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Ejaculate-Hormonal Traits in the Leopard Cat (*Felis bengalensis*) and Sperm Function as Measured by In Vitro Penetration of Zona-Free Hamster Ova and Zona-Intact Domestic Cat Oocytes

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ABSTRACT Electroejaculate traits and circulating follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone concentrations were analyzed in adult leopard cats (*Felis bengalensis*), a rare felid species indigenous to east Asia. The ability of leopard cat sperm to bind and penetrate zona-free hamster ova and zona-intact domestic cat oocytes in vitro was examined as a means of testing sperm function. The influence of culture media [Biggers, Whitten, Whittingham (BWW) vs. modified Krebs Ringer bicarbonate (mKRB)], seminal plasma removal, and swim-up separation on sperm motility, sperm morphology, and oocyte penetration also were assessed. Sperm treatments included dilution of raw semen (DR), ejaculate centrifugation, and either resuspension (NS) or swim-up processing (SU). The percentage of oocytes penetrated (penetration rate) and the number of penetrated sperm/oocyte (penetration index) were determined. Ejaculates from each male consisted of at least a 50% sperm motility rating, and hormone concentrations in individual males were unrelated to any ejaculate trait measured concurrently on the same day. The SU technique improved ($P < 0.05$) percent sperm motility and the proportion of structurally normal sperm compared to DR and NS treatments. Leopard cat spermatozoa were capable of binding to and penetrating hamster ova and domestic cat oocytes; however, penetration was influenced by culture medium and seminal processing. In the hamster assay, a higher ($P < 0.05$) penetration rate and penetration index were achieved when mKRB was used for gamete incubation instead of BWW. NS processing also increased ($P < 0.05$) overall penetration compared to DR and SU. In the cat oocyte assay, zona penetration rate was similar ($P > 0.05$) in the DR, NS, and SU aliquots; however, the zona penetration index was increased ($P < 0.05$) by the NS compared to the DR and SU treatments. This study 1) provides baseline ejaculate and endocrine norms for the leopard cat, 2) demonstrates that leopard cat sperm undergo nuclear decondensation in hamster ova and penetrate zona-intact domestic cat oocytes, 3) indicates that seminal plasma removal enhances leopard cat sperm fertilizing ability and ovum penetration, and 4) suggests that heterologous oocyte penetration is effective for assessing factors influencing fertilization and sperm function in this nondomestic felid.

Key Words: FSH, LH, Testosterone, Sperm morphology

INTRODUCTION

The Felidae family consists of 37 extant species, 36 of which are classified as threatened or endangered by extinction (CITES, 1973). Although certain nondomestic felids thrive reproductively in natural or zoological settings, many do not (O'Brien et al., 1983). Historically, reproductive success in these species has been measured by the production of live offspring. To provide a prospective approach, we have focused on developing fertility assessment techniques for both zoo-maintained and free-ranging felids (Howard et al., 1986). Laboratory analysis has allowed making general quantitative and qualitative estimates of electroejaculate norms and endocrine patterns for a variety of nondomestic felids (Wildt et al., 1983, 1986, 1987a,b, 1988; Howard et al., 1984). These studies have demonstrated that: 1) repeated electroejaculation reveals hierarchal differences in overall semen characteristics within a given population of males; 2) both semen

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characteristics and certain hormonal patterns, especially circulating testosterone concentrations, are species-specific; and 3) many species produce high proportions of structurally abnormal spermatozoa, which appear, in part, related to a loss in genetic diversity. These semen and hormonal data, however, are not a direct measure of fertility or an index of sperm viability.

The zona-free hamster ovum penetration assay, introduced by Yanagimachi (1972), has been used to study sperm function in a variety of species (for review, see Howard et al., 1990b), including humans (Yanagimachi et al., 1976; Rogers, 1985). Although the hamster bioassay determines the ability of a spermatozoon to fuse with the vitellus and undergo nuclear decondensation within the ooplasm, the test fails to measure penetration potential through the zona pellucida. Defective sperm–zona interactions are important causes of infertility in mice and humans (Kot and Handel, 1987; Mahadevan et al., 1987). One alternative or complimentary approach is the use of zona-intact immature ovarian oocytes. Domestic cat ovaries are readily available from veterinary or neuter clinics.

Recently, we demonstrated that domestic cat spermatozoa are capable of penetrating zona-free hamster ova in vitro, and penetration rates are higher in normospermic than teratospermic males (Howard et al., 1988, 1990b). Similar results were observed using zona-intact domestic cat oocytes, an approach that provided additional information on zona binding affinity between cat populations (Howard et al., 1990b). Thus these bioassays appear valuable for studying the potential impact of teratospermia on sperm–ovum interaction, an important observation since one survey revealed that ejaculates in 20 of 28 felid species average 36–84% structurally abnormal spermatozoa (Howard et al., 1984).

Our ultimate objective is to compare the utility of the hamster ovum and cat oocyte penetration assays for studying gamete function and evaluating fertility potential in rare felid species. The present study concerned the leopard cat (*Felis bengalensis*), a small (2–5 kg body weight) felid whose natural habitat extends through much of Asia (Lekagul and McNeely, 1977). In the wild and in captivity, leopard cats breed throughout the year and usually produce one to four kittens after a gestation of 65–72 days (Dathe, 1968; Lekagul and McNeely, 1977). Recent studies have demonstrated that the female leopard cat is responsive to exogenous gonadotropins and that follicular oocytes fertilize in vitro after coculture with processed, conspecific spermatozoa (Goodrowe et al., 1989). The purpose of the present study was to define electroejaculate and endocrine norms for the male leopard cat and then to assess the ability of leopard cat spermatozoa to bind and penetrate zona-free hamster ova and zona-intact domestic cat oocytes.

MATERIALS AND METHODS

Animals

Four male leopard cats were maintained at the National Institutes of Health Animal Center in Poolesville, Maryland, and were studied concurrently from April through August. All animals were captive-bred, unproven breeders ranging from 11 to 13 years of age and from 2.3 to 4.2 kg in body weight. Leopard cats were housed under similar conditions in individual cement and wire enclosures (212 cm high × 135 cm wide × 275 cm deep) containing a suspended squeeze cage (90 cm high × 60 cm wide × 70 cm deep) to facilitate anesthesia induction and postanesthesia recovery. Windows in the holding area exposed all animals to approximately 12 hr of natural daylight/day, 10 of these hours being supplemented with artificial illumination (from 0700 to 1700 hr). All males had ad libitum access to water and were fed a commercial, nondomestic feline diet (Nebraska Brand Feline Diet, North Platte, NE) daily. Males had no visual access to each other; however, all were in aural and olfactory proximity to one another and to adult females maintained as singletons in the same building.

