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Sperm Capacitation in the Domestic Cat (*Felis catus*) and Leopard Cat (*Felis bengalensis*) As Studied With a Salt-stored Zona Pellucida Penetration Assay

J.C. ANDREWS,[1,2] J.G. HOWARD,[1] B.D. BAVISTER,[2] AND D.E. WILDT[1]
[1]*National Zoological Park, Smithsonian Institution, Washington, DC;* [2]*Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin*

ABSTRACT The ability of domestic cat or leopard cat spermatozoa to penetrate zonae pellucidae (ZP) of salt-stored, domestic cat oocytes was examined as an assay for sperm capacitation. Ovarian oocytes were recovered after ovariectomy and matured in vitro for 18–36 h. Following removal of cumulus cells, the oocytes were used fresh, or stored (4°C, 0.5–24 weeks) in a HEPES-buffered hypertonic salt solution. Electroejaculated, washed sperm ($2–4 \times 10^6$ sperm/ml) were preincubated for 1.0 h (38°C, 5% CO_2 in air) and then co-incubated (2×10^5 sperm/ml) with fresh or stored oocytes for 6.0 h. Gametes were incubated in a protein-free, modified Tyrode's solution (TLP–PVA) or in the same medium containing 4.0 mg/ml bovine serum albumin (BSA; TALP–PVA). Treatments were compared for percentage ZP penetration (defined as sperm heads reaching more than halfway through the ZP) as an index of sperm capacitation. In both the domestic cat and leopard cat, there was no difference ($P > 0.05$) in sperm penetration of fresh ZP (domestic cat, 42.5 ± 5.4%; leopard cat, 38.6 ± 2.8%) or stored ZP (domestic cat, 32.4 ± 4.2%; leopard cat, 27.6 ± 2.3%). Sperm incubated in protein-free medium (TLP–PVA) were less capable ($P<0.05$) of ZP penetration (domestic cat, 14.6 ± 5.9%; leopard cat, 7.9 ± 3.0%) than sperm incubated in medium TALP–PVA containing BSA (domestic cat, 60.3 ± 5.9%; leopard cat, 58.4 ± 3.0%). These data indicate that (1) albumin facilitates capacitation and ZP penetrating ability of cat spermatozoa; (2) domestic cat ZP appear to lack a block to heterospecific penetration by "foreign" (leopard cat) sperm; and (3) penetration of stored domestic cat ZP can be used as an index of sperm capacitation in the domestic cat and the leopard cat.

Key Words: Protein-free medium, BSA, In vitro sperm penetration

INTRODUCTION

Spermatozoa must undergo capacitation (Austin, 1951; Chang, 1951) and functional (physiological) acrosome reactions before zona pellucida (ZP) penetration can occur (Austin and Bishop, 1958; Meizel, 1978; Yanagimachi, 1981; Koehler, 1981; Bavister, 1986). Penetration of intact ZP can be used as a definitive endpoint for sperm capacitation during in vitro fertilization (IVF) (Andrews and Bavister, 1989; Bavister, 1986, 1990). In practice, however, this approach is limited because ZP penetration is monospermic, due to the block to polyspermic fertilization, which is predominantly mediated through the cortical granule reaction (Wolf, 1981). Although IVF clearly demonstrates that capacitation has occurred, it only gives information on a single sperm per egg. Since the ratio of sperm to eggs during IVF is often 10,000–20,000:1, penetration of fresh ZP via IVF is not necessarily an efficient strategy for quantifying sperm capacitation (Bavister, 1986).

The salt-stored ZP penetration assay, developed in the hamster (Yanagimachi et al., 1979; Boatman et al., 1988), rabbit (Fayrer-Hosken and Brackett, 1987), and the human (Franken et al., 1988; Yoshimatsu et al., 1988) is an attractive approach for evaluating sperm capacitation in vitro. In the hamster and the human (species in which the block to polyspermy is primarily at the level of the ZP), salt storage destroys the ability of eggs to mount the cortical reaction, so that numerous sperm can reach the perivitelline space (Yanagimachi et al., 1979; Boatman et al., 1988; Yoshimatsu et al., 1988). Salt-stored ZP also retain the ability to distinguish between capacitated and noncapacitated spermatozoa (Boatman et al., 1988; Uto et al., 1988). The mean number of sperm in the perivitelline space (PVS) provides a quantitative index of capacitation (Boatman et al., 1988). We have demonstrated that the sensitivity of the salt-stored ZP penetration assay was improved above IVF of living eggs by up to 63-fold in the hamster (mean number of sperm in the PVS = 63.5; Boatman et al., 1988). In addition, salt-stored ZP can be conveniently banked for later use.

