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Male reproductive traits, semen cryopreservation, and heterologous in vitro fertilization in the bobcat (*Lynx rufus*)

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Abstract

There is limited information on bobcat ejaculate traits and sperm cryopreservation and fertilizing ability. Bobcats were electroejaculated under general anesthesia in November (autumn) and April (spring), and endocrine and sperm traits were characterized. Testosterone (mean ± SEM: 0.90 ± 0.15 ng/mL) was not different between sampling times, but cortisol (average: 13.95 ± 1.73 μg/dL) was significantly higher in April. Average number of spermatozoa was $10.0 \pm 3.4 \times 10^6$ sperm/ejaculate, with values being significantly higher in April. Sperm motility (average 55.7 ± 5.8% motile sperm) was not different between sampling times. The proportion of normal spermatozoa in the ejaculate (average: 14.7 ± 2.1%) was significantly higher in April, but the percentage of spermatozoa with intact acrosomes (average: 43.7 ± 3.8%) was significantly higher in autumn. Spermatozoa were cryopreserved in a Tes-Tris–based diluent (TEST) or Biladyl, both containing 20% egg yolk and 4% glycerol. Diluted sperm were loaded into straws, refrigerated using a programmable thermoblock with a dry chamber, frozen in nitrogen vapors, thawed, and incubated in F-10 medium with 5% fetal bovine serum for up to 3 h. After cryopreservation in TEST, there were about 50% motile sperm upon thawing, and survival was high during incubation post-thaw. Cryopreservation in Biladyl led to similar results, but motility decreased substantially during incubation post-thaw. Bobcat spermatozoa fertilized domestic cat oocytes matured in vitro. Fertilization rates were higher for sperm collected in April and cryopreserved in TEST (46%) than for those cryopreserved using Biladyl (<3%). Fertilized oocytes cleaved in culture, and some (27%) reached the morula stage. This study has allowed us to gain further baseline information on bobcat reproduction, explore sperm cryopreservation conditions, and show that fertilizing capacity can be tested using in vitro–matured cat oocytes. These results will be important for future conservation efforts.

Keywords: Bobcat; Cryopreservation; Fertilization; Spermatozoa; Testosterone

1. Introduction

Bobcats (*Lynx rufus*) are found mainly in the United States, but they also occur in northern Mexico and southern Canada [1]. The bobcat lives in a variety of habitats, including subtropical coastal swamps in the southeast, arid areas in the northwest, and temperate

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forests in the north of the United States [1–3]. In Mexico, bobcats can be found in dry scrub and grassland and in tropical dry forest [4]. The total bobcat population in the United States was estimated in the early 1980 s to be between 725,000 and 1 million. Loss of habitat is regarded as the primary threat to bobcats, and the species is legally hunted in the three countries. In the past 20 years, bobcats have been the most heavily harvested and traded of the cat species [3]. It has been reported that more than 50,000 skins were exported from the United States in 2006 alone [4].

The bobcat is a species of ''least concern'' according to the Red List of Threatened Species [4], and it is included in category 5a of global and category 4 of America's regional ranking of cat species vulnerability established by the IUCN/SSC Cat Specialist Group [3]. It is also included on CITES Appendix II.

Adult bobcats are reproductively active until death [5]. Males show seasonal reduction in spermatogenic activity during summer and early autumn, although such reduction may be absent in older males. The breeding season varies with latitude, longitude, altitude, and climatic variations [5]. Breeding may happen throughout the year, although a peak occurs from December to August, with most births in April and May [1,5]. Early breeders may have a second litter late in the year [1,5]. Bobcats are polygynous [6].

Baseline information on bobcat reproductive traits is scarce. Captive breeding, mainly in zoos, reveals that many individuals fail to reproduce under these conditions. Only three kittens have been reportedly born in the past 12 months in registered centers [7]. In addition to its inherent conservation value, the bobcat may be regarded as an important model for phylogenetically close species such as the critically endangered Iberian lynx (*Lynx pardinus*) or the Eurasian lynx (*Lynx lynx*), which has suffered severe population declines in certain European areas. The use of related species as models for threatened ones has been widely recognized [8,9].

The use of cryopreserved spermatozoa has been a major focus in assisted reproduction for the study and management of wild populations [10–12]. Semen cryopreservation allows the transport of male genetic information within or between populations without requiring the actual translocation of animals [9], and this has been recognized as a valuable approach for *in situ–ex situ* linkage [9,13]. Species and individuals differ in the ability of their spermatozoa to survive cryopreservation, and there is a need to understand the underlying basis of such differences [14,15]. Cryopreservation results in a decrease in the proportion of motile spermatozoa and in the proportion of sperm cells with an intact acrosome [16]. Both parameters are essential for sperm fertilizing ability [17–20]. In many felids, the high proportion of abnormal spermatozoa appears to be related to the ability of spermatozoa to survive cryopreservation; this trait is also an important determinant of fertilizing ability [21].

Basic semen analyses provide only limited information regarding the sperm's ability to participate in fertilization [17,22]. Efforts have been directed in various species to examine the fertilizing ability of spermatozoa. Although the ideal means to test for fertilizing ability is artificial insemination, in endangered species, or in species for which a limited number of individuals are kept in captivity, this approach is not feasible and other tests are required. The functional capacity of spermatozoa can thus be examined using salt-stored or live heterologous oocytes that have been matured in vitro. In wild felids, recent studies have demonstrated the capacity of spermatozoa to fertilize oocytes recovered from domestic cats matured in vitro [23–25], but no studies exist examining fertilizing ability of bobcat spermatozoa.

The aims of this study were thus to (1) characterize bobcat semen traits, (2) analyze the ability of bobcat spermatozoa to survive in vitro, (3) assess two different semen cryodiluents, evaluating sperm motility and acrosome integrity, for cryopreservation of spermatozoa from this species, and (4) evaluate the fertilizing capacity of cryopreserved bobcat spermatozoa using heterologous in vitro fertilization of domestic cat oocytes matured in vitro.