Anesthesia and Blood and Semen Collection

Each animal was electroejaculated at 1–2 week intervals. At these times, anesthesia was induced with 2 mg xylazine (Rompun; Haver-Lockhart, Shawnee, KS) and 75 mg ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, NJ) injected intramuscularly. After a surgical plane of anesthesia was achieved, a blood sample (6–7 ml) was collected by jugular venipuncture immediately before electroejaculation onset (pre-EE) and immediately after (post-EE). The interval between xylazine-ketamine HCl administration and the pre-EE blood sample averaged (±S.E.M.) 17.3 ± 2.8 min, and the interval from pre-EE to post-EE averaged 27.2 ± 3.1 min. Samples were centrifuged 1 hr after collection, and the recovered sera were stored at −20°C until assayed for hormones.

A rectal probe (1 cm diameter × 13 cm length) with three longitudinal electrodes and an AC electroejaculator (P.T. Electronics, Boring, OR) were used to collect semen (Wildt et al., 1983; Howard et al., 1986). A regimented electroejaculation protocol was used whereby each male received a total of 80 electrical stimuli according to previously described domestic cat protocols (Wildt et al., 1983; Howard et al., 1990a). A total of 24 ejaculates was collected (six ejaculates/male).

Semen Analysis and Processing

The ejaculate from each male was evaluated immediately at ×250 using phase-contrast microscopy for subjective estimates of the percentage of motile spermatozoa and sperm progressive status. Sperm status was classified as the type of forward progressive movement of the cell graded from 0 (no movement) to 5

(rapid, steady forward progression) (Wildt et al., 1983; Howard et al., 1986). To determine an overall sperm assessment rating with equal emphasis on sperm motility and status, a sperm motility index (SMI) was calculated (SMI = [sperm progressive status × 20] + [% sperm motility] ÷ 2). Ejaculate volume was measured, and the concentration of spermatozoa/ml of ejaculate was determined using a standard hemacytometer method (Wildt et al., 1983; Howard et al., 1986). Motile spermatozoa/ejaculate ($\times 10^6$) was calculated by multiplying ejaculate volume times sperm concentration/ml times percent sperm motility. Sperm morphology was assessed by fixing ejaculate aliquots (20 μl) in 1% glutaraldehyde (Wildt et al., 1983; Howard et al., 1986) followed by phase-contrast microscopic examination of 200 spermatozoa/aliquot at ×1,000. Cells were categorized as normal or as having one of the following structural deformities: macrocephaly, microcephaly, bicephaly, tricephaly, mitochondrial sheath aplasia (including partial or complete aplasia of mitochondrial sheath), coiled flagellum, bent midpiece with or without a cytoplasmic droplet, bent flagellum with or without a cytoplasmic droplet, proximal or distal cytoplasmic droplet.

Sperm samples containing at least a 50% motility rating were transferred to 1.5 ml conical tubes (Sarstedt Inc., Princeton, NJ) and slowly diluted with either 150 μl Biggers, Whitten, and Whittingham (BWW) medium containing 3 mg/ml bovine serum albumin (BSA) (Biggers et al., 1971) or modified Krebs Ringer bicarbonate (mKRB) medium containing 4 mg/ml BSA (Niwa et al., 1985). The constituents of each of these media have been reviewed recently (Howard et al., 1990a,b).

To evaluate the effects of semen handling on sperm function, leopard cat ejaculates were processed as described recently for the domestic cat (Howard et al., 1990a,b). In brief, this involved dividing each undiluted raw (UR) ejaculate into three aliquots: 1) diluted raw (DR); 2) non-swim-up (NS); and 3) swim-up (SU). The NS and SU aliquots were centrifuged (300*g*, 8 min), and the supernatant was discarded. Fresh medium (50 μl) was layered gently onto each sperm pellet. For the NS aliquot, the spermatozoa were resuspended immediately in the layered supernatant, whereas, in the SU aliquot, spermatozoa were allowed to migrate for 1 hr into the fresh medium layer. After a 1 hr incubation (25°C), each of the three sperm suspensions was evaluated for sperm concentration, motility, progressive status, and morphological integrity before being analyzed further in the hamster ovum or cat oocyte penetration assay.

Hormone Radioimmunoassays

A heterologous double-antibody radioimmunoassay (RIA), previously developed and validated for the domestic cat (Chakraborty et al., 1979, Howard et al., 1990a), was validated for measuring luteinizing hormone (LH) in leopard cat sera. The LH antibody was a rabbit antibovine antiserum (JJR 5; source, Dr. J.J. Reeves), the iodinated tracer was highly purified ovine LH (LER-1056-C2; source, Dr. L.E. Reichert, Jr.), and the standard was canine LH (LER-1685-1; source, Dr. L.E. Reichert, Jr.). Recovery estimates after subtracting endogenous serum LH from the assay of 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ng canine LH added to leopard cat serum were 0.30, 0.45, 1.12, 1.87, 4.18, and 7.76 ng LH, respectively ($y = 0.97x + 0.06$; $r = 0.99$; $P < 0.001$). Serial dilutions of serum pools were parallel to the standard curve using LER-1685-1. Minimum assay sensitivity was 1.5 ng/ml, and all samples were analyzed in a single RIA that had an intraassay coefficient of variation of 12.8% (n = 6).

A double-antibody RIA previously adapted for nondomestic felids (Brown et al., 1988, 1989) and most recently validated for the domestic cat (Howard et al., 1990a) was validated for measuring follicle-stimulating hormone (FSH) in leopard cats. The antibody was rabbit antiovine FSH (JAD-LER 178; source, Dr. J.A. Dias), the iodinated tracer was a highly purified ovine FSH (LER-1976-A2; source, Dr. L.E. Reichert, Jr.), and the standard consisted of an ovine FSH preparation (NIH-FSH-S8; source, National Institutes of Health). Recovery estimates after substracting endogenous serum FSH from the assay of 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, and 100.0 ng FSH were 2.10, 3.35, 5.77, 11.83, 25.16, 49.91, and 87.12 ng FSH, respectively ($y = 0.88x + 1.51$; $r = 0.99$; $P < 0.001$). Inhibition curves for serum pools were parallel to the standard curve using NIH-FSH-S8. Cross-reactivity was <3% for 200 ng LH (NIH-LH-S18), growth hormone (NIH-GH-S11), prolactin (NIH-PRL-S12), and GnRH. Sensitivity was 5.0 ng/ml, and the intraassay coefficient of variation was 3.7% (n = 7).