Although IVF has been achieved in the domestic cat (*Felis catus*) (Hamner et al., 1970), Indian desert cat (*Felis silvestris*) (Pope et al., 1989), leopard cat (*Felis bengalensis*) (Goodrowe et al., 1990), puma (*Felis concolor*) (Miller et al., 1990), and tiger (*Panthera tigris*) (Donoghue et al., 1990), information on the physiology of gametes in any felid species is very limited. Studying the basic biological mechanisms underlying felid sperm

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Address reprint requests to Dr. Jane C. Andrews, Department of Veterinary Science, University of Wisconsin-Madison, 1655 Linden Drive, Madison, WI 53706.

fertilizing ability is imperative for understanding the comparative similarities and differences among species and eventually for developing artificial breeding techniques. There are 37 felid species, all of which except the domestic cat are listed as endangered or threatened by extinction.

Howard and Wildt (1990) recently demonstrated the ability of leopard cat sperm to penetrate heterologous, zona-intact domestic cat oocytes following a 1-h sperm preincubation and 3-h sperm–egg co-incubation in the presence of bovine serum albumin (BSA). This penetration assay using fresh, ZP-intact oocytes was also effective for determining the detrimental impact of teratospermia on sperm function in the domestic cat (Howard et al., 1991).

The present experiments were designed to evaluate the utility of salt-stored domestic cat eggs for assessing ZP penetration by domestic cat and leopard cat sperm. The effect of BSA on capacitation of electroejaculated, washed sperm and gamete interaction was also determined.

In the past, this type of research has been hindered because of the need to include impure commercial preparations of BSA in culture media to maintain sperm motility and facilitate capacitation. As a control medium for the present studies, we used a chemically defined (protein-free) culture medium that supports sperm motility in the absence of albumin without inducing sperm capacitation (Bavister, 1981a; Andrews and Bavister, 1989).

MATERIALS AND METHODS

Animals

Adult, male domestic cats and leopard cats were housed as previously described (Howard and Wildt, 1990; Howard et al., 1990). Briefly, animals were maintained at the National Institutes of Health Animal Center (NIHAC; Poolesville, MD) and exposed to approximately 12 h of natural daylight/day, 10 h of which were supplemented with artificial lighting. All animals were captive-bred, unproven breeders ranging from 4 to 13 yr of age and 2.3 to 4.2 kg in body weight. Male domestic cats were provided a dry commercial cat food (Purina Cat Chow, Ralston Purina Co., St. Louis, MO) ad libitum. Male leopard cats were fed a commercial nondomestic feline diet (Nebraska Brand Feline Diet, North Platte, NE) daily. All cats were given water ad libitum.

Culture and Salt Storage Media

All media and stock solutions were prepared with ultrapure water obtained by reverse osmosis followed by purification with a Milli-Q system (Millipore Corporation, Bedford, MA). Water was tested for contaminants with the hamster sperm bioassay (Bavister and Andrews, 1988). The medium for sperm preincubation and sperm–egg co-incubation consisted of a modified Tyrode's solution (TLP) containing 1 mg/ml polyvinyl alcohol (PVA, average molecular weight = 10,000; Sigma Chemical Co., St. Louis, MO) in place of BSA (TLP-PVA: Bavister, 1981b, 1989). For sperm capacitation, 4 mg/ml of BSA (Fraction V; Sigma Chemical Co.) was added to the medium used for sperm preincubation and sperm–egg co-incubation and was designated as TALP-PVA (Bavister, 1981b, 1989). Sodium pyruvate (P; 0.1 mM final concentration) was added to both culture media immediately before use.

