresh homologous (mean SMI = 78.1) and thawed heterologous (mean SMI = 85.4) spermatozoa, respectively. One of 21 oocytes (from LD2) inseminated with conspecific fresh sperm (SMI = 75.9) pro-

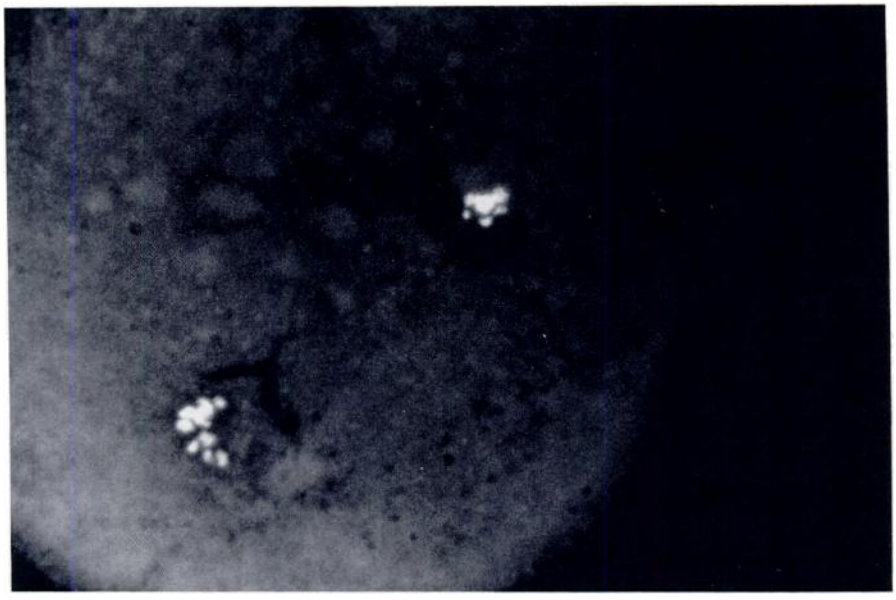

FIG. 1. Mature metaphase II puma oocyte with first polar body, following Hoescht-staining. ~×1000.

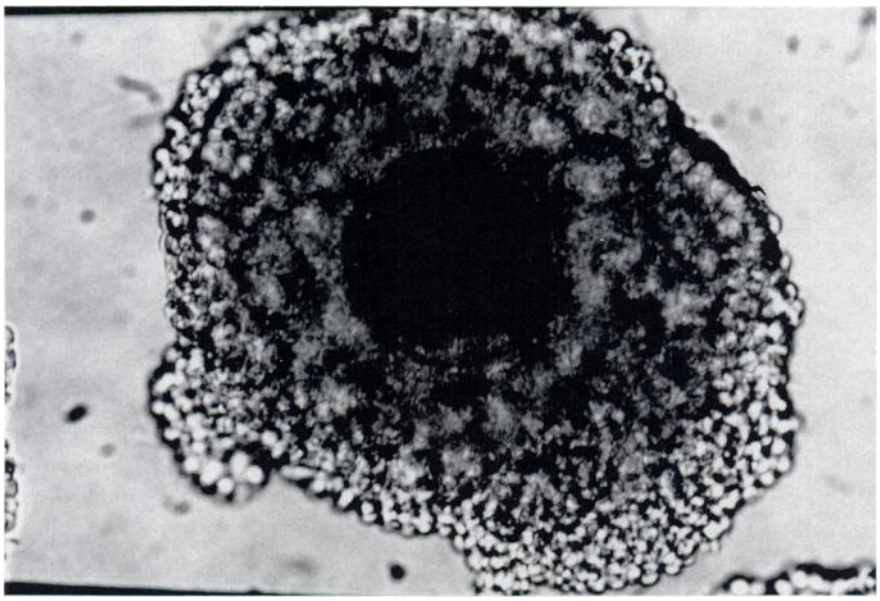

FIG. 2. Immature oocyte collected from snow leopard (SL1). Oocyte is darkly pigmented and cumulus cell layer is tightly compacted (quality classification = excellent). ~×200.