Testosterone was measured using a double-antibody radioimmunoassay ^{125}I kit (Radioassay Systems Laboratory, Carson, CA), previously used for the domestic cat (Howard et al., 1990a) and for a variety of nondomestic felid species (Wildt et al., 1987a,b, 1988; Brown et al., 1988, 1989). The first and second antibodies were rabbit antitestosterone-19-carboxymethyl-ether-BSA and goat antirabbit gamma globulin, respectively. In validation tests, serial dilutions of serum pools were parallel to the standard curve, and the recoveries of testosterone after subtracting endogenous hormone from the assay of 0.05, 0.125, 0.25, 0.5, 1.25, and 2.5 ng testosterone were 0.08, 0.15, 0.29, 0.48, 1.12, and 2.47 ng, respectively ($y = 0.96x + 0.02$; $r = 0.99$; $P < 0.001$). Inter- and intraassay coefficients of variation were 8.9% (n = 6) and 2.4% (n = 7), respectively, and the assay sensitivity was 0.1 ng/ml.

Zona-Free Hamster Ovum Assay

A total of 16 electroejaculates (four per male) was used to assess the affinity of leopard cat spermatozoa for zona-free hamster ova. Bioassay procedures, includ-

ing the hormonal stimulation of hamsters and oviductal recovery of ova, have been described recently (Howard et al., 1988, 1990b). Hamster ova were collected and incubated with 0.1% hyaluronidase (Type I-S; Sigma Chemical Co.) and 0.1% trypsin (Sigma Chemical Co.) to remove cumulus cells and zonae pellucidae, respectively. Following three washes in fresh medium, ova (20 ova/drop) were placed in a sterile tissue culture dish (35 × 10 mm) containing a 20 μl drop of the diluted sperm suspension (2×10^5 motile sperm cells/drop). The medium was covered with sterile, lightweight paraffin oil (Fisher Scientific Co., Fair Lawn, NJ) and incubated for 3 hr in a 5% CO_2 in air, humidified environment (37°C).

After coincubation, loosely bound spermatozoa were removed from the vitelli surface of each ovum by a series of three washings. Ova were mounted on a siliconized microscopic slide as described previously (Howard et al., 1990b). Using phase-contrast microscopy (×400), hamster ova were examined and scored for: 1) sperm–ovum interaction, 2) numbers of bound spermatozoa/ovum, 3) morphological structure of bound spermatozoa, and 4) ovum penetration. Sperm–ovum interaction was determined as the percentage of inseminated ova with spermatozoa either binding the vitelline membrane or penetrating the vitellus and having at least one swollen sperm head. Spermatozoa remaining on the vitelline membrane following three washes were recorded as bound. The morphological integrity of spermatozoa bound to the vitelline membrane also was determined by examining 100 spermatozoa bound to five to ten ova and then classifying according to normal or abnormal structure. Ovum penetration was defined by the presence of a swollen or decondensed sperm head, with a corresponding flagellum in the vitellus. The penetration results were expressed as 1) percent penetration rate, which was the number of ova penetrated divided by the total number of ova inseminated times 100, and 2) a penetration index, calculated as the total number of decondensed sperm heads divided by the total number of ova inseminated.

Zona-Intact Domestic Cat Ovum Assay

Eight electroejaculates (two per male) were used to study the interaction between leopard cat spermatozoa and domestic cat ovarian oocytes. The recovery, processing, and incubation of cat oocytes obtained from antral follicles have been described recently (Howard et al., 1990b). In brief, ovaries from ovariohysterectomized domestic cats were maintained in physiological saline at 5°C for 1–3 hr before processing. Based on results from the hamster ovum assay described above and previous domestic cat studies (Howard et al., 1988, 1990b), it was determined that mKRB was the medium of choice. Ovaries were punctured repeatedly with a 22 gauge needle to release cumulus–oocyte complexes into mKRB. Only oocytes with homogeneously dark vitelli and tightly compacted corona radiata and cumulus cell masses were used (Goodrowe et al., 1988). Oocytes were cultured for 16 hr in fresh mKRB medium under lightweight paraffin oil (Fischer Scientific Co.) in a 5% CO_2 in air, humidified environment (37°C). To remove cumulus cells, oocytes were transferred to mKRB medium containing 0.2% hyaluronidase (Type I-S; Sigma Chemical Co.) for 15 min (37°C), washed three times, and then manipulated individually using a siliconized micropipette (~ 170 μm diameter).

For insemination, a 20 μl aliquot of each processed sperm suspension (2×10^5 motile sperm cells/aliquot) from each leopard cat was placed in a 35 × 10 mm culture dish, and 20 domestic cat oocytes were added. Following a 3 hr coincubation, ova were washed three times in fresh mKRB medium using a fine-bore micropipette to remove loosely bound spermatozoa. Using differential interference-contrast microscopy (×320), each oocyte was examined using a micromanipulator-holding pipette for maneuvering the oocyte during examination (Howard et al., 1990b). Spermatozoa remaining on the zona pellucida were recorded as bound, whereas spermatozoa within the zona pellucida in the same focal plane as the vitellus were classified as penetrated. Sperm–zona interaction (defined as the percentage of ova with spermatozoa binding to or penetrating the zonae pellucidae) was used as an index of the functional ability of spermatozoa to bind to and penetrate the zonae pellucidae of heterologous oocytes. The percent zona penetration rate was defined as the number of ova with spermatozoa penetrating the zonae pellucidae divided by the total number of ova inseminated times 100. A zona penetration index was calculated as the total number of spermatozoa penetrating the zonae pellucidae divided by the number of ova inseminated.