The ovary transport and oocyte recovery medium was Dulbecco's phosphate-buffered saline (Paul, 1975), containing 1 mg/ml PVA (PBS–PVA), 50 μg/ml penicillin (Sigma Chemical Co.) and 50 μg/ml streptomycin (Sigma Chemical Co.). Ovarian oocytes were matured in either Tissue Culture Medium 199 (TCM 199; Gibco Chemical Co., Grand Island, NY) containing 0.25 mM pyruvate (Sigma Chemical Co.), 10.0% heat-inactivated estrus cow serum (obtained from CALS experimental herd, UW-Madison), 10 μg/ml follicle-stimulating hormone (FSH, Vetrepharm, London, Ontario) and 10 μg/ml gentamicin sulfate (Sigma Chemical Co.) or in modified Eagle's medium (C-MEM; Schroeder and Eppig, 1984) containing 0.23 mM pyruvate (Irvine Scientific, Santa Ana, CA), 1% heat-inactivated fetal calf serum (FCS; Gibco Chemical Co., Grand Island, NY), 3 mg/ml BSA (Fraction V; Sigma Chemical Co.), 1 μg/ml FSH (NIADDK-oFSH-17 AFP-6446C), and 1 μg/ml luteinizing hormone (LH; NIADDK-oLH-25 AFP-5551B). The salt storage solution consisted of 0.5 M $(NH_4)_2SO_4$, 0.75 M $MgCl_2$, 0.2 mM $ZnCl_2$, and 0.1 mg/ml PVA (Boatman et al., 1988), with 40 mM HEPES buffer (pH 7.4). Silicone oil (Aldrich Chemical Co, Milwaukee, WI) was extracted (Bavister, 1989) and used as an overlay of the culture media for sperm preincubation, sperm–egg co-incubation and oocyte maturation.

Egg Preparation

Domestic cat ovaries were obtained immediately postmortem or after ovariohysterectomy, placed in PBS–PVA, and maintained at 4°C for 1–4 h before processing. The ovarian follicles were punctured and aspirated with a pulled glass pipette tip (Unopette; VWR Scientific, Chicago, IL) attached to a 1-ml Drummond syringe (Fisher Scientific, Pittsburgh, PA) containing PBS–PVA. Oocytes with intact plasma membranes and ZP and uniform, darkly pigmented or slightly granular cytoplasm (Fig. 1) were washed 3 times in PBS–PVA and matured in equilibrated (5% CO_2 in air, 38°C) maturation medium (as described above) for 18–36 h. After maturation, the cumulus cells were mechanically removed by pipetting, and the oocytes were used either immediately (fresh) or after salt storage (stored) for 0.5–24 weeks. Only high-quality oocytes with uniform, darkly pigmented cytoplasm were used fresh. A portion of these eggs (20%) as well as eggs of suboptimal quality (granular and/or mottled cytoplasm) were pooled, matured in vitro, salt-stored, and used later as stored eggs. The latter were removed from the salt solution and rinsed twice (1.0 h/rinse) in equilibrated (protein-free) TLP–PVA before insemination.

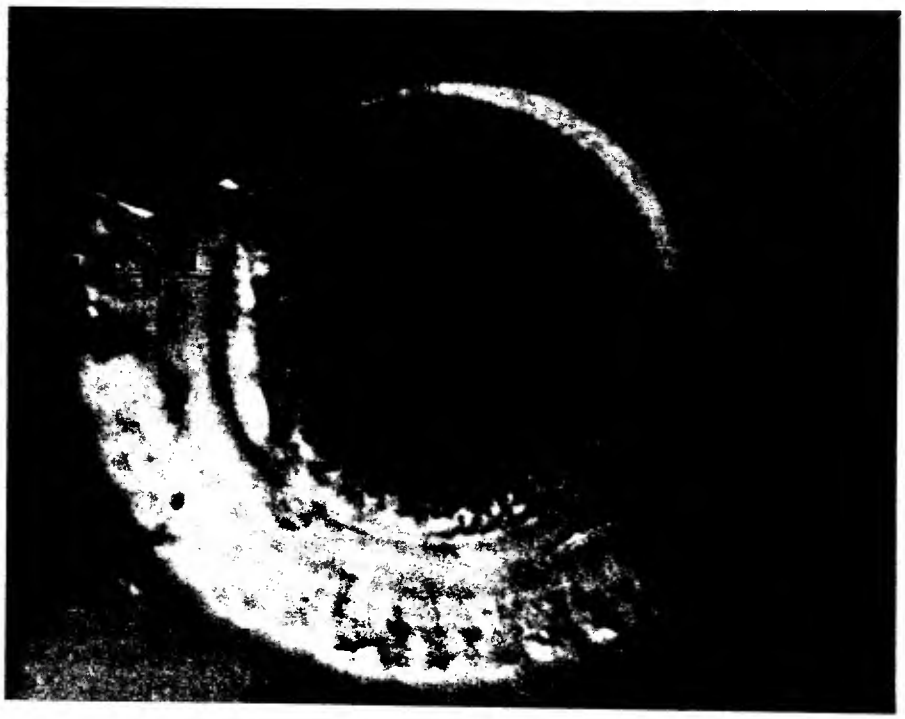

Fig. 1. A salt-stored domestic cat egg with zona pellucida (ZP) penetrated by leopard cat spermatozoa (magnification = ×740; 7.4 mm = 10 μm). Three sperm are indicated with arrows, one in the outer (O) half and two are in the inner (I) half of the ZP.