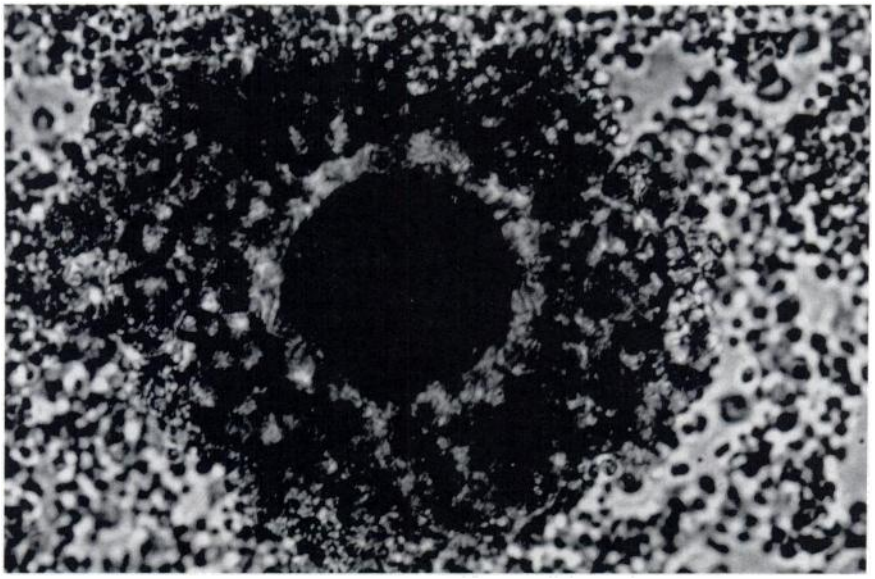

FIG. 3. Snow leopard oocyte after 48 h of culture in the presence of FSH and LH. Oocyte retains its dark pigment and cumulus cells have expanded during culture (quality classification = excellent). ~×200.

duced a 4-cell embryo. Although LD4 was in poor condition before dying of chronic medical problems, 12 fair-to-excellent quality oocytes were recovered, and 2 pronuclei were observed in 1 oocyte after coculture with fresh leopard sperm (SMI = 80.2).

Jaguar (Panthera onca). Twenty-one oocytes were recovered from a 12-yr-old animal in poor condition. The majority of the oocytes were fair-to-excellent quality, and one third of these advanced to metaphase II in culture. The ovaries of a 17-yr-old jaguar in fair condition yielded no oocytes.

Snow Leopard (Panthera uncia). A total of 32 oocytes were recovered from a snow leopard in fair condition. Most were classified as excellent, and 53.3% matured to metaphase II. When inseminated with thawed, domestic cat sperm, 27.8% were fertilized on the basis of 2 pronuclei present.

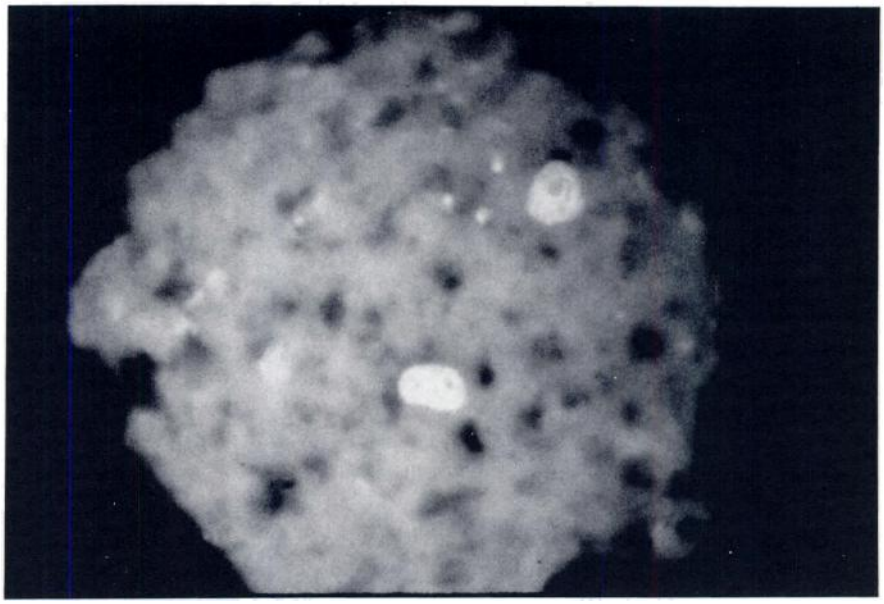

FIG. 4. Hoescht-stained leopard oocyte demonstrating evidence of fertilization by the presence of 2 pronuclei. ~×400.