Statistical Analysis

In the sperm treatment study and the hamster ovum penetration study, each animal was considered a complete replicate (block) for each sperm treatment (UR, DR, NS, and SU groups) and medium (BWW and mKRB) combination. Medium was assigned within animal, whereas treatment was assigned within ejaculate. Analysis of the domestic cat oocyte study was based on a randomized complete block design, with each male considered as one complete replicate (block) for each sperm treatment. Similarly, hormonal data were assessed using a completely randomized block design, with an individual animal considered as a replicate for each blood sampling collection time (pre-EE and post-EE). All data were analyzed using a general linear models program (SAS, 1986). Dependent variables presented as percentages were analyzed using raw and arc–sin-transformed data. Because analysis of variance revealed no interpretive difference between the raw and transformed data, results were presented as least squares means ± standard error of the means

TABLE 1. Seminal Traits Including the Incidence of Morphologically Normal and Abnormal Spermatozoa in Leopard Cats (n = 24 Ejaculates)

Ejaculate volume (μl)	148.3 ± 14.9[a]
Sperm concentration ($\times 10^6$/ml)	37.0 ± 5.4
Sperm motility (%)	73.8 ± 2.6
Sperm status[b]	3.5 ± 0.1
Sperm motility index[c]	72.3 ± 2.4
Motile spermatozoa/ejaculate ($\times 10^6$)	4.0 ± 0.7
Structurally normal spermatozoa (%)	65.4 ± 2.0
Structurally abnormal spermatozoa (%)	
Macrocephalic	0.6 ± 0.1
Microcephalic	0.0 ± 0.0
Bicephalic	0.0 ± 0.0
Tricephalic	0.0 ± 0.0
Mitochondrial sheath aplasia	0.0 ± 0.0
Coiled flagellum	3.6 ± 0.7
Bent midpiece with droplet	10.7 ± 1.4
Bent midpiece without droplet	2.8 ± 0.6
Bent flagellum with droplet	1.6 ± 3.0
Bent flagellum without droplet	6.0 ± 1.2
Proximal cytoplasmic droplet	3.4 ± 0.6
Distal cytoplasmic droplet	5.9 ± 1.8

[a]Values are means ± S.E.M.
[b]Sperm status was based on a scale of 0–5; 5 = most rapid forward progression.
[c]Sperm motility index was an average of 1) percent sperm motility and 2) sperm status × 20.

(S.E.M.) of raw values. When a significant F value was calculated ($P < 0.05$), differences among means were determined by a least significant difference (LSD) multiple-comparison procedure. Correlation coefficients were calculated between specific hormonal data and ejaculate characteristics.

RESULTS

Leopard Cat Ejaculate Characteristics and Hormone Concentrations

Semen containing motile spermatozoa was obtained from each leopard cat at each electroejaculation. All ejaculates met the minimum motility criteria, allowing subsequent processing and coculture with either hamster ova or domestic cat oocytes. Based on a total of 24 electroejaculates, mean ejaculate characteristics are provided in Table 1. Fewer than 35% of all spermatozoa were pleiomorphic, and the most prevalent abnormality included cells with a bent midpiece containing a cytoplasmic droplet within the bent angle. Sperm head defects (including macro- or microcephaly and bi- or tricephaly) affected fewer than 1% of all cells.

Seminal traits, including sperm motility, progressive status, motility index, and structural morphology, were influenced ($P < 0.01$) by processing treatment but not by dilution medium ($P > 0.05$). Therefore, results for the BWW and mKRB subgroups in the zona-free hamster ovum assay were pooled for the comparisons presented in Table 2. Diluting raw ejaculate (DR) or resuspending the ejaculate without swim-up processing (NS) reduced ($P < 0.05$) sperm motility ratings and the number of structurally normal spermatozoa. The decrease in the proportion of normal spermatozoa resulted from ~70% increase in the number of cells with a bent flagellum and a ~10% increase in those with a deformed midpiece. Increased flagellar bending occurred both in structurally normal cells and in spermatozoa afflicted with a proximal or distal cytoplasmic droplet. When compared to undiluted raw ejaculate (UR) or the DR and NS aliquots, the SU technique improved ($P < 0.05$) sperm motility, progressive status, and sperm motility index. Compared to DR and NS treatments, SU processing also increased ($P < 0.05$) the proportion of structurally normal spermatozoa.

Hormone concentrations within individual males fluctuated randomly among times of semen collection and were not correlated ($P > 0.05$) with any ejaculate characteristic measured concurrently on the same day. The maximum ranges in individual hormone concentrations within a given male over different semen collection days were FSH 21.3–44.1 ng/ml, LH 0.5–9.3 ng/ml, and testosterone 1.5–5.8 ng/ml. Occasionally, modest differences were observed between the pre-EE and post-EE blood sample taken on the same day from an individual, but overall mean FSH, LH, and testosterone concentrations did not vary ($P > 0.05$) between the pre-EE (24.9 ± 1.5 ng/ml, 3.0 ± 0.5 ng/ml, 3.3 ± 0.4 ng/ml, respectively) and post-EE (25.4 ± 1.7 ng/ml, 2.9 ± 0.4 ng/ml, 3.2 ± 0.3 ng/ml, respectively) collection periods. The overall correlation coefficients between simultaneous measurements of serum FSH and LH ($r = -0.3$), FSH and testosterone ($r = -0.2$), and LH and testosterone ($r = -0.2$) were nonsignificant ($P > 0.05$).

Zona-Free Hamster Ovum Assay

Spermatozoa from each leopard cat male were capable of interacting with zona-free hamster ova as demonstrated by high sperm–ovum interaction values (Table 3). Neither culture medium nor the DR, NS, and SU sperm treatments influenced ($P > 0.05$) the ability of spermatozoa to interact with zona-free hamster ova. Culture medium also had no effect ($P > 0.05$) on the mean number of bound spermatozoa/ovum. Although all inseminants contained a standardized sperm concentration (2×10^5 motile cells/drop), the mean number of bound sperm/ovum varied ($P < 0.01$) among the DR, NS, and SU groups, with the SU treatment resulting in fewer total and morphologically normal spermatozoa bound. With one exception (microcephalic spermatozoa), each type of sperm abnormality was observed bound to ova (Table 4). However, regardless of sperm treatment, bound spermatozoa were proportionally more structurally normal than the percentages measured in the original inseminants (Table 4).