Semen Collection

Spermatozoa were obtained by electroejaculation using a standardized procedure (Wildt et al., 1983; Howard and Wildt, 1990; Howard et al., 1990). Briefly, males were anesthetized with tiletamine hydrochloride–zolazepam (Telazol; 4.5 mg/kg im, A.H. Robbins, Richmond, VA), and a probe (1 cm in diameter × 13 cm in length; P.T. Electronics, Boring, OR) with three longitudinal electrodes was inserted into the rectum. A sterile polyethylene, 5.0-ml collection vial (Nalge Co., Rochester, NY; cat. #6250-0005) was placed over the penis, and 80 electrical stimuli of 2–5 V and 20–100 mA were administered with an AC, 60-Hz sine wave stimulator (P.T. Electronics, Boring, OR).

Sperm Preparation

Semen was evaluated immediately for volume, sperm concentration, percentage motility, and forward progressive motility (×250, phase-contrast microscopy; Wildt et al., 1983; Howard et al., 1990). An aliquot of each ejaculate was fixed in 1% glutaraldehyde for assessment of sperm morphology at ×1,000 magnification (Howard et al., 1990). Ejaculates that contained at least 4×10^6 total motile sperm with an average progression rating of 3.0 (0–5, low to high) and greater than 60% structurally normal sperm/ejaculate, were transferred to a 1.5-ml conical microcentrifuge tube (Sarstedt Inc., Princeton, NJ). Each ejaculate was diluted 1:1 with equilibrated (5% CO_2 in air, 38°C) TLP–PVA, held at room temperature (23°C) and protected from light. The diluted semen sample was centrifuged at 300*g* for 8 min, and the resulting supernatant was carefully removed and discarded. The sperm concentration of the pellet was determined using a hemocytometer. The washed spermatozoa were preincubated at 4×10^6 motile sperm/ml for 1 h in 100-μl drops of equilibrated (5% CO_2 in air, 38°C) TLP–PVA or TALP–PVA overlaid with 4 ml of silicone oil in 35-mm Petri dishes (Falcon #1008). Because of a limited ejaculation volume on 1 day, sperm from one domestic cat male was preincubated at a concentration of 2×10^6 sperm/ml. Sperm motility was evaluated at 0.0 and 7.0 h of incubation (i.e., after 1 h of preincubation and 6 h of sperm–egg co-incubation).

In Vitro ZP Penetration

Prior to sperm collection, IVF dishes (Falcon #1007) were prepared. Two drops (95 μl/drop) of TALP–PVA (+BSA) and two drops of TLP–PVA (−BSA) were placed in each dish, overlaid with 10 ml of silicone oil and equilibrated at 38°C in 5% CO_2 in air for 1.0 h before egg addition. The stored or fresh eggs from each female were kept separate (in different drops of holding medium) and distributed equally between the drops of TALP–PVA (+BSA) and TLP–PVA (−BSA) in a 2 × 2 factorial design. Each 95-μl drop of medium contained 2–14 eggs, the absolute number depending on the number of acceptable eggs obtained from each female/day. Five μl of the corresponding preincubated sperm suspension (see Sperm Preparation) from the domestic cat

TABLE 1. Experiment I: Experimental Design for Evaluation of the Effect of Albumin on Fresh and Salt-Stored Domestic Cat Zonae Pellucidae Penetration by Domestic Cat or Leopard Cat Spermatozoa†

			No. of males		No. of days		No. of females		No. of eggs	
Medium	BSA	Type of eggs	DC	LC	DC	LC	DC	LC	DC	LC
TALP-PVA	+	FRESH	2	3	3	3	5	5	48	57
	+	STORED	2	3	3	3	8	7	49	38
Total pooled for ANOVA†:							13	12	97	95
TLP-PVA	−	FRESH	2	3	3	3	5	5	59	58
	−	STORED	2	3	3	3	8	7	47	44
Total pooled for ANOVA*,†:							13	12	106	102

†Electroejaculated, washed domestic cat (DC) or leopard cat (LC) spermatozoa were preincubated for 1.0 h ($2–4 \times 10^6$ sperm/ml) and subsequently co-incubated with eggs for 6.0 h (2×10^5 sperm/ml).
*The ANOVA demonstrated no difference ($P > 0.05$) between STORED and FRESH groups, so these eggs were pooled and the effect of albumin on ZP penetration evaluated (ANOVA).