2. Materials and methods

2.1. Animals

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD1201/2005, which conforms to European Union Regulation 2003/65. Procedures also adhered to the Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences and recommended by the journal.

Bobcat males (3 to 8 years old) kept at the Zoobotánico de Jerez (Jerez de la Frontera, Andalusia, Spain) were electroejaculated between spring 2004 and autumn 2007. A total of four males were used in 13 electroejaculation attempts. Two of the males were of proven fertility. All the males were kept in enclosures with visual, olfactory, and auditory contact with

conspecific males and females. The enclosures had natural vegetation and illumination, shelves, and hiding places. The animals received a balanced diet consisting of chicken and beef and, once a week, live pigeons as enrichment.

2.2. Sperm collection and evaluation

Semen collection was performed under surgical anesthesia using an intramuscular administration of tiletamine hydrochloride plus zolazepam (10 mg/kg body weight; Zoletil; Virbac, Carros, France). Animals received no food for 12 to 24 h and no liquid for 2 h before anesthesia. The level of anesthesia achieved allowed for a general examination and semen collection by electroejaculation. Occasionally, anesthesia was supplemented with isoflurane despite the risk of sphincter relaxation and urine contamination. In any case, urine contamination was rare, and when it occurred, the sample was discarded. Body weight and testicular dimensions were obtained before electroejaculation. Testes weight was calculated as described [26]. Relative testes weight was calculated as total testes weight/body weight. Blood samples were collected and sera analyzed for testosterone and cortisol in a commercial laboratory using a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Electroejaculation was carried out as described previously [17]. A rectal probe (17.9 cm length and 13.5 mm diameter; P.T. Electronics, Boring, OR, USA) with three longitudinal electrodes (35.8 mm) was placed in the rectum above the prostate and bulbourethral glands and voltage applied. A total of 100 electrical stimulations were delivered using a 60-Hz sine-wave stimulator (P.T. Electronics). Stimulations from 2 to 5 V were delivered in sets of 10 stimulations each, and the total sequence was divided into four series [17]. Semen was collected in a warm, sterile polypropylene, 25-mL cup (Lab-Center, Madrid, Spain). Semen collected in each series was evaluated immediately for volume, by measuring it with a micropipette, and for pH, using test strips (Medi-Test Combi 9; Machereg-Nagel, Düren, Germany). A semen sample was placed between a slide and coverslip (prewarmed to 37 °C) and examined under phase-contrast optics to assess the percentage of motile spermatozoa and the quality of motility (using a scale from 0 to 5, where 0 is no movement and 5 represents rapid, linear forward movement [27]). A sperm motility index (SMI) was calculated as follows: [% motile sperm + (quality × 20)]/2 [27].

In each series, 5 μL semen was fixed with 45 μL 1% glutaraldehyde solution in 0.165 M cacodylate buffer, pH 7.3. This sample was used to estimate sperm concentration by using a hemocytomer. A sample of 10 μL semen was fixed in 250 μL 4% paraformaldehyde in 110 mM Na_2HPO_4 and 2.5 mM NaH_2PO_4 buffer solution (pH 7.4), stored at 4 °C and later processed to assess sperm morphology and acrosomal integrity (see later).

The remaining semen sample was diluted with an equal volume of a HEPES-buffered Ham's F-10 medium (catalog no. 99168; Irvine Scientific, Izasa, Barcelona, Spain), supplemented with modified Ham's F-10 Gentamicin-50X (catalog no. 90127; Irvine Scientific), which was supplied lyophilized and contains 0.05 M glutamine, 0.05 M pyruvate and gentamicin sulfate (1:50 dilution; final concentration 10 μg/mL). Ham's F-10 media was also supplemented with L-glutamine (Sigma, Madrid, Spain; 2 mM final concentration), pyruvate (Sigma; 1 mM final concentration), triple-antibiotic mix (Sigma: penicillin, 130 IU/mL; streptomycin, 130 μg/mL; neomycin, 260 μg/mL, final concentrations) and 5% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco Invitrogen, Barcelona, Spain). Diluted semen was stored at room temperature pending completion of the electroejaculation series and later used for cryopreservation.

Paraformaldehyde-fixed sperm samples were stained with Coomassie brilliant blue [28,29] and used for assessments of sperm morphology and acrosome integrity. For staining, samples were centrifuged 3 times during 8 min at 1700 × *g*. After the first and second centrifugations, the supernatant was removed, discarded, and 250 μL 0.1 M ammonium acetate solution was added. After the third centrifugation, the pellet was diluted in 40 μL of the ammonium acetate solution and smeared onto two slides, which were dried at room temperature. Slides were flooded with 50 μL Coomassie blue solution (0.22% Coomassie brilliant blue in 50% methanol and 10% glacial acetic acid) for 90 s, washed with distilled water, dried at room temperature, and mounted with DPX mountant (BDH, Madrid, Spain). For each sample, a total of 100 spermatozoa were microscopically examined for morphology and an additional 100 spermatozoa for acrosome integrity using bright-field microscopy at ×1000 magnification.

