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Assessment of semen quality, sperm cryopreservation and heterologous IVF in the critically endangered Iberian lynx (*Lynx pardinus*)

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Abstract. Semen traits and factors affecting sperm cryopreservation were assessed in the Iberian lynx (*Lynx pardinus*), a species regarded as the most endangered felid in the world. For cryopreservation, semen was washed, resuspended in a Tes–Tris-based diluent (TEST) or a Tris-based diluent (Biladyl), both with 20% egg yolk and 4% glycerol, loaded into straws, cooled to 5°C using an automated programmable system and frozen on nitrogen vapour. Heterologous IVF of *in vitro*-matured domestic cat oocytes was used to test the fertilising ability of cryopreserved spermatozoa. Electroejaculates from five males were obtained. Characterisation of the electroejaculates revealed mean (± s.e.m.) values of $3.3 \pm 0.6 \times 10^6$ total spermatozoa, $73.6 \pm 4.6\%$ motile spermatozoa, $23.7 \pm 4.0\%$ morphologically normal spermatozoa and $40.7 \pm 2.3\%$ spermatozoa with intact acrosomes. After thawing a higher percentage of motile spermatozoa was seen in TEST than in Biladyl ($34.0 \pm 6.2\%$ *v.* $7.5 \pm 4.8\%$, respectively; $P < 0.05$); however, there were no differences in the percentage of intact acrosomes between the two diluents. Iberian lynx spermatozoa fertilised domestic cat oocytes *in vitro*, with higher fertilisation rates observed for spermatozoa cryopreserved in TEST than in Biladyl, although the difference did not reach statistical significance ($20.5 \pm 4.5\%$ *v.* $11.5 \pm 6.8\%$, respectively). There were positive significant relations between the fertilisation rates and both the percentage of normal spermatozoa and the percentage of spermatozoa with an intact acrosome before cryopreservation ($P = 0.04$). This first report of the collection and cryopreservation of Iberian lynx semen and analysis of fertilising ability is an important step in the development of assisted reproductive techniques for this critically endangered felid species.

Additional keywords: assisted reproductive technologies, felid, genome resource bank, teratospermia.

Introduction

The Iberian lynx is 'critically endangered' (International Union for Conservation of Nature (IUCN) 2008) and is regarded as the world's most endangered felid and the most threatened carnivore in Europe (Nowell and Jackson 1996). Although the species was once distributed throughout the Iberian peninsula (Rodríguez 2007), at present approximately 200 individuals are confined to the south of Spain, distributed in two isolated, reproductively viable populations with low levels of genetic variability (Johnson *et al*. 2004), one in Doñana and the other in Sierra Morena (Rodríguez and Delibes 2003; Rodríguez 2007). Recent reports suggest the presence of the Iberian lynx in central Spain (Alda *et al*. 2008).

For decades, considerable work has focused on the ecology and *in situ* conservation of this species with an *ex situ* conservation programme starting in 2004 (Vargas *et al*. 2007). One of the main objectives of captive breeding is to conserve 85% of the genetic variability existing at the beginning of the programme and to maintain it for a period of 30 years. To achieve this, approximately 60–70 Iberian lynx (32 founders plus individuals born in the captive breeding programme) will be bred in captivity (Vargas *et al*. 2008) before reintroductions are attempted in or after 2010.

Conservation of the Iberian lynx, using both *in situ* and *ex situ* approaches, may benefit from a variety of assisted reproductive technologies (Wildt 1990; Pukazhenthi and Wildt 2004;

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Pope *et al*. 2006; Pukazhenthi *et al*. 2006*a*; Swanson 2006). In particular, the collection, preservation and use of spermatozoa is important for the establishment of a genetic resource bank, which, in combination with other techniques, such as AI, *in vitro* oocyte maturation, fertilisation and culture, and embryo transfer, would allow gene flow to be established between isolated populations, including *in situ* and *ex situ* linkage, without removing individuals from the wild or displacing a captive animal (Pukazhenthi *et al*. 2006*a*; Swanson *et al*. 2007). The general feasibility of this approach has already been demonstrated for the cheetah (Wildt *et al*. 1997) and is currently being developed for other cat species (Swanson *et al*. 2007).