Leopard cat spermatozoa also were capable of penetrating hamster ova as demonstrated by the presence of decondensed sperm heads within the ooplasm (Fig. 1). The overall penetration index and penetration rate

TABLE 2. Influence of Dilution, Centrifugation, and Swim-Up Separation on Sperm Motility and Structural Morphology in Leopard Cat Ejaculates Used for Co-Incubation With Zona-Free Hamster Ova and Zona-Intact Domestic Cat Oocytes

	Sperm treatment			
	Undiluted raw	Diluted raw	Non-swim-up	Swim-up
Zona-free hamster ovum assay[1]				
Sperm motility (%)	77.5 ± 1.5[a]	69.1 ± 1.5[b]	66.9 ± 1.5[b]	82.8 ± 1.5[c]
Sperm status	3.5 ± 0.1[a]	3.1 ± 0.1[b]	3.5 ± 0.1[a]	4.0 ± 0.1[c]
Sperm motility index	74.1 ± 1.7[a]	65.5 ± 1.7[b]	69.1 ± 1.7[b]	81.7 ± 1.7[c]
Normal spermatozoa (%)	65.4 ± 2.0[a]	51.0 ± 2.0[b]	50.0 ± 2.0[b]	62.4 ± 2.0[a]
Zona-intact domestic cat oocyte assay[2]				
Sperm motility (%)	68.3 ± 2.1[a]	60.0 ± 2.1[b]	61.9 ± 2.1[b]	78.9 ± 2.1[c]
Sperm status	3.6 ± 0.1[a]	2.6 ± 0.1[b]	3.1 ± 0.1[b]	3.8 ± 0.1[a]
Sperm motility index	69.8 ± 1.9[a]	56.3 ± 1.9[b]	62.2 ± 1.9[b]	76.3 ± 1.9[c]
Normal spermatozoa (%)	63.2 ± 2.0[a]	48.0 ± 2.0[b]	49.0 ± 2.0[b]	60.0 ± 2.0[a]

[1]Values are means ± pooled S.E.M. of combined media treatment groups (BWW and mKRB). Each value is based on 16 ejaculates from four male leopard cats (four ejaculates/male).
[2]Values are means ± pooled S.E.M. of mKRB medium group. Each value is based on eight ejaculates from four males (two ejaculates/male).
[a,b,c]Within rows, means with different superscripts differ ($P < 0.05$).

TABLE 3. Influence of Culture Medium and Seminal Processing on Penetration of Zona Pellucida-Free Hamster Ova by Leopard Cat Spermatozoa

	Total number of ova	Sperm–ovum interaction (%)[1]	Number of bound sperm/ovum	Total number of normal bound sperm/ovum	Penetration index[2]	Penetration rate (%)[3]
Medium						
BWW medium						
Diluted raw	160	100.0 ± 0.1[a4]	42.6 ± 6.4[a,b]	30.4 ± 3.2[a,b]	0.03[a]	3.1[a]
Non-swim-up	160	98.8 ± 0.1[a]	28.4 ± 6.4[a,c]	20.8 ± 3.2[a,c]	0.06[a]	6.9[a]
Swim-up	160	96.3 ± 0.1[a]	14.0 ± 6.4[c]	10.8 ± 3.2[c]	0.03[a]	2.5[a]
mKRB medium						
Diluted raw	160	97.5 ± 0.1[a]	51.2 ± 6.4[b]	38.9 ± 3.2[b]	0.09[a,b]	8.1[a,b]
Non-swim-up	160	93.1 ± 0.1[a]	34.3 ± 6.4[b,c]	26.1 ± 3.2[b,c]	0.19[b]	14.4[b]
Swim-up	160	90.1 ± 0.1[a]	21.2 ± 6.4[c]	17.4 ± 3.2[c]	0.08[a,b]	8.1[a,b]
Overall						
BWW medium	480	98.3 ± 0.1[a]	28.3 ± 5.7[a]	20.7 ± 5.0[a]	0.04[a]	4.2[a]
mKRB medium	480	93.6 ± 0.1[a]	35.6 ± 5.7[a]	27.6 ± 5.0[a]	0.12[b]	10.2[b]
Overall						
Diluted raw	320	98.8 ± 0.1[a]	46.9 ± 4.5[a]	34.5 ± 2.7[a]	0.06[a]	5.6[a]
Non-swim-up	320	95.9 ± 0.1[a]	31.4 ± 4.5[b]	23.4 ± 2.7[b]	0.13[b]	10.6[b]
Swim-up	320	93.2 ± 0.1[a]	17.6 ± 4.5[c]	14.0 ± 2.7[c]	0.05[a]	5.3[a]

[1]Sperm–ovum interaction was the percentage of ova with spermatozoa either binding to the vitelline membrane or penetrating the vitellus and demonstrating swollen sperm heads.
[2]Penetration index was equal to the total number of decondensed sperm heads divided by the number of ova inseminated.
[3]Penetration rate was the number of ova with decondensed sperm heads divided by the total number of ova inseminated multiplied by 100.
[4]Values are means ± S.E.M.
[a,b,c]Within columns of each group, values with different superscripts differ ($P < 0.05$).

were 0.08% and 7.2%, respectively. Penetration was influenced ($P < 0.05$) by culture medium; the use of mKRB increased the penetration index by threefold and the penetration rate by 2.5-fold compared to BWW (Table 3). Penetration rate also was affected by sperm treatment, with the NS aliquots demonstrating a greater ($P < 0.05$) mean penetration index and penetration rate than the DR or SU groups. A higher ($P < 0.05$) incidence of polyspermic penetration, which is reflected in the penetration index value, was detected following gamete incubation in mKRB compared to BWW (Table 3). Similarly, a higher incidence of polyspermy was observed in the NS aliquots than in the DR and SU groups.