Sperm morphology (Fig. 1A) was categorized as either normal or with abnormalities in the head (e.g., macrocephalic, microcephalic, bicephalic), midpiece (e.g., abnormal or bent) or the rest of the flagellum (e.g., abnormal or bent). The presence of cytoplasmic

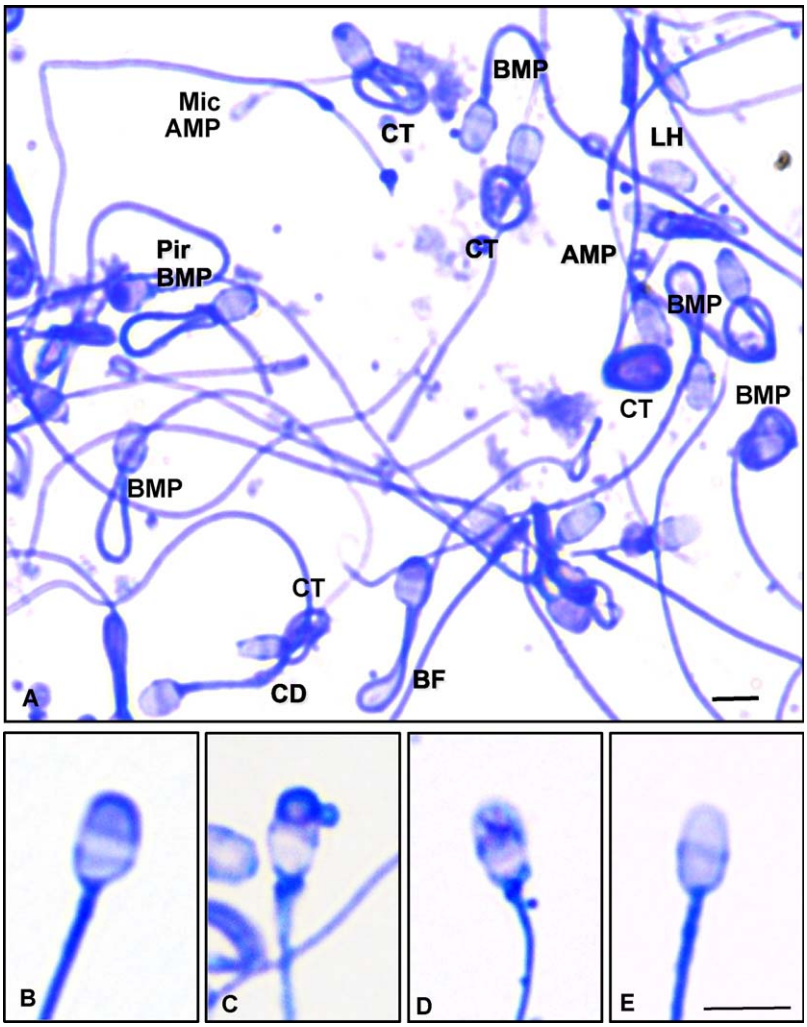

Fig. 1. Bobcat spermatozoa stained with Coomassie brilliant blue and examined using bright-field optics (total magnification ×1000). (A) Sperm morphology: N, normal; Mic, microcephalic; Pir, piriform head; AMP, abnormal midpiece; BMP, bent midpiece; BF, bent flagellum; CT, coiled tail; LH, loose (detached) head. (B–E) Acrosome status: (B) intact normal acrosome, (C) intact abnormal acrosome, (D) damaged acrosome, (E) missing acrosome. Scale bars = 5 μm.

droplets was noted but was not categorized as a separate type of abnormality; it was instead used as an indicator of sperm maturation. In addition, the presence of coiled tails and loose heads was recorded.

Acrosomal integrity (Fig. 1B–E) was classified as (1) intact normal (sperm with uniform dark staining over the acrosome region with no staining of the post-acrosomal region); (2) intact with abnormal acrosome (sperm with a uniform staining of the acrosomal region and with a darker staining of a section of the apical region); (3) damaged (sperm with patches without stain in the acrosomal region); (4) nonintact or missing (sperm with absence of acrosomal staining or staining only in the equatorial segment) [30].

2.3. Sperm incubation in medium containing different sera

The ability of bobcat spermatozoa to survive in vitro was examined by incubating them in HEPES-buffered modified Ham's F-10 medium, supplemented with Ham's F-10 Gentamicin-50X, L-glutamine, pyruvate, and triple-antibiotic (as described above) and with either 5% (v/v) heat-inactivated FBS (Gibco) or 5% (v/v) bobcat serum; the latter was inactivated by heating at 56 °C for 30 min. Both sera were filtered through a 0.22-μm filter before use.

Semen aliquots (7.5 μL), taken from Series 2 of the electroejaculation, were diluted in 67.5 μL F-10

medium with FBS or bobcat serum, placed in 1.5-mL Eppendorf tubes, and incubated in a thermoblock at 37 °C under air. At 0, 1, and 2 h, samples were taken and examined under phase-contrast optics for assessment of motility and fixed in 4% paraformaldehyde solution for subsequent staining with Coomassie brilliant blue and assessment of acrosome integrity.

2.4. Sperm cryopreservation

Semen samples from each male, diluted in HEPES-buffered Ham's F-10 with 5% FBS, having similar quality (i.e., that did not differ by more than 10% in percentage of motile spermatozoa) were pooled. Semen pools from each individual were centrifuged at 300 to 700 × *g* (depending on semen quality) for 10 min, the supernatant discarded, the volume of the loose pellet measured using a micropipette, and cryodiluent added very slowly (1:4, v:v), drop by drop, at room temperature.

Two different cryodiluents were used: (a) TEST, which consists of 4.83% Tes, 1.15% Tris, 0.4% glucose, 200 IU penicillin/mL, 200 μg streptomycin/mL, 20% egg yolk, and 4% glycerol; and (b) Biladyl, which is a commercial diluent (Minitüb, Tiefenbach, Germany) containing 2.42% Tris, 1% fructose, 1.38% citric acid, 20% egg yolk, and 4% glycerol; this diluent was supplemented with an antibiotic cocktail containing tylosin, gentamicin, spectinomycin, and lincomycin (Minitüb). Biladyl is supplied in two solutions, A and B, with 0 and 14% glycerol, respectively. The solutions were mixed to obtain a 4% final concentration of glycerol.

Straws of 0.25 mL (Minitüb) were cut in half with a pair of scissors, and the half with the cotton seal was used. A total of 50 μL sperm suspension in cryodiluent was loaded into the short straws at room temperature, and straws were then closed with a thermal sealer (ERSA; Minitüb). Straws were placed inside a 5-mL cryovial for refrigeration. Aliquots with semen in each cryodiluent were also refrigerated for evaluation of motility and acrosome integrity at the end of refrigeration.