There have been various studies on the collection, characterisation and cryopreservation of felid spermatozoa. Methods for cryopreserving spermatozoa have been examined, although there is still a considerable need for basic cryobiological studies of diverse species (Leibo and Songsasen 2002). Cryopreservation is directly lethal to a significant proportion (usually approximately 50%) of spermatozoa in a typical semen sample (Watson 2000). In general terms, thawed spermatozoa also have a high percentage of damaged acrosomal membranes (Swanson and Wildt 1997). Past experience has demonstrated that spermatozoa from different species vary considerably in their cryosensitivity, with differences also apparent between individuals (Yu *et al*. 2002). Thus, there is a need to adjust cryopreservation protocols to suit different species. Studies on domestic cat spermatozoa have used Tris-citric acid- or Tes–Tris (TEST)-based cryodiluents with variable results: some studies have reported better cryosurvival with Tris-based cryodiluents, whereas others have reported better survival with TEST-based diluents (Luvoni 2006). There have been no prior studies into the cryopreservation of spermatozoa from the Iberian lynx.

The preparation of samples for cryopreservation involves dilution in culture medium (e.g. Ham's F-10) and subsequent centrifugation (Pukazhenthi *et al*. 2002; Swanson *et al*. 2006; Thiangtum *et al*. 2006; Stoops *et al*. 2007). Evaluation of sperm survival has relied on incubations of fresh or cryopreserved spermatozoa in culture medium with fetal bovine serum, followed by the examination of sperm motility and acrosome integrity over time (Thiangtum *et al*. 2006). These parameters may serve as good indicators of sperm survival during gamete coculture for IVF or after AI. In some species, such as the clouded leopard, homologous serum (rather than bovine serum) is preferred (Pukazhenthi *et al*. 2002). Because sperm survival during incubation may be poor for some species, improvements using alternative media or homologous serum deserve further examination.

Although several parameters can be assessed in the laboratory to evaluate the quality of semen samples, they provide only a limited estimate of sperm function. On the other hand, IVF provides a useful test of sperm function, but the collection of oocytes from endangered species is not always possible. However, oocytes from domestic cats are readily available and could be used to assess the functionality of Iberian lynx spermatozoa, provided that the spermatozoa are able to interact with and penetrate domestic cat oocytes, as has been shown for other non-domestic felids such as the leopard cat (Howard and Wildt 1990; Andrews *et al*. 1992), tiger (Donoghue *et al*. 1992*a*), cheetah (Donoghue *et al*. 1992*b*; Roth *et al*. 1995), snow leopard (Roth *et al*. 1994), fishing cat (Thiangtum *et al*. 2006), Pallas' cat (Swanson *et al*. 2006) and ocelot (Stoops *et al*. 2007).

The aims of the present study were to: (*1*) characterise Iberian lynx semen traits; (*2*) analyse the ability of Iberian lynx spermatozoa to survive *in vitro* under various conditions; (*3*) assess cryopreservation methods, evaluating effects on sperm motility and acrosome integrity; and (*4*) evaluate the fertilising capacity of cryopreserved Iberian lynx spermatozoa using heterologous IVF of domestic cat oocytes.

Materials and methods

Animals

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD1201/2005, which conforms to European Union Regulation 2003/65. Five Iberian lynx males (3–6 years old), kept at the 'El Acebuche' Iberian lynx captive breeding centre (Doñana National Park, Huelva, Spain), were electroejaculated under general anaesthesia in late November–early December, just before the beginning of the breeding seasons of 2005 or 2006. The breeding season spans from December to February, with most matings taking place in January. All males were originally trapped in the wild and, after suitable quarantine periods, were kept under seminatural conditions in 550-m^2 individual enclosures with visual, olfactory and auditory contact with conspecific males and females. The enclosures had natural mixed Mediterranean vegetation. Animals were fed a balanced diet, consisting largely of commercially bred live rabbits and rabbit carcasses with vitamin supplements. Of the five males used in the present study, four were subsequently found to be fertile.

Sperm collection and evaluation

Semen collection (once for each male) was performed under anaesthesia using an intramuscular injection of either tiletamine hydrochloride plus zolazepam ($10\,mg\,kg^{-1}$ bodyweight; Zoletil; Virbac, Carros, France) or a combination of ketamine hydrochloride ($5\,mg\,kg^{-1}$; Imalgene 1000; Merial, Lyon, France) plus medetomidine hydrochloride ($50\,\mu g\,kg^{-1}$; Domtor; Orion Pharma, Espoo, Finland). The effects of medetomidine were reversed by injection of atipamezole hydrochloride ($250\,\mu g\,kg^{-1}$; Antisedan; Pfizer, Madrid, Spain).

Bodyweight and testicular dimensions were determined before electroejaculation. Testes weight was calculated from the volume of each testis as described by Harcourt *et al*. (1995), specifically:

$$\text{Weight (g)} = (\text{Left testis (cm}^3) + \text{Right testis (cm}^3)) \times 1.1$$

where 1.1 is the conversion factor for calculating the weight of body tissue from its volume. For each testis, volume $= 4/3 \times \pi \times \text{length}/2 \times \text{width}_1/2 \times \text{width}_2/2$ (Harcourt *et al*. 1995), where width_1 and width_2 are two breadths of the ellipsoid.