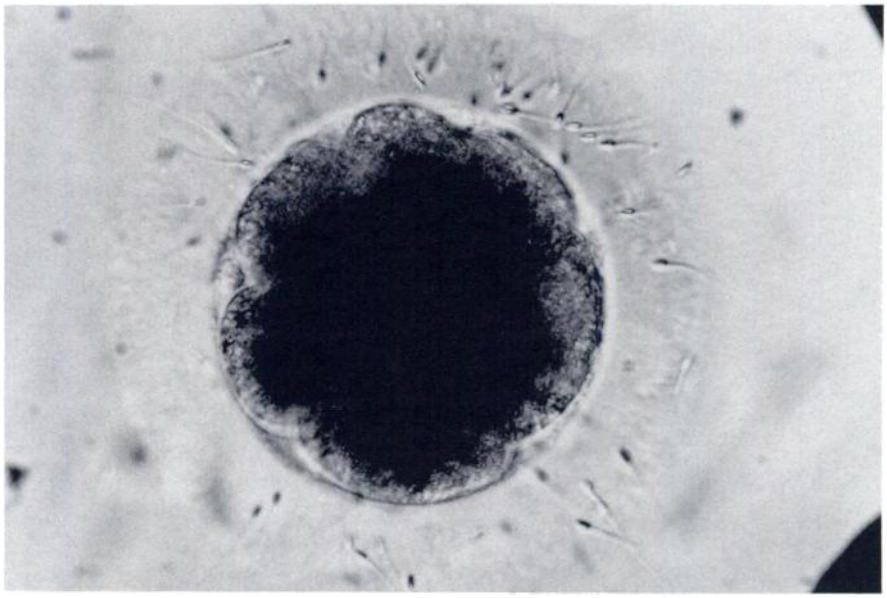

FIG. 5. Hybrid 8-cell embryo resulting from insemination of a puma oocyte with thawed, domestic cat spermatozoa. ~×400.

Puma (Felis concolor). Ovaries from 2 young individuals (P1, P2) yielded more than 100 oocytes of which only 4.7% were degenerate. In contrast, no oocytes were recovered from an aged female (P3) in poor health. Of all fair-to-excellent quality oocytes, 43.8% demonstrated evidence of in vitro maturation. On one occasion (P1), 5 oocytes were inseminated with thawed puma spermatozoa, and 2 were judged fertilized by the presence of 2 polar bodies. After insemination with thawed, domestic cat sperm, 7 of 36 puma oocytes were fertilized and, on two occasions, cleaved embryos resulted (Fig. 5). One embryo arrested at the 2-cell stage, but 2 others developed to the 16-cell stage as determined by Hoescht-staining after 72 h in culture.

Cheetah (Acinonyx jubatus). All donors were in poor condition due to age and/or hepatic and renal failure. A total of 45 oocytes were recovered of which 73.3% were

degenerate. This occurred despite a death-to-oocyte recovery interval of 24 h in 3 of 4 females. Of the 12 oocytes cultured for maturation, only one advanced to metaphase II.

Clouded Leopard (Neofelis nebulosa). A single pair of ovaries was obtained from one aged individual in poor condition. Two immature oocytes were isolated, neither of which matured in culture.

Bobcat (Lynx rufus). Ovaries were recovered from a young, free-ranging bobcat killed by being struck by a car. This female was dead for more than 12 h before the ovaries were excised and shipped over a 12-h interval to the laboratory. A total of 102 oocytes were recovered of which approximately half were rated as fair-to-good quality. Of the 51 oocytes cultured, 15.7% matured to metaphase II.