Temperature was lowered from 20 to 5 °C over a period of 120 min using a programmable dry block (ThermoStat plus 5352; Eppendorf, Hamburg, Germany) fitted with a dry thermoblock chamber (Combi-Box; Eppendorf). A rack was placed inside the chamber to hold both the semen aliquots for evaluation at the end of refrigeration and the 5-mL cryovial containing the straws. An external digital thermometer with two thermocouples was used to monitoring temperature in the chamber and inside the cryovial.

At the end of refrigeration, straws were removed from the cryovial and frozen by using a two-step method [12]: straws were placed horizontally on a metal rack, 7.5 cm above the surface of liquid nitrogen in a Styrofoam box, and held for 1 min. Straws were then lowered quickly to a second level in the metal rack, which was 2.5 cm above the surface of liquid nitrogen, and kept for 1 min before plunging them in liquid nitrogen. Frozen straws were placed inside cryovials for storage and transferred to and kept in a liquid nitrogen tank.

2.5. Sperm thawing and assessment

Straws were thawed by exposing them to air for 10 s and immersing them in a water bath at 37 °C for 30 s. Each straw's content was poured into a sterile 1.5-mL microtube prewarmed to 37 °C. Immediately after thaw, the sperm suspension was diluted 1:3 (v:v), drop by drop, with a Tyrode's solution containing 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, penicillin and streptomycin (100 μg/mL, each) and 0.6% (w/v) BSA (Fatty acid free; Calbiochem, Madrid, Spain). One aliquot of this sperm suspension was used for in vitro fertilization with a final sperm concentration adjusted to 1.5×10^5 to 4×10^5 motile sperm/mL, placed in 50- 100-μL drops covered with mineral oil in 35-mm culture dishes. The remaining spermatozoa were incubated at 38.5 °C under 5% CO_2 in air, and subsamples were assessed at various times (0, 1, 2 h) for motility and acrosome integrity as described above.

2.6. Oocyte collection and in vitro maturation

Ovaries from domestic cats were obtained from ovariohysterectomies carried out in local veterinary clinics and were transported to the laboratory at 5 °C in 15-mL vials with 5 mL of sterile 0.9% (w/v) NaCl solution supplemented with penicillin-streptomycin (100 μg/mL each). Samples were processed according to Gómez et al. [31] with some modifications. The ovaries were sectioned longitudinally with a scalpel blade and sliced with two 21-gauge needles to release cumulus-oocyte complexes (COCs) from follicles. For oocyte collection, a HEPES-buffered TCM-199 medium (Sigma), supplemented with 15 mM HEPES, 15 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 0.4% (v/v) BSA (Sigma, A-8412), and penicillin and streptomycin (100 μg/mL each), was used. After several washes in medium, grade I (excellent quality) to grade III (fair)

COCs were selected and transferred to 4-well culture dishes (Nunclon; Nalgene, Nunc International, Roskilde, Denmark) containing 500 μL TCM-199 supplemented with 25 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 1.12 mM cysteine, 0.4% (w/v) BSA (Fraction V; Sigma, A-9418), 25 ng EGF/mL [32], ovine follicle-stimulating hormone/luteinizing hormone (FSH/LH; Sigma, 10 μg/mL each), 1 μg estradiol/mL, and penicillin and streptomycin (100 μg/mL each). Dishes were incubated for 24 h at 38.5 °C under 5% CO_2 in air and maximum humidity.

2.7. In vitro fertilization

After in vitro maturation, oocytes were co-incubated with frozen-thawed bobcat spermatozoa. Oocytes (10 to 20 per drop) were placed in 50- to 100-μL drops of Tyrode's solution supplemented with 15 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, penicillin and streptomycin (100 μg/mL each), and 0.6% (w/v) BSA (Fatty acid free; Calbiochem) [31,33]. Oocytes were co-incubated with 1.5×10^5 to 4×10^5 motile spermatozoa/mL under mineral oil in an atmosphere of 5% CO_2 in air and maximum humidity at 38.5 °C for 18 to 20 h.

2.8. In vitro culture

After gamete coculture, oocytes were transferred to HEPES-buffered Tyrode's medium and aspirated in and out of a micropipette tip to remove cumulus cells and unattached spermatozoa. Presumptive zygotes were transferred to 4-well culture dishes with 500 μL Tyrode's solution containing 1% (v/v) minimum essential medium (MEM) nonessential amino acids, 0.3% (w/v) BSA (fatty acid free), supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, and penicillin and streptomycin (100 μg/mL each) (IVC-1 medium) [31,33]. Incubation was carried out under 5% CO_2 in air and maximum humidity at 38.5 °C. Cleavage was evaluated 44 to 48 h after insemination, and embryo culture was finalized at this time point (with some exceptions; see later). Noncleaved oocytes were stained with Hoechst 33342 (prepared in PBS and further diluted 1:10, v:v, in glycerol to reach a final concentration of 10 μg/mL) to assess maturation and fertilization status and the number of spermatozoa bound to the zona pellucida. Hybrid embryos generated with spermatozoa from each male, and that were cryopreserved in TEST, were allowed to progress in culture to assess development. For embryo incubation, a three-step culture system was used [33]. Briefly, after cleavage evaluation, embryos were transferred to IVC-2 medium (similar to IVC-1, but also containing 2%, v/v, MEM essential amino acids) until day 5 of culture. Then, embryos were transferred to IVC-3 medium (similar to IVC-2, but with 10%, v/v, of FBS instead of BSA) until Day 7. Day of fertilization was considered as Day 0. At the end of incubation, hybrid embryos were also stained and examined to count the total number of nuclei.

2.9. Experimental design and statistical analyses

Data were analyzed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Results are presented as means ± SEM. A value of $p < 0.05$ was regarded as statistically significant. Semen traits from ejaculates collected in autumn or spring were compared using nonparametric Mann-Whitney U-test.