Electroejaculation was performed following a procedure described by Howard (1993). A lubricated rectal probe (length 17.9 cm, diameter 13.45 mm; PT Electronics, Boring, OR, USA)

with three longitudinal electrodes (35.7 mm in length) was placed in the rectum, with the electrodes at the level of the prostate and bulbourethral glands, and voltage applied. A total of 100 electrical stimulations was delivered using a 60-Hz sine-wave stimulator (PT Electronics). Stimuli were delivered in sets of 10 stimulations at each voltage (range 2–5 V), with the total sequence divided into four series (first series: 2, 3, 4 V; second series: 3, 4, 5 V; third series: 4, 5 V; fourth series: 4, 5 V). Semen was collected in prewarmed sterile 25-mL polypropylene cups (catalogue no. 430130; Lab Center, Madrid, Spain).

Semen collected in each series was evaluated immediately for volume (using a micropipette) and pH (using test strips; Medi-Test Combi 9; Machereg-Nagel, Düren, Germany). A semen sample was placed on a slide, covered with a coverslip (prewarmed to 37°C) and examined under phase contrast optics to assess the percentage of motile spermatozoa and the quality of movement using a scale of 0–5, where 0 is no movement and 5 represents rapid, linear forward movement. Motility values were used to calculate a sperm motility index (SMI), using the formula (% motile sperm + (quality × 20))/2.

In addition, after each electroejaculation series, 5 μL semen was fixed with 45 μL of 1% glutaraldehyde solution in 0.165 M cacodylate buffer, pH 7.3. This fixed sperm suspension was used to estimate sperm concentration using a haemocytometer. Furthermore, a sample of 10 μL semen was fixed in 250 μL of 4% paraformaldehyde in 110 mM Na_2HPO_4 and 2.5 mM NaH_2PO_4 buffer solution (pH 7.4) and later stained with Coomassie blue (Larson and Miller 1999) to assess sperm morphology and acrosomal integrity. Fixed samples were stored at 4°C until further processing.

Semen samples were diluted with an equal volume of Ham's F-10 containing fetal bovine serum (FBS) unless indicated otherwise (see below) and stored at room temperature (22°C) pending completion of the electroejaculation series and later used for cryopreservation.

For Coomassie blue staining (Larson and Miller 1999), paraformaldehyde-fixed samples were centrifuged for 8 min at 1700*g*, the supernatant removed and discarded and replaced with 250 μL of 0.1 M ammonium acetate solution (pH 9.0) in distilled water. This step was repeated twice. After the second wash, the pellet was resuspended in approximately 50 μL ammonium acetate. The sperm suspension was smeared onto two different slides and allowed to dry at room temperature. After drying, slides were stained with 50 μL Coomassie blue stain solution (0.22% Coomassie brilliant blue R-250; Sigma, Madrid, Spain) in 50% methanol (BDH, Madrid, Spain) and 10% glacial acetic acid (Panreac, Barcelona, Spain) for 90 s and rinsed with distilled water, dried at room temperature and preserved by placing a coverslip over a drop of mounting medium (DPX mountant for microscopy; BDH).

Sperm morphology was assessed by categorising spermatozoa as either normal or with abnormalities in the head (macrocephalic, microcephalic, bicephalic), midpiece (abnormal or bent) or the rest of the flagellum (abnormal, coiled flagellum, bent tail, tightly coiled tail). A total of 100 spermatozoa was counted and classified. The presence of cytoplasmic droplets was determined, but these were not categorised as separate abnormalities and were, rather, used as an indicator of sperm maturation. In addition, the presence of coiled tails (which may potentially result from cold shock) was recorded. Acrosomal integrity was assessed in a total of 100 spermatozoa and was classified as: (*1*) intact normal, spermatozoa with uniform dark staining over the acrosomal region with no staining of the postacrosomal region; (*2*) intact with abnormal acrosome, spermatozoa with uniform staining of the acrosomal region and with a darker staining of all or part of the apical region; (*3*) damaged, unstained patches in the acrosomal region; or (*4*) non-intact, acrosomal staining absent or staining only in the equatorial segment (Pukazhenthi *et al*. 2006*b*).