(Experiment I) or leopard cat (Experiment II) was added to each IVF drop (final drop volume was 100 μl). In one case, domestic cat sperm were preincubated at 2×10^6 sperm/ml; therefore, 10 μl of sperm was added to 90-μl IVF drops. Sperm (2×10^5 sperm/ml) and fresh or stored domestic cat eggs were co-incubated for 6.0 h (38°C, 5.0% CO_2 in air).

Following co-incubation, the eggs were fixed in a 2% glutaraldehyde/2% formaldehyde solution and evaluated for penetration using differential interference contrast microscopy (×400). Assessment categories included the proportion of eggs with (1) sperm heads in the outer one-half of the ZP (ZP_{out}); (2) sperm heads more than one halfway through the ZP (ZP_{in}); (3) more than one sperm head greater than halfway through the ZP (poly-ZP_{in}); (4) one or more sperm heads in the perivitelline space (PVS); and (5) more than one sperm in the PVS (poly-PVS). In addition, the mean number of sperm in ZP_{out}, or in ZP_{in} plus the PVS, was evaluated.

Experimental Design and Statistical Analysis

Each treatment set (fresh or stored ZP ± BSA) was duplicated 0–4 times within a day using eggs from different females as available (Table 1); each experiment was replicated over 3 days using sperm from one male for each day. A total of 2 domestic cat males and 3 leopard cat males was used. The stored or fresh eggs from each female were distributed equally and randomly among treatments (Table 1).

The percent ZP penetration data were analyzed by female (n = 13, Experiment I; n = 12, Experiment II) using an analysis of variance (ANOVA) with an arcsin transformation for the 5 categories of ZP penetration. The data concerning the mean number of sperm per ZP were analyzed by female with an ANOVA and a $\log_{10}$ transformation. The standard errors and probabilities were calculated using the type III MS as an error term. Fresh vs. stored eggs were used as the whole plot and ±BSA as the split plot.

RESULTS

Experiment I (Domestic Cat Sperm and Eggs)

No interaction or differences between fresh and stored percentage ZP penetration were observed within the five penetration categories ($P > 0.05$; Table 2; ±BSA treatments remained pooled). Therefore, data for fresh and stored eggs were combined and analyzed for the effect of BSA on sperm capacitation as determined by ZP penetration. Domestic cat spermatozoa incubated in the presence of BSA penetrated more ($P < 0.05$) ZP than sperm incubated in the absence of BSA (Table 3). A large number of spermatozoa remained in the outer half of the ZP in both the presence and absence of BSA, although the values were different (Table 3). When BSA was present throughout incubation, 60.3% of the eggs were ZP_{in} penetrated, 43.0% were poly ZP_{in} penetrated, 44.3% contained PVS sperm, and 28.9% were poly-PVS penetrated (Table 3). By contrast, few spermatozoa reached the inner half of the ZP and/or the PVS in the absence of BSA (14.6% ZP_{in}, 9.7% poly ZP_{in}, 9.6% PVS, 3.5% poly-PVS; Table 3; $P < 0.05$) compared with the BSA-containing counterparts.

When the data were analyzed for the mean number of spermatozoa with heads embedded in the ZP, no interaction or difference ($P > 0.05$) between stored and fresh ZP was observed; therefore, stored and fresh ZP data were pooled and evaluated for the effect of BSA on the mean number of sperm within the ZP and PVS. The outer half of the ZP contained more ($P < 0.05$) spermatozoa when BSA was present than when it was absent, although the increase was less than twofold (Table 4). There was an approximately sixfold increase in the number of sperm penetrating more than halfway through the ZP in the presence of BSA than in its absence ($P < 0.05$). There was no difference ($P > 0.05$) in the mean number of sperm in the PVS of stored (0.58 ± 0.14) vs. fresh (0.58 ± 0.11) eggs (±BSA remained pooled); the mean number of sperm in the PVS

TABLE 2. Percentage Penetration of Fresh and Salt-Stored Domestic Cat Zonae Pellucidae by Domestic Cat or Leopard Cat Spermatozoa†

Penetration category[a]	Experiment I[b,*] (domestic cat)		Experiment II[b,*] (leopard cat)	
	Fresh ($\bar{X}$% ±SE)	Stored	Fresh ($\bar{X}$% ±SE)	Stored
ZP_{out}	79.7 ± 7.3	87.8 ± 5.7	88.0 ± 3.3	95.5 ± 2.7
ZP_{in}	42.5 ± 5.4	32.4 ± 4.2	38.6 ± 2.8	27.6 ± 2.3
Poly-ZP_{in}	29.1 ± 5.0	23.7 ± 3.9	27.6 ± 5.0	26.2 ± 4.1
PVS	30.6 ± 4.7	23.2 ± 3.8	15.6 ± 6.5	23.1 ± 5.3
Poly-PVS	14.7 ± 3.4	17.7 ± 2.7	8.7 ± 5.5	21.2 ± 4.5