Two-way repeated-measures ANOVA (RMANOVA) was used to analyze changes in motility and acrosome integrity over time both during incubation in Ham's F-10 medium with different sera and throughout the cryopreservation process in TEST or Biladyl. When RMANOVA showed statistically significant differences, the effect of each variable was tested while controlling for the other variable.

Before analyzing fertilization and cleavage rates, differences between and within ejaculates of males were analyzed using ANOVA. Fertilization rates for spermatozoa cryopreserved in TEST or Biladyl were analyzed using the nonparametric Wilcoxon signed rank test for paired samples. Possible relations between motility and acrosome integrity in fresh, refrigerated, frozen-thawed, and post-thaw incubated samples and between these parameters and fertilization or cleavage rates were analyzed by nonparametric Spearman's rank correlation.

3. Results

3.1. Phenotypic and semen traits of male bobcats

Between April 2004 and November 2007, 13 electroejaculations were carried out on four different males. Spermic ejaculates were obtained on 11 occasions (85%). Averages of semen traits were calculated for samples collected in November (autumn) or April (spring) (Table 1).

Serum testosterone and cortisol levels were on average 0.90 ± 0.15 ng/mL and 13.95 ± 1.73 μg/dL, respectively. There were no significant differences

Table 1
Testicular, endocrine, and seminal traits of bobcats (*Lynx rufus*).

	Average	November (autumn)	April (spring)
Number of ejaculates	13	9	4
Age (years)	5.85 ± 0.46	6.00 ± 0.67	5.50 ± 0.29
Body mass, kg	10.93 ± 0.63	11.14 ± 0.72	10.45 ± 1.36
Total testes weight, g	2.06 ± 0.21	2.02 ± 0.22	2.14 ± 0.52
Relative testes weight, $\times 10^{-4}$	1.96 ± 2.10	1.92 ± 0.26	2.05 ± 0.4
Testosterone, ng/mL	0.90 ± 0.15	0.79 ± 0.09	1.33 ± 0.70
Cortisol, μg/dL	13.95 ± 1.73	12.49 ± 1.79*	19.80 ± 1.10*
Ejaculate volume, μL	362.69 ± 84.06	368.33 ± 119.06	350.00 ± 82.06
Semen pH	7.78 ± 0.09	7.70 ± 0.10	7.95 ± 0.18
Sperm concentration, $\times 10^6$ sperm/mL	24.40 ± 7.87	10.82 ± 2.91*	60.59 ± 12.44*
Total number of spermatozoa, $\times 10^6$	10.01 ± 3.40	4.51 ± 2.11*	24.69 ± 4.78*
Motility, %	55.71 ± 5.76	50.48 ± 5.25	67.92 ± 14.07
Quality of motility, scale 0 to 5	2.71 ± 0.23	2.45 ± 0.21	3.30 ± 0.46
SMI	54.94 ± 4.84	49.78 ± 4.42	66.99 ± 10.52
Morphologically normal sperm, %	14.67 ± 2.07	11.63 ± 1.91*	21.78 ± 1.61*
Sperm with intact acrosome, %	43.66 ± 3.79	47.96 ± 4.23*	33.60 ± 4.17*

Note: Seminal traits correspond with whole ejaculate. Results are means ± SEM. Relative testes weight = total testes weight/body weight. SMI = (% sperm motility + [20 × quality of motility])/2.
*Statistical differences between columns ($p < 0.05$).

between testosterone levels in November and April, but differences existed between time of sampling for cortisol, with significantly higher values in spring (Table 1).

Semen volume and sperm concentration were low, and there were significant differences in sperm concentration between the two collection times. Ejaculates contained an average of 4.5×10^6 total spermatozoa in November and 25×10^6 total spermatozoa in April; these differences were statistically significant. An average value of 56% motile sperm was found, with 50% motile sperm in November and 68% motile spermatozoa in April; these differences were not significant (Table 1).

Bobcats produced a very high proportion of pleomorphic spermatozoa (average of 15% normal spermatozoa). There were, on average, 12% morphologically normal sperm in November (range, 4% to 17%) and 22% normal sperm in April (range, 20% to 25%), the difference being statistically significant. The proportion of spermatozoa with an intact acrosome was, on average, 44%. Differences between November (48% intact acrosomes) and April (34% intact acrosomes) were statistically significant (Table 1).

3.2. Effect of sera

Incubation of bobcat spermatozoa in HEPES-buffered Ham's F-10 medium supplemented with inactivated bobcat serum resulted in no loss of motility during a 2-h incubation period, whereas incubation in the same medium with FBS showed a reduction in sperm motility over time (Fig. 2). Comparison between medium with different sera showed a higher sperm motility index for medium with bobcat serum than that for medium with FBS at the end of incubation (55.0 vs. 35.0 SMI, respectively), although differences did not reach statistical significance. On the other hand, there were no significant changes in acrosome integrity over time or between media with different sera (35.0% vs. 38.0 % sperm with intact acrosomes at 2-h incubation) (Fig. 2).

3.3. Cryopreservation of bobcat spermatozoa

Three of the four bobcats produced semen of enough quality for cryopreservation (SMI of 55 or above), with a total of 7 of 11 (64%) spermic ejaculates used. There were differences in SMI (range, 52.0 to 84.7) and acrosome integrity (range, 26.8% to 41.3%) among ejaculates.