Serval (Felis serval). The ovaries of a single, middle-aged serval in good health yielded 33 oocytes, most of which (78.8%) were rated fair-to-excellent quality. Of the 24 oocytes cultured, 62.5% matured, and 37.5% were fertilized when inseminated with thawed, domestic cat spermatozoa (SMI = 86.5).

Geoffroy's Cat (Felis geoffroyi). One pair of ovaries was recovered from a middle-aged female in good condition. All 45 oocytes were rated as fair-to-excellent quality, and ~23% of these matured in vitro and were fertilized (on the basis of 2 pronuclei) after being inseminated with thawed, domestic cat spermatozoa (SMI = 95.0).

Temminck's golden cat (Felis temminckii). No oocytes were isolated from an aged individual in poor condition.

Leopard cat (Felis bengalensis). Two leopard cat donors were in poor condition as a result of age-related medical problems. Of the 22 oocytes recovered immediately from freshly received ovaries, 8 (36.4%) were degenerate. However, 4 of 13 cultured oocytes matured in vitro, and 2 of these (15.4%) were fertilized after insemination with fresh, homologous spermatozoa (SMI = 95.0).

DISCUSSION

In many zoological collections, nondomestic felid populations consist of older animals, many of which have never produced offspring. This genetic under-representation generally is attributable to poor reproduction caused by a limited gene pool (inbreeding), sexual incompatibility, or stress related to suboptimal captive habitat [3]. It is not unrealistic to consider salvaging genetic material from rare species using extraordinary strategies like in vitro maturation and fertilization. Recent successes in laboratory and livestock animals provides considerable incentive. Perhaps the most remarkable findings are those of Schroeder et al. [24] who demonstrated that ovarian oocytes collected from mice that had been dead for 6 or more h remain capable of maturing and fertilizing in vitro. When transferred, these embryos develop into healthy offspring. In light of these results and given more detailed studies on optimizing maturational events, the rescue of rare felid oocytes is a realistic possibility. In addition to having conservation biology potential, this approach improves our fundamental understanding of oocyte maturation and gamete coculture requirements across a taxon of related species. These efforts are complicated by the rarity of the test species and the requirement that most raw data must be collected opportunistically, often from older and/or medically compromised individuals. Although our sample size within species was relatively limited, we were able to draw several major conclusions.

First, it is possible to recover ovarian oocytes from an array of felid species, even from ovaries stored ex situ in a physiological medium for 36 h. We previously have demonstrated that domestic cat follicular oocytes remain capable of maturing to metaphase II when recovered within 24–32 h of initial storage [9]. Second, although impossible to confirm with present data, there may be some species differences with respect to oocyte recovery, maturation, and IVF efficiency using the domestic cat system. This variability probably is related, in part, to the age and/or poor health conditions of many of the individuals used in the present study. For example, when young or middle-aged healthy females were used (i.e., puma, snow leopard, serval), oocyte quality and maturation/IVF rate appeared more comparable to that measured in the domestic cat [9]. However, although almost half of the females in the study were in poor health, 12 of 18 (66.7%) yielded oocytes and the incidence of in vitro maturation reached as high as 48.9% (Tiger, T2). Likewise, it also was possible to recover oocytes from older females that were in fair-to-good health. In general, for the species evaluated, individuals older than 12 yr probably could be considered reproductively senescent [25]. However, oocytes (n = 2–86) were recovered from 11 of 19 females 12–18 yr of age. There was no apparent increase in the incidence of oocyte degeneration with increasing age.

Our findings of considerable intra- and interspecies variability in oocyte recovery and in vitro maturation and fertilization were not surprising in light of earlier studies in this taxon using in vivo-matured oocytes. For example, although the leopard cat and domestic cat are of the same physical size, the leopard cat—subjected to the same gonadotropin regimen as the domestic cat—produces fewer high-quality follicular oocytes and a lower incidence of in vitro fertilization [4, 11]. Additionally, unlike inseminated domestic cat oocytes which thrive in culture [10, 12, 13], a high proportion of leopard cat oocytes (45%) degenerate within 30 h of being placed in culture [11]. Oocyte recovery number, oocyte quality, and IVF rate in the tiger [15] are comparable to the domestic cat [4]. In contrast, embryo cleavage rate in vitro is much lower in the puma, perhaps because this species produces many pleiomorphic spermatozoa [14]. Regardless, these observations in the context of the present results indicate that there may be some species-specific norms that may perturb the rapid adaptation

of in vitro oocyte maturation or fertilization methods across all species.