†Electroejaculated, washed spermatozoa were preincubated for 1.0 h ($2–4 \times 10^6$ sperm/ml) and subsequently co-incubated with eggs for 6.0 h (2×10^5 sperm/ml).
[a]Categories include the percentage of eggs with (1) sperm heads less than halfway through ZP (ZP_{out}); (2) sperm heads greater than halfway through ZP (ZP_{in}); (3) more than one sperm greater than halfway into the ZP (Poly ZP_{in}); (4) one or more sperm in the PVS (PVS); (5) more than one sperm in the PVS (Poly-PVS).
[b]Experiments I and II were not directly comparable because they are independent data sets collected on separate days.
*There was no difference ($P > 0.05$) in stored and fresh ZP penetration by domestic cat or leopard cat spermatozoa (±BSA treatments were grouped together).

TABLE 3. Percentage Penetration of Domestic Cat Zonae Pellucidae by Domestic Cat or Leopard Cat Spermatozoa in the Presence and Absence of Albumin†

Penetration category[b]	Experiment I[a] (domestic cat)		Experiment II[a] (leopard cat)	
	+BSA ($\bar{X}$% ±SE)	−BSA	+BSA ($\bar{X}$% ±SE)	−BSA
ZP_{out}	94.8 ± 5.2*	72.6 ± 5.2**	99.0 ± 3.3*	84.7 ± 3.3**
ZP_{in}	60.3 ± 5.9*	14.6 ± 5.9**	58.4 ± 3.0*	7.9 ± 3.0**
Poly-ZP_{in}	43.0 ± 5.5*	9.7 ± 5.5**	51.1 ± 4.6*	2.7 ± 4.6**
PVS	44.3 ± 5.3*	9.6 ± 5.3**	35.4 ± 5.4*	3.3 ± 5.4**
Poly-PVS	28.9 ± 3.4*	3.5 ± 3.4**	29.3 ± 4.7*	0.6 ± 4.7**

†Studies I and II were not directly comparable because the data were collected on different days.
[a]Values are means ±SEM of combined fresh and stored eggs.
[b]See Table 2 for definitions of sperm penetration categories.
Values within species and row with different superscripts *,** differ ($P < 0.05$).

was greater ($P < 0.05$) in the presence of BSA (1.02 ± 0.16) than in its absence (0.14 ± 0.16) (data not shown).

The variance attributable to (1) the length of time the eggs were salt-stored, or (2) the type of oocyte maturation protocol used (see Materials and Methods) is nested within the variance attributable to day, fresh*stored, day*fresh*stored, and female (day*fresh*stored). Since the F values indicated that there was no effect of, or interaction between, these variables (data not shown), the effect of salt-storage time, or the type of oocyte maturation, was not investigated further.

Experiment II (Leopard Cat Sperm and Domestic Cat Eggs)

No interaction or difference was observed in the various penetration categories for fresh vs. stored ZP (Table 2); therefore, fresh and stored ZP were pooled to examine the impact of BSA on heterologous IVF. Spermatozoa incubated in the presence of BSA penetrated more ($P < 0.05$) ZP in all penetration categories compared with sperm incubated in the absence of BSA. As observed in Experiment I, the presence or absence of BSA had little influence on the ability of leopard cat sperm to enter the outer half of the domestic cat ZP (Table 3; +BSA = 99.0%; −BSA 84.7%). However, penetration of the ZP_{in} was greatly influenced by BSA. In the presence of albumin, 58.4% were ZP_{in} penetrated, 51.1% were poly 1/2 ZP_{in} penetrated, 35.4% contained PVS sperm, and 29.3% were poly-PVS penetrated. By contrast, less than 8.0% ZP_{in} penetration occurred in the absence of albumin (Table 3).