There was no significant decrease in motility after refrigeration or after freezing and thawing in spermatozoa diluted in TEST, but significant differences were observed after thawing and incubation for 3 h (Fig. 3A). On the other hand, there were no significant differences in percentage of intact acrosomes after freezing and thawing or during incubation after thawing (Fig. 3B). For spermatozoa diluted in Biladyl, a difference in motility was noted between fresh and thawed sperm ($p < 0.05$) with no further significant declines during incubation after thawing (Fig. 3A). As for acrosome

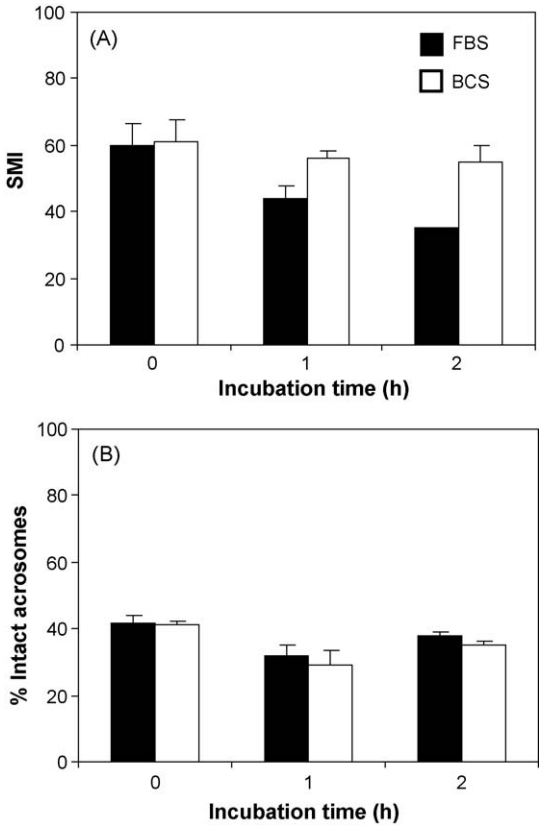

Fig. 2. Effect of fetal bovine serum or bobcat serum on incubation of bobcat spermatozoa. (A) Sperm motility index (SMI) and (B) percentage of intact acrosomes of bobcat spermatozoa during incubation in Ham's F-10 medium containing inactivated bobcat serum (BCS) or inactivated fetal bovine serum (FBS) (N = 3).

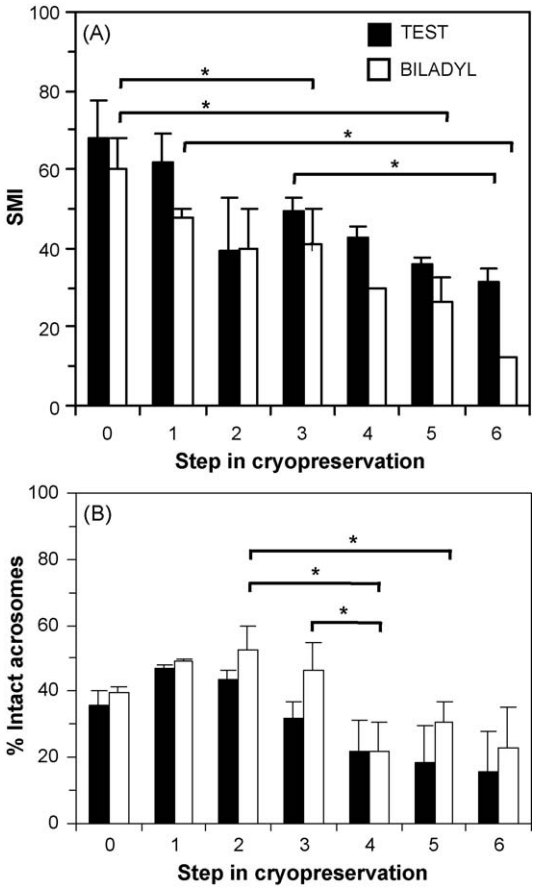

Fig. 3. Sperm cryopreservation of bobcat spermatozoa using TEST or Biladyl, both containing 20% egg yolk and 4% glycerol. (A) Sperm motility index (SMI) and (B) percentage of sperm with intact acrosomes at different steps of the cryopreservation process. 0, fresh semen pool, after dilution with HEPES-buffered Ham's F-10 containing 5% fetal bovine serum; 1, after centrifugation and addition of cryodiluent; 2, at the end of refrigeration; 3, after thawing and dilution in Ham's F-10 with 5% fetal bovine serum; 4, 5, and 6, after thawing and 1, 2, and 3 h of incubation, respectively, at 38.5 °C under 5% CO_2/air. Asterisks indicate differences ($p < 0.05$) between cryopreservation steps identified by the ends of each horizontal line (N = 3).

integrity, there was a decrease after thawing in relation to values recorded before freezing ($p < 0.05$) (Fig. 3B).

There were some apparent differences between cryodiluents from the beginning of the cryopreservation process (68.2 vs. 60.0 SMI in TEST and Biladyl, respectively), which seemed to remain after thawing (49.2 vs. 41.3 SMI) and after incubation for 1 h (42.5 vs. 30.0 SMI); however, none reached significance. Overall, cryopreservation in TEST resulted in better motility than that with Biladyl (31.7 ± 3.3 vs. 12.5 ± 0.0 SMI after 3-h incubation post-thaw, respectively), although significant differences were not revealed (Fig. 3A).

It has been argued that percentages of normal sperm and acrosome integrity in fresh semen may be better predictors of sperm survival post-thaw than is initial sperm motility [12]. We therefore examined possible relations between sperm traits in fresh semen and motility after cryopreservation. No relation was found between percentage of normal spermatozoa in fresh semen and motility post-thaw. On the other hand, significant relations were observed between percentage of intact acrosomes in fresh semen and after refrigeration with SMI after thawing and 1 h post-thaw.