Zona-Intact Domestic Cat Oocyte Assay

Leopard cat spermatozoa were capable of binding to and penetrating the zona pellucida of domestic cat oocytes (Fig. 2), and, with one exception, the data were unaffected by sperm processing treatment (Table 5). Although zona penetration rate was not influenced, the zona penetration index was increased ($P < 0.05$) by

TABLE 4. Morphological Characteristics of Inseminated Spermatozoa Compared to Spermatozoa Bound to Zona-Free Hamster Ova in Leopard Cats

	Sperm treatment					
	Diluted raw		Non-swim-up		Swim-up	
	Inseminant	Bound	Inseminant	Bound	Inseminant	Bound
Normal spermatozoa (%)	51.0 ± 2.0[a1]	73.6 ± 1.2[c]	50.0± 2.0[a]	74.6 ± 1.2[c]	62.4 ± 2.0[b]	79.8 ± 1.2[c]
Abnormal spermatozoa (%)						
Microcephalic	0.3 ± 0.2	0.0 ± 0.0	0.5 ± 0.2	0.0 ± 0. 0	0.1 ± 0.1	0.0 ± 0.0
Coiled flagellum	3.8 ± 0.7	1.9 ± 0.6	4.3 ± 1.2	1.8 ± 0.5	0.6 ± 0.3	0.8 ± 0.4
Bent midpiece with droplet	10.2 ± 2.0	1.5 ± 0.3	9.6 ± 1.5	1.2 ± 0.6	6.5 ± 0.8	0.8 ± 0.3
Bent midpiece without droplet	4.8 ± 1.0	7.1 ± 1.6	5.5 ± 1.1	5.8 ± 1.3	7.5 ± 1.5	4.8 ± 1.5
Bent flagellum with droplet	7.3 ± 2.6	0.4 ± 0.2	7.3 ± 2.9	1.3 ± 1.2	7.0 ± 2.5	0.1 ± 0.1
Bent flagellum without droplet	18.8 ± 2.8	13.1 ± 3.2	20.5 ± 2.7	14.5 ± 3.6	13.7 ± 1.8	13.5 ± 3.4
Proximal cytoplasmic droplet	1.9 ± 0.6	1.1 ± 0.6	1.3 ± 0.4	0.4 ± 0.3	0.9 ± 0.3	0.1 ± 0.1
Distal cytoplasmic droplet	1.9 ± 0.6	1.3 ± 0.6	1.0 ± 0.4	0.4 ± 0.2	1.3 ± 0.6	0.1 ± 0.1

[1]Values are means ± S.E.M. of combined media groups (BWW and mKRB).
[a,b,c]Within the inseminant and bound category for normal spermatozoal morphology, means with different superscripts differ ($P < 0.05$).

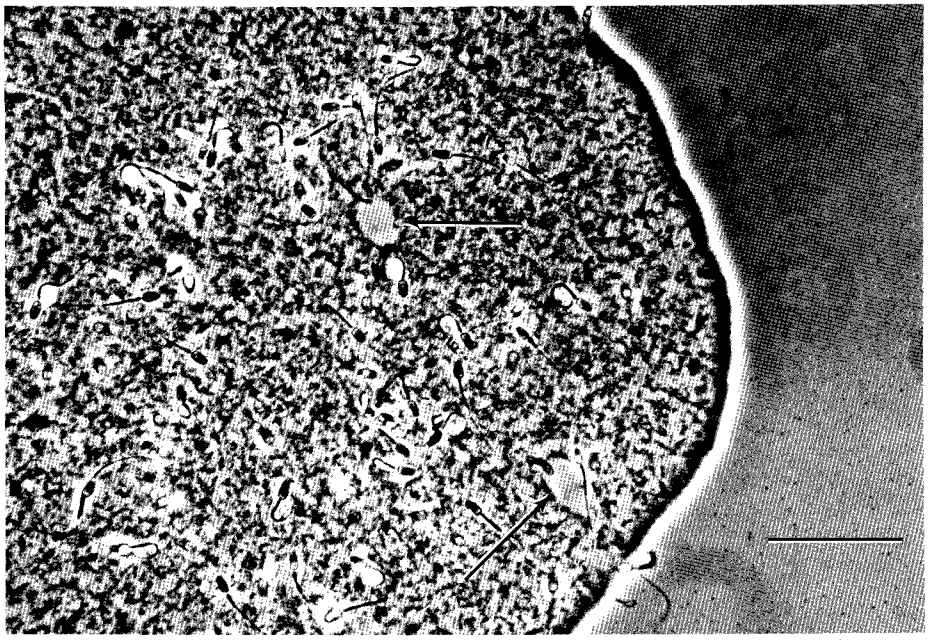

Fig. 1. Penetration of a zona-free hamster ovum by leopard cat spermatozoa. Multiple swollen sperm heads (arrows) with attached flagellae are visible in the vitellus of the ovum. Bar = 40 μm.

24–44% by the NS treatment compared to the DR and SU treatments (Table 5).

Data on the Basis of Individual Males

Because of the limited number of animals available for study, little emphasis was placed on potential differences among individual males. However, one important observation appeared worthy of note. During the study, there were no apparent differences in ejaculate characteristics among males except that male 4 consistently produced fewer structurally normal spermatozoa/ejaculate than males 1–3 (Table 6). For male 4, the penetration rates of hamster ova were 60–79% less and domestic cat oocytes 39–61% less than were measured in males 1–3.

DISCUSSION

Although the domestic cat and leopard cat are of the same genus *Felis* and electroejaculate volume and sperm motility ratings were comparable, domestic cats subjected to the same electroejaculation protocol as leopard cats consistently produce greater sperm concentrations (mean range 167–361 × 10^6/ml) and more motile spermatozoa/ejaculate (mean range 17–24 ×

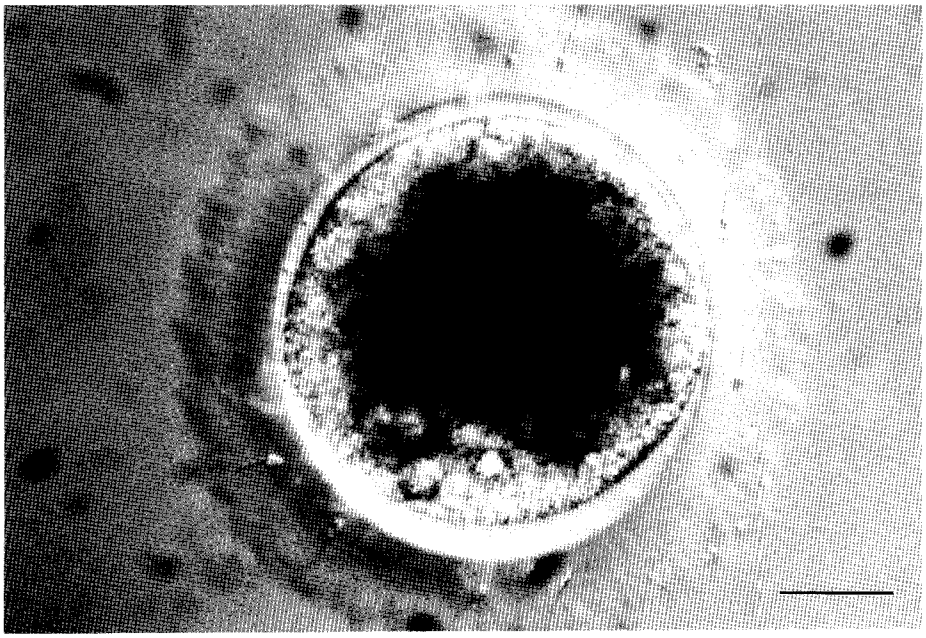

Fig. 2. Penetration of a zona-intact domestic cat ovum. Leopard cat spermatozoa are visible within the zona pellucida. Bar = 30 μm.