There was no difference ($P > 0.05$) in the mean number of leopard cat sperm between stored and fresh eggs when the ZP_{out} and ZP_{in} plus PVS penetration were evaluated. Although there was no difference ($P > 0.05$) in the average number of sperm in the outer half of the ZP in the ±BSA treatments, the mean number of sperm reaching more advanced stages of ZP penetration (>½ ZP) was enhanced by approximately 20-fold over the control (−BSA; $P < 0.05$) when BSA was present (Table 4). The only category in which there was

TABLE 4. Number of Domestic Cat or Leopard Cat Sperm in Domestic Cat Zonae Pellucidae in the Presence or Absence of Albumin

Penetration category	BSA	Experiment I[a,b] (domestic cat)	Experiment II[a,b] (leopard cat)
ZP_{out}	+	5.3 ± 0.3**	13.9 ± 3.1**
	−	3.6 ± 0.3*	10.8 ± 2.9**
ZP_{in}	+	2.6 ± 0.5*	3.9 ± 0.4*
	−	0.4 ± 0.5*,**	0.2 ± 0.4**

[a]Studies I and II were not directly comparable because they were independent data sets collected on separate days.
[b]Values are means ±SEM of combined fresh and stored eggs.
Within a study and penetration category, values with different superscripts *,** differ ($P < 0.05$).

a significant difference ($P < 0.05$) between fresh versus stored eggs was in the mean number of leopard cat sperm in the PVS, in the presence of albumin (fresh, +BSA = 0.63 ± 0.44; stored, +BSA = 3.5 ± 0.39); in the absence of BSA, the mean number of sperm in the PVS of fresh and stored eggs was identical (0.04 ± 0.39 and 0.04 ± 0.39 respectively; data not shown).

Since there was no effect of, or interaction between, day, fresh*stored, day*fresh*stored, and female (day*fresh*stored), the effect of salt-storage time, or the type of oocyte maturation (nested within these variances), was not further investigated (data not shown).

DISCUSSION

Our results demonstrate that stored domestic cat ZP were as penetrable by felid spermatozoa as fresh ZP. Therefore, as found in other species (hamster: Yanagimachi et al., 1979; Boatman et al., 1988; rabbit: Fayrer-Hosken, 1987; human: Franken et al., 1988; Yoshimatsu et al., 1988), we have confirmed that the salt-stored ZP penetration assay can be used as an alternative to conventional IVF as a valuable indicator for domestic cat and leopard cat sperm capacitation and the acrosome reaction.

When our data were evaluated on the basis of (1) five categories of sperm penetration into the ZP (Table 3), and (2) the mean number of sperm/ZP (Table 4), the results clearly indicated that BSA facilitated domestic cat and leopard cat sperm capacitation. Approximately 6- and 20-fold, respectively, more sperm progressed to the inner half of the ZP in the presence of BSA than in its absence. By contrast, the differential between ±BSA for eggs with sperm terminating in the ZP_{out} was only 1.3–1.5 and was only significant for the homologous domestic cat system. Without this protein, sperm from these two species remained relatively efficient at penetrating the outer layer of the ZP. However, BSA appeared to be vital for deeper ZP penetration. Taken together, our results indicate that sperm penetration into the outer half of the ZP may not accurately reflect sperm capacitation in the domestic cat or the leopard cat. Because the outer half of the cat ZP appeared more diffuse and porous than the inner half (Fig. 1), it is possible that many sperm, including those of inferior quality, become embedded in this region. Until it has been determined that all spermatozoa in the outer half of the ZP are robust and have undergone the physiological processes of sperm capacitation and the acrosome reaction (rather than sperm death), penetration of the inner half of the ZP should be considered as a more rigorous index of felid sperm capacitation.

An obligate role for serum albumin in supporting sperm capacitation in vitro has been demonstrated in the mouse, rat, and golden hamster (Miyamoto and Chang, 1973; Hoppe and Whitten, 1974; Bavister, 1981a). In addition to its role in supporting capacitation, serum albumin is a potent stimulator of the acrosome reaction (Meizel, 1978; Bavister, 1986; Andrews and Bavister, 1989). Although numerous hypotheses have been formulated to explain the capacitation and acrosome reaction inducing properties of albumin (Davis, 1980; Davis et al., 1980; Langlais et al., 1981; Aonuma et al., 1982; Andrews and Bavister, 1989), the biochemical mechanisms underlying the facilitation of these processes remain unclear. This is due, in part, to the dual requirements for albumin in culture media for supporting sperm capacitation/acrosome reactions and for maintenance of sperm viability. In the present experiments, it was possible to maintain domestic cat and leopard cat sperm viability in the absence of albumin in a chemically defined (protein-free) culture medium (TLP–PVA). Since we now know that this medium fails to support sperm capacitation and/or the acrosome reaction efficiently (Tables 3 and 4), it will be possible in future studies to probe the mechanisms of cat sperm capacitation and the acrosome reaction in the absence of a ubiquitous protein such as BSA.