3.4. *In vitro fertilization of in vitro–matured domestic cat oocytes with frozen-thawed bobcat spermatozoa*

It was found that bobcat spermatozoa could fertilize zona-intact, live, in vitro–matured oocytes from the domestic cat (Fig. 4). Percentages of fertilization varied slightly between males and considerably between the time of the year of semen collection, and between

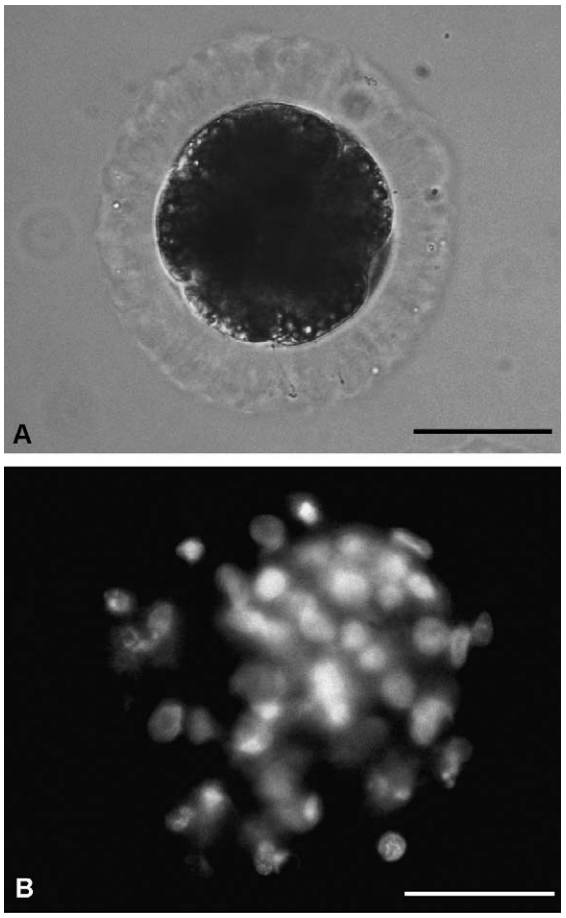

Fig. 4. Hybrid embryos resulting from heterologous in vitro fertilization using bobcat spermatozoa cryopreserved in TEST and in vitro–matured domestic cat oocytes. (A) An embryo of about eight cells examined by phase-contrast optics 44 h postinsemination; (B) a morula-stage embryo after staining with Hoechst 33342 and examined under fluorescence microscope after 7 days of in vitro culture. Day of fertilization was considered as Day 0. Scale bars = 50 μm.

cryodiluents. Spermatozoa from males collected in April (spring) and cryopreserved in TEST showed high fertilization rates: 45 of a total of 98 (46%) mature oocytes were fertilized (Table 2). However, the spermatozoa collected in November (autumn), and cryopreserved in TEST, showed that only 5 of a total of 29 (17%) mature oocytes were fertilized. In addition, although only a limited comparison was possible between sperm samples cryopreserved in TEST and Biladyl, it was clear that spermatozoa cryopreserved in Biladyl achieved very low fertilization rates. It was found that spermatozoa cryopreserved in TEST fertilized 50 of 127 (39.3%) mature oocytes, whereas those cryopreserved in Biladyl fertilized 1 of 34 (2.9%) mature oocytes. Fertilized oocytes cleaved in culture. Percentages of cleavage over mature oocytes were 24.4% (31 of 127) for spermatozoa cryopreserved in TEST and 2.9% (1 of 34) for those cryopreserved in Biladyl.

On two occasions, hybrid embryos resulting from spermatozoa cryopreserved in TEST were cultured in vitro until Day 7 to explore if progression occurred up to the blastocyst stage. Maximum development reached by these hybrid embryos was the morula stage. Of a total of 26 embryos cultured until Day 7, 1 was found at the 4-cell stage, 18 contained between 16 and 30 nuclei, and 7 embryos developed to the $\geq$32-cell stage.

We have detected positive and significant relations between the proportion of spermatozoa with intact acrosomes after thawing and the proportion of fertilized oocytes and the proportion of cleaved oocytes.

4. Discussion

This study has allowed us to characterize bobcat ejaculates obtained through electrostimulation under general anesthesia, examine cryopreservation of spermatozoa using two different diluents, and assess sperm function after cryopreservation by using heterologous in vitro fertilization of domestic cat oocytes matured in vitro.

A wide range of total number of spermatozoa per ejaculate was observed (0.2×10^6 to 33.5×10^6 sperm). Overall, these values were low when compared

Table 2
Heterologous fertilization of in vitro–matured cat oocytes with bobcat (*Lynx rufus*) spermatozoa cryopreserved in TEST with 20% egg yolk and 4% glycerol ($N = 3$).

	November (autumn)	April (spring)
Number oocytes fertilized/total number of oocytes (%)	5/59 (8.5)	45/122 (36.9)
Number oocytes fertilized/number mature oocytes (%)	5/29 (17.2)	45/98 (46.0)
Number spermatozoa bound/mature oocyte	0.79	2.65
Number cleaved oocytes/number fertilized oocytes (%)	5/5 (100)	26/45 (57.8)
Number cleaved ooyctes/number mature oocytes (%)	5/29 (17.2)	26/98 (26.5)

Note: Sperm concentration was 1.5×10^5 to 4×10^5 motile cells/mL. Gametes were co-incubated for 18 to 20 h. The percentage of cleaved oocytes was evaluated after 44 to 48 h in culture postinsemination. Noncleaved oocytes were stained with Hoechst 33342 (10 μg/mL) to retrospectively assess maturation and fertilization status.

with those recorded for other medium-size felids (e.g., ocelot [25]; fishing cat [24]; serval [34]). Nevertheless, total sperm numbers in this study (average of 10×10^6 spermatozoa/ejaculate) were higher than those described for this species in earlier studies (an estimated average of 0.78×10^6 total spermatozoa/ejaculate [17]). In addition, they are also higher than those recorded in related lynx species such as the Iberian lynx, *Lynx pardinus* (1.48×10^6 to 5.37×10^6 total spermatozoa; unpublished data) or the Eurasian lynx, *Lynx lynx* (1.6×10^6 to 2.9×10^6 total spermatozoa [35]). Differences between our results and those by other authors could relate to difference in the age of animals, time of sampling, or conditions in which animals were kept. It seemed that differences were in part due to time of the year when ejaculates were collected (see later) but also, in our case, to differences between males with one particular male producing high sperm numbers.