TABLE 5. Influence of Seminal Processing on Penetration of Zona Pellucida-Intact Domestic Cat Oocytes by Leopard Cat Spermatozoa

	Total number of ova	Sperm-zona interaction (%)[1]	Number of bound sperm per ovum	Zona penetration index[2]	Zona penetration rate[3] (%)
Sperm treatments					
Diluted raw	160	80.0 ± 0.1[4]	3.3 ± 0.8	1.4[a]	51.3
Non-swim-up	160	78.8 ± 0.1	2.3 ± 0.8	2.5[b]	60.0
Swim-up	160	69.4 ± 0.1	1.8 ± 0.8	1.9[a]	53.1
Overall	480	76.1 ± 0.1	2.5 ± 0.7	1.9	54.8

[1]Sperm-zona interaction was the percentage of ova with spermatozoa binding to or penetrating the zonae pellucidae.
[2]Zona penetration index was equal to the total number of spermatozoa penetrating the zonae pellucidae divided by the number of ova inseminated.
[3]Zona penetration rate was the number of ova with spermatozoa penetrating the zonae pellucidae divided by the total number of ova inseminated times 100.
[4]Values are means ± S.E.M. Each value is based on 8 ejaculates from 4 males (2 ejaculates/male).
[a,b,c]Within columns, values with different superscripts differ ($P < 0.05$).

10^6) (Howard et al., 1990a,b). The diminished number of spermatozoa in the leopard cat ejaculate may be illustrative of nondomestic felids in general, because low concentrations are observed in the electroejaculates of the cheetah (27×10^6/ml), clouded leopard (28×10^6/ml), tiger (32×10^6/ml), lion (3×10^6/ml), leopard (46×10^6/ml), and puma (22×10^6/ml) (Wildt et al., 1983, 1986, 1987a,b, 1988). Unlike most of the latter species, however, and similar to most domestic cat populations, the leopard cat produced a relatively high proportion of structurally normal spermatozoa, which was almost identical to that reported for normospermic domestic cats (Howard et al., 1990a).

Basal FSH and LH concentrations in leopard cats mimicked almost exactly those levels measured in normospermic domestic cats. In contrast, testosterone production in leopard cats was two- to fourfold greater than in normospermic domestic cats and eight- to ninefold greater than in teratospermic domestic males. Another unique dissimilarity between the two species was the ability of leopard cats to sustain testosterone concentrations during the ~45 min anesthesia–electro-

TABLE 6. Sperm Morphology and Penetration Rate (%) of Zona-Free Hamster Ova and Zona-Intact Domestic Cat Oocytes in Individual Male Leopard Cats*

	Individual male leopard cats			
	1	2	3	4
Normal spermatozoa (%)	68.8 ± 1.9	76.6 ± 1.8	67.9 ± 1.5	48.3 ± 2.2
Zona-free hamster ovum assay				
Overall				
BWW medium	2.5	4.2	10.0	0.8
mKRB medium	15.0	8.3	13.3	4.2
Overall				
Diluted raw	6.3	7.5	7.5	1.3
Non-swim-up	15.0	7.5	18.8	3.8
Swim-up	5.0	3.8	8.8	2.5
Overall penetration rate	8.8	6.3	11.7	2.5
Zona-intact domestic cat oocyte assay				
Overall				
Diluted raw	65.0	60.0	45.0	35.0
Non-swim-up	87.5	80.0	45.0	27.5
Swim-up	65.0	75.0	50.0	22.5
Overall penetration rate	72.5	71.7	46.7	28.3

*Values are based on a total of six ejaculates/male.

ejaculation interval. Carter et al. (1984) first reported that serum testosterone levels decline ~78% in anesthetized cats bled over an 84 min period. The fall in testosterone secretion is unrelated to any simultaneous increase in adrenal glucocorticoid production. Since then, two other studies have reported that peripheral testosterone concentrations decrease by a similar magnitude in conscious male domestic cats during the first 30–60 min of blood sampling (Goodrowe et al., 1985) and in anesthetized-electroejaculated cats subjected to a 40 min bleeding interval (Howard et al., 1990a) for reasons that remain unknown. The lack of a similar response in leopard cats handled similarly to the domestic cats of the Carter et al. (1984) and Howard et al. (1990a) studies reaffirmed our previous suggestions (Wildt et al., 1988) that some felid species have evolved strikingly different endocrine mechanisms for controlling and sustaining gonadal function.

Adding tissue culture media to the raw ejaculate of the leopard cat increased pleiomorphic sperm numbers by increasing the proportion of cells with a bent flagellum or midpiece. Similar flagellar derangement has been described in epididymal ram (Amann et al., 1982) and rat (Cooper, 1986) spermatozoa and in ejaculated bull (Lindahl and Drevius, 1964) and human (Makler et al., 1981) spermatozoa exposed to hypotonic medium in vitro. Drevius (1963) proposed that hypotonic conditions alter the plasma membranes of bull spermatozoa, which causes osmotic swelling and induces flagellar bending and coiling. We retrospectively analyzed the osmolality of leopard cat seminal plasma and the BWW and mKRB diluting media using a vapor pressure osmometer. The osmolality of leopard cat seminal plasma was 326 mmol/kg, similar to that reported for domestic cat seminal plasma (Johnston et al., 1988). In contrast, the osmolality of both media ranged from 275 to 280 mmol/kg, suggesting that osmotic stress may be a cause of increased flagellar bending after tissue culture dilution. SU processing, however, negated most of the detrimental effects of dilution by increasing the number of normal spermatozoa compared to the DR and NS treatment groups.