Interestingly, few oocytes were recovered from either the cheetah or clouded leopard and the in vitro oocyte maturation rates were low for these species. Although we received a total of 6 cheetah ovaries within 24 h of removal, only 45 oocytes were recovered and half of these were degenerate. Only one of the cheetah oocytes advanced to metaphase II in vitro. This low efficiency may have been related to the poor health status and greater age of the donors. But it also is well established that the cheetah [26–28] and, to a lesser extent, the clouded leopard [29] are less genetically diverse than other Felidae species. We have suggested that the history of inbreeding revealed by the cheetah's genetic uniformity contributes to its overall poor reproductive performance as well as to increased disease susceptibility [20, 26–28, 30, 31]. The present results illustrate a possible female component to the cheetah's reproductive difficulties that may be a consequence of inbreeding depression. This area deserves much further study, especially because most captive felid populations are highly inbred [32]. If genetic variability does, in part, influence reproductive success in the Felidae family, as has been suggested recently [4], then the utility of biotechniques like in vitro maturation and fertilization will be partially dependent on genetic diversity within individuals and species populations.

IVF rates of 15.4–46.2% were achieved in 9 females (representing 7 species) and were comparable to the average incidence of fertilization (36%) in similarly treated domestic cat oocytes. However, only 3 of 357 inseminated oocytes from the nondomesticated species cleaved to at least the 4-cell stage compared to a rate of 33% for the domestic cat [9]. The low cleavage rate may have been the result of inadequate cytoplasmic maturation (cytoplasmic maturation normally is required for spindle formation and pronuclei migration [33]). Perhaps the use of thawed spermatozoa also compromised fertilization. However, we recently observed equally high embryo cleavage rates using in vivo-matured tiger oocytes inseminated with either fresh (69.4%) or thawed (70.2%) spermatozoa (Donoghue, Johnston, and Wildt, unpublished data). It was possible that the prolonged interval between ex situ recovery and initiation of oocyte recovery disrupted normal oocyte maturation. A recent study examining the effect of holding temperature on bovine oocytes before maturation indicated that fertilization rates are reduced significantly when ovaries are held at 4°C compared to 22°C [34]. It is possible that holding felid ovaries at 4°C for an extended period disrupted microtubule and microfilament integrity for future spindle formation, thus compromising early embryogenesis.

Hybridization between different felid species apparently is not an unusual phenomenon; approximately 30 different types of hybrids have resulted from the crossbreeding of felid species maintained in captivity [35]. Howard and Wildt [16] have demonstrated recently that leopard cat spermatozoa are capable of binding to and penetrating zona-intact domestic cat oocytes in vitro. On nine occasions in our study, we inseminated oocytes from 6 different nondomestic felid species with frozen/thawed domestic cat spermatozoa, and some oocytes fertilized in all species. These results support the theory that gametes from different felid species can interact to the point of cross-species sperm binding and zona penetration. Furthermore, the present study extends earlier findings by demonstrating that this interaction can include male pronuclear formation as well as embryo cleavage. Most importantly, on one occasion, two 16-cell embryos formed from the insemination of in vitro-matured puma oocytes with domestic cat spermatozoa. This suggests that the Felidae family could serve as an excellent model taxon for studying zona-receptor mechanisms associated with sperm penetration and cross species-specific limitations to sperm-oocyte interaction and early embryogenesis.

In summary, we have documented the feasibility of recovering large numbers of oocytes from young to middle-aged females representing a broad cross-section of the Felidae family. Many of these oocytes can be induced to mature in vitro, and although the efficiency of the process appears to decrease in aged animals in poor health, a few oocytes remain salvageable from some of these individuals. Furthermore, regardless of donor status, a portion of the cultured oocytes fertilize in vitro with homologous or heterologous spermatozoa. Studies are in progress that examine in more detail the influence of age, health, ovarian transit time, and shipping temperature on in vitro oocyte maturation and fertilization.

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