The proportion of motile spermatozoa in the ejaculate of bobcats (average of 56% motility) was similar to that found in an earlier report [17]. This value is also similar to the proportion of motile sperm recorded in the Eurasian lynx (55% motile sperm during the breeding season [35]) but lower than those in the Iberian lynx (about 80% before the breeding season; unpublished data) or in other medium-size cats such as the caracal (85% [17]), serval (73% [34]), fishing cat (73% [24]), or ocelot (83% [25]).

Bobcat ejaculates contained a low proportion of normal spermatozoa (a range of 4% to 25%), which is similar to values seen in an earlier report on this species [17] and were not different than values in the Eurasian or Iberian lynx ([35] and unpublished data, respectively). These observations place lynx species in the lower end of the range of felid species with morphologically normal sperm [21]. The proportion of spermatozoa with intact acrosomes in bobcat ejaculates ranged between 27% and 60%, and it is similar to that observed in Eurasian and Iberian lynx ([35] and unpublished data, respectively), although is far from values in other medium-size cats such as the fishing cat, which shows ejaculates with 83% to 94% spermatozoa with intact acrosomes [24].

A comparison of ejaculates collected in November (autumn), just before the beginning of the reproductive season, and April (spring), toward the end of the season, allowed us to identify changes in semen traits. In November, ejaculates contained less spermatozoa and a lower proportion of morphologically normal sperm although there was a higher proportion of sperm with intact acrosomes. Nevertheless, there were no differences in testes weight, or relative testes weight, which indicate that increases in testes weight (if any) predate increases in sperm output. Seasonal changes in sperm numbers and semen quality have been described in a number of species, including felids, and they are known also to affect sperm function (Arabian leopard [36]; Eurasian lynx [35]; Pallas' cat [37]). Management of captive breeding programs may require reproductive health assessments, including semen collection and evaluation, to monitor the potential reproductive capacity of males. This is carried out before the reproductive season. Thus, based on the results of this study, such semen assessments should take into account that ejaculate quality before the beginning of the reproductive season may be below the optimum achieved during the peak of the mating season. In addition, nothing is known about semen traits in free-ranging bobcats, so it is not possible to speculate how ejaculate traits in captive animals compare with those in free-ranging individuals.

We examined the survival of bobcat spermatozoa in a HEPES-buffered F-10 medium supplemented with either fetal bovine serum or bobcat serum (both inactivated and filtered before use). Because this medium is usually employed with other felid spermatozoa (ocelot [25]; tiger [38,39]; puma and lion [39]; cheetah [40]), we sought to assess whether any of these sera were adequate for dilution of bobcat spermatozoa before preparation for cryopreservation. No significant decreases in sperm motility, or acrosome integrity, were seen during incubation of up to 2 h, and no significant differences were noted between sera, although bobcat serum appeared to afford better survival. Further studies are needed to clarify if bobcat serum does indeed result in better survival and would thus be the best option as medium supplement.

Bobcat spermatozoa were successfully cryopreserved using a protocol previously employed for other felids [25,29]. Average proportion of motile sperm upon thawing was high after cryopreservation in TEST containing 20% egg yolk and 4% glycerol, with about 50% sperm motility and an SMI ~50 after thawing. This was better than sperm survival after cryopreservation using the same method in the Iberian lynx (about 34% motile sperm and an SMI of 43 upon thawing; unpublished data) or ocelot (25% motile sperm, SMI 40 [25]), and it was similar to results in other medium-size cats (fishing cat, 38% to 65% motile sperm, SMI 51 to 65 [24]; tigrina, SMI 49 [41]). Although rather low, the percentage of intact acrosomes recorded for bobcat sperm after thawing was consistent with values found in other medium-size cats (Iberian lynx, 39%, unpublished data; fishing cat, 30% to 50% [24]; ocelot, 40% to 50%

[25]). Cryopreservation in Biladyl led to results that were similar to those observed with TEST, but there was a tendency toward a higher deterioration of motility during incubation after thawing.

Heterologous in vitro fertilization assays revealed that cryopreserved bobcat spermatozoa were able to fertilize domestic cat oocytes in vitro. Fertilization rates were high, with about 46% of mature oocytes being fertilized by spermatozoa collected in April and cryopreserved in TEST. When compared with other species, these results were similar to those found with Pallas' cat spermatozoa [23] but lower than the fertilization rates observed employing similar conditions with spermatozoa from fishing cat [24] or ocelot [25], for which rates between 62% and 70% were found. Interestingly, despite a lack of significant differences post-thaw between bobcat sperm samples cryopreserved in TEST or Biladyl in either sperm motility or acrosome integrity, heterologous fertilization revealed that sperm cryopreserved in the latter diluent exhibited reduced fertilizing ability. This is an important result because it highlights the need to assess cryopreservation methods by functional tests. Furthermore, it reveals that spermatozoa can be assessed in the laboratory when it is unlikely that other tests such as artificial insemination or homologous in vitro fertilization could be employed for this purpose.

After heterologous fertilization, hybrid embryos cleaved during in vitro culture. The percentages of mature and fertilized oocytes that cleaved in culture after fertilization with bobcat spermatozoa were higher than those obtained with spermatozoa from the related Iberian lynx (14% and 56%, respectively; unpublished data) but were lower than results obtained in other medium-size felids (fishing cat, 53% cleavage of mature oocytes [24]; ocelot, 64% cleavage of mature oocytes [25]).

In conclusion, the current work has allowed us to undertake a characterization of bobcat ejaculates and assess sperm cryopreservation in this species, together with heterologous in vitro fertilization of thawed spermatozoa. Our results revealed that (1) it is possible to collect and cryopreserve semen from captive bobcats, with best results for cryopreservation obtained with TEST containing 20% egg yolk and 4% glycerol; (2) semen traits are similar to those of other medium-size felids and better than semen traits in related lynx species; (3) there are no differences between inactivated fetal bovine serum and homologous serum when added to the HEPES-buffered F-10 medium that is used to incubate and assess sperm survival in vitro; (4) frozen-thawed bobcat sperm function can be assessed by heterologous in vitro fertilization using in vitro–matured domestic cat oocytes.

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