This study provides evidence that leopard cat spermatozoa are capable of penetrating heterologous oocytes in vitro. Spermatozoa from one other species of nondomestic felid, the Siberian tiger (*Panthera tigris altaica*), have been shown to penetrate zona-free hamster ova (Post et al., 1987; Byers et al., 1987, 1989). In this species, this bioassay was effective for assessing various preincubation and coculture conditions and sperm functionality. Optimal ovum penetration by tiger spermatozoa was observed after a 2 hr preincubation at 37°C and a 3 hr coincubation (Byers et al., 1987, 1989). Because the medium used in the tiger studies was BWW, it is possible that tiger spermatozoa are similar to leopard cat and domestic cat spermatozoa and would experience greater penetration rates if mKRB medium was used. The observation that leopard cat spermatozoa readily bind and penetrate the zona pellucida of domestic cat oocytes was not surprising since these species are able to hybridize naturally (O'Brien, unpublished observation). The ability of spermatozoa from species further removed on the evolutionary tree to fertilize domestic cat oocytes remains to be tested.

The functional ability of leopard cat spermatozoa to penetrate zona-free hamster ova and bind and penetrate zona-intact domestic cat oocytes was almost identical to that observed in normospermic domestic cats (Howard et al., 1988, 1990b). Of particular interest was the similarity in sperm–ovum interaction, number of sperm bound/ovum, penetration index, and penetration

rate between species within bioassays. In the context of the cat oocyte assay, this suggests that spermatozoa from both species are equally susceptible to the mechanisms controlling the number of spermatozoa physically bound to the oocytes. In the domestic cat study, spermatozoa from normospermic males were three to six times more likely to penetrate zona-free hamster ova and almost four times more likely to penetrate zona-intact cat oocytes than spermatozoa from teratospermic ejaculates (Howard et al., 1990b). Because the incidence of sperm pleiomorphisms was the primary difference between these two cat populations, we concluded that the most significant factor dictating nuclear decondensation within the hamster ovum and binding and penetration of zona-intact oocytes was the morphological integrity of the spermatozoa in the preprocessed ejaculate. The data from the individual leopard cats appeared to support these earlier observations; the only male consistently producing higher proportions of abnormal spermatozoa also consistently demonstrated the lowest penetration rates of hamster ova and domestic cat oocytes.

Deleterious effects of seminal plasma on the hamster ovum assay and homologous in vitro fertilization systems have been observed (Kanwar et al., 1979; Rogers, 1985). Chang (1957) first demonstrated that previously capacitated spermatozoa could be "decapacitated" by treatment with seminal plasma. Components in seminal plasma absorb onto, or integrate within, the sperm plasma membrane, and removing or altering these substances triggers the capacitation event by sensitizing the membrane and causing the acrosome reaction (Yanagimachi, 1988). These seminal plasma factors normally are lost in the female reproductive tract during the prefertilization interval or in vitro during routine spermatozoal processing and incubation (Yanagimachi, 1988). In the domestic cat, removing seminal plasma from the spermatozoa is not a prerequisite for sperm–oocyte interaction; neither heterologous nor homologous ovum penetration in vitro is enhanced by centrifuging the semen and decanting the seminal plasma (Howard et al., 1990b). Likewise, the presence of seminal plasma does not affect the ability of tiger spermatozoa to penetrate zona-free hamster ova (Byers et al., 1987, 1989). In contrast, removing seminal plasma from leopard cat spermatozoa enhanced penetration of hamster ova and domestic cat oocytes twofold compared to the noncentrifuged sperm treatment. Therefore, it appears that leopard cat spermatozoa are sensitive to the presence of seminal plasma.

SU processing increased sperm motility, status, and proportion of structurally normal spermatozoa in leopard cat inseminants. Our results confirm previous observations that the SU technique improves a sperm population by separating cells on the basis of motility and structural morphology (Russell and Rogers, 1987). This improvement, however, had no impact on ovum penetration rates in either bioassay, which may be related to the observation that this technique reduced the total number of spermatozoa in the SU aliquots. Although all inseminants contained a standardized concentration of motile spermatozoa, SU samples contained fewer total spermatozoa/inseminant because these aliquots had higher motility ratings and fewer immotile cells. In the SU treatments, fewer leopard cat spermatozoa were bound to the vitelline membrane of hamster oocytes and to the zonae pellucidae of domestic cat oocytes compared to the DR and NS groups. This suggested that the diminished number of spermatozoa in the SU inseminants had a reduced chance of colliding with or being attracted to oocytes. Therefore, it appears that removing the seminal plasma from leopard cat semen maximizes sperm binding and ovum penetration. In contrast, the SU processing technique offers no advantages and, in fact, appears to reduce the total number of viable spermatozoa available for insemination, a problem particularly relevant in a species, such as the leopard cat, that tends to produce low sperm concentrations in the raw ejaculate.

The penetration index and rate were increased when leopard cat spermatozoa and hamster oocytes were preincubated and coincubated in mKRB compared to BWW medium, similar to that observed in normospermic domestic cats (Howard et al., 1990b). Therefore, it appears that spermatozoa from these two felid species were equally sensitive to medium composition and that the slightly greater concentration of BSA and sodium pyruvate in mKRB facilitated sperm fusion and penetration of the zona pellucida. Data now available from two felid studies suggest that there is greater sperm–ovum interaction with mKRB than with BWW, which is interesting in that BWW is the traditional medium used with the zona-free hamster ovum penetration assay.

In summary, this study has provided some baseline ejaculate and endocrine norms for the leopard cat. This species deserves further attention because of its endangered status and also because, unlike the majority of nondomestic felids, it produces relatively high proportions of structurally normal spermatozoa. Leopard cat spermatozoa bind and/or penetrate heterologous oocytes in vitro at a rate similar to that observed in the closely related domestic cat, suggesting that fertilization strategies between these species are extremely conserved. Both heterologous bioassays provided complimentary results, and both offered different types of information on sperm affinity and ability to penetrate oocyte investments. These tests appear particularly valuable for understanding basic gamete physiology in species in which "controlled" and classical experimental studies are restricted by the limited numbers of available animals. Assuming that spermatozoa capable of binding and penetrating a heterologous oocyte will interact similarly with a homologous ovum, these bioassays also offer opportunities for developing and improving artificial approaches to animal propagation.

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