Because salt storage destroys the block to polyspermy (Yanagimachi et al., 1979; Boatman et al., 1988), we expected the stored ZP to exhibit a greater incidence of polyspermic penetration than the fresh eggs. However, our results showed that there was no difference in the percentage polypenetration (Table 2) or in the mean number of sperm per egg between stored and fresh eggs, except in the case of leopard cat sperm PVS penetration: significantly more leopard cat sperm were in the PVS of stored eggs than in fresh eggs (see Results). This increase in PVS penetrability of stored eggs by leopard cat sperm may have been associated with the elimination of the block to polyspermy by salt storage. In contrast, Gelwicks et al. (1990) observed an increased incidence of polyspermy (as determined by penetration of the plasma membrane) when domestic cat oocytes contained intact germinal vesicles compared with mature ova that had undergone germinal vesicle breakdown (66% vs. 23% polyspermy, respectively). It also has been suggested that the optimal in vitro oocyte maturation interval is 30–48 h in the domestic cat (Johnston et al., 1989). Therefore, our relatively high frequency of polyspermy with fresh ZP may be attributable to our relatively short oocyte maturation interval of 18–36 h. Alternatively, the high inci-

dence of poly-ZP penetration may be normal in the cat, since the level at which the block to polyspermy occurs is unknown. The cat may be like the rabbit (another induced ovulator), in which the block is at the level of the plasma membrane, where supernumerary sperm are often found in the perivitelline space (Braden et al., 1954; Kuzan et al., 1984). In the pig, the block to polyspermy is established in the inner region of the ZP shortly after egg activation by spermatozoa, but accessory sperm do penetrate the outer, more diffuse regions of the ZP of both fertilized and unfertilized eggs (Thibault, 1959; Hunter, 1977). This is interesting, since the bilayered pig ZP is morphologically similar to the cat ZP observed in the present study with the light microscope (Fig. 1). By contrast, polypenetration of the outer half of the cat ZP may have resulted from an excessive concentration of spermatozoa in the fertilization medium (2×10^5 sperm/ml). Additional studies are warranted to determine the physiological significance of our polyspermic fresh ZP penetration data.

Hamner et al. (1970) demonstrated that ejaculated domestic cat sperm require 0.5–24 h of incubation in utero before they acquire the capacity to fertilize cat ova in vitro. Bowen (1977) demonstrated that ductus deferens cat sperm could fertilize domestic cat oviductal ova in vitro without incubation in the female reproductive fluids in vivo. More recently, Niwa et al. (1985) demonstrated that epididymal domestic cat sperm could penetrate domestic cat ZP within 20 min postinsemination, and swollen sperm heads are observed in as little as 30 min after insemination. Goodrowe et al. (1988) assessed cat ZP penetration by electroejaculated sperm and suggested that optimal domestic cat sperm capacitation is achieved within 3 h of incubation in vitro. However, unlike in the present study, the depth of sperm penetration into the ZP was not evaluated. In our pilot studies, we observed that most domestic cat sperm co-cultured with cat eggs for 3 h remained in ZP_{out}; spermatozoa reached the PVS only after 6 h of sperm/egg co-incubation. The present studies demonstrated that domestic cat and leopard cat sperm capacitation (as determined by sperm penetration of ZP_{in}) can occur within 7 h of incubation (1 h preincubation plus 6 h sperm/egg co-incubation). However, additional studies must be conducted to determine the actual timing of felid sperm capacitation both within and among species. Utilization of the salt-stored ZP penetration assay, and a protein-free culture medium that maintains sperm viability but that does not support cat sperm capacitation efficiently (TLP–PVA), will facilitate examination of the biological mechanisms and kinetics of cat sperm capacitation.

Our results also confirmed the data of Howard and Wildt (1990) by demonstrating that leopard cat spermatozoa can penetrate fresh domestic cat ZP. Leopard cat spermatozoa penetrated fresh and salt-stored domestic cat ZP with equal efficiency. This was interesting because it implied that the ZP receptor on felid sperm and the ligand on the ZP may be conserved across felid species; therefore, a functional block to heterospecific ZP penetration may not exist. Because of this nonspecificity, it may be possible to use the penetration of salt-stored domestic cat ZP by nondomestic cat sperm in place of conventional IVF as a tool for studying sperm function in other endangered felid taxa.

Understanding the basic biological mechanisms underlying felid sperm fertilizing ability will aid in developing assisted breeding techniques including artificial insemination, IVF, in vitro oocyte maturation, and cryopreservation. Each of these strategies may contribute to maintaining genetic diversity and promoting species survival.

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