Sperm incubation in different media and sera

The ability of Iberian lynx spermatozoa to survive *in vitro* was tested using HEPES-containing Ham's F-10 (Irvine Scientific, Izasa, Barcelona, Spain) or HEPES-containing TCM-199 media (Sigma) with either FBS or Iberian lynx serum. Ham's F-10 medium was supplemented with modified Ham's F-10 Gentamicin-50X (Irvine Scientific), which was supplied lyophilised and containing 0.05 M glutamine, 0.05 M pyruvate and gentamicin sulfate (1 : 50 dilution; final concentration 10 μg mL^{-1}). Both Ham's F-10 and TCM-199 were supplemented with L-glutamine (Sigma; 2 mM final concentration), pyruvate (Sigma; 1 mM final concentration) and a triple antibiotic mix (Sigma; final concentrations 130 IU mL^{-1} penicillin, 130 μg mL^{-1} streptomycin and 260 μg mL^{-1} neomycin). Media were also supplemented with 5% heat-inactivated FBS (Gibco Invitrogen, Barcelona, Spain). Medium F-10 was alternatively supplemented with heat-inactivated Iberian lynx serum. For inactivation, serum was incubated at 56°C for 30 min and was filtered through a 0.22-μm filter before use.

Semen aliquots (7.5 μL), taken from Series 2 of the electroejaculation procedure, were diluted in 67.5 μL of each medium, placed in Eppendorf tubes and incubated in a dry thermoblock at 37°C under air. At different intervals (0, 1 and 3 h), samples were taken and examined under phase contrast optics to assess motility and were fixed in 4% paraformaldehyde solution for subsequent staining with Coomassie blue and evaluation of acrosomal integrity.

Sperm cryopreservation

After collection and initial evaluation, semen was diluted 1 : 1 (v/v) in HEPES-containing modified Ham's F-10. Each sample was kept at room temperature (22°C) until the end of semen collection for each male. After collection, fractions with similar quality were pooled. Diluted semen pools were centrifuged at 300–700*g* (depending on semen quality) for 10 min, the supernatant discarded, the volume of the loose pellet measured using a micropipette and cryopreservation diluent added very slowly (1 : 4, v/v), drop by drop, at room temperature.

For sperm cryopreservation, two different cryodiluents were examined: (*1*) TEST, a diluent containing 4.83% Tes, 1.15% Tris, 0.4% glucose, 200 IU mL^{-1} penicillin, 200 μg mL^{-1} streptomycin, 20% egg yolk and 4% glycerol; and (*2*) Biladyl, a commercial diluent (Minitüb, Tiefenbach, Germany) with 2.42% Tris, 1% fructose, 1.38% citric acid, 20% egg yolk and 4% glycerol. The Biladyl diluent was further supplemented with

an antibiotic cocktail containing tylosin, gentamicin, spectinomycin and lincomycin (Minitüb). Biladyl actually consists of two solutions, Solution A (containing no glycerol) and Solution B (containing 14% glycerol). We used these solutions to prepare a one-step diluent, mixing both solutions in such a way that the final concentration of glycerol was 4%.

A total of 50 μL sperm suspension in cryodiluent was loaded into short straws at room temperature (approximately 22°C). Before loading, 0.25-mL straws (Minitüb) were cut in half with a pair of scissors and the half with the cotton-sealed end was used. After loading, straws were closed with a thermal sealer (ERSA; Minitüb). Straws were finally placed inside a 5-mL cryovial for refrigeration. An aliquant with semen in cryodiluent was placed in a microtube and used for evaluation of motility and acrosome integrity at the end of refrigeration.

Refrigeration, from 20°C to 5°C over a period of 120 min, was performed using a programmable dry block (ThermoStat plus 5352; Eppendorf, Hamburg, Germany). The dry block was used with a dry thermoblock chamber (CombiBox; Eppendorf), into which a microtube rack was placed to hold both the sample of diluted semen for evaluation and the 5-mL cryovial containing the straws. An external digital thermometer with two thermocouples was fitted for accurate monitoring of temperature; one thermocouple was placed inside the chamber and the other was placed inside one straw filled with cryodiluent alone, which, in turn, was placed inside the 5-mL cryovial. The dry block has a temperature control capacity ranging from −5°C to 99°C; both the temperature and the run time can be programmed individually, with up to four temperature levels and four time phases adjustable in succession. Thus, the run time for the linear transition between two temperature levels can be programmed individually and this, in turn, determines the curve (in $^{\circ}C\,min^{-1}$) for temperature descent. Initial trials revealed that the actual temperature inside the CombiBox chamber (as measured by the external thermometer) was different from that displayed in the dry block control panel and measured by its in-built thermocouple; this was probably due to the slow cooling by convection inside the CombiBox chamber. We therefore ran a series of tests to identify the best programme that would result in the desired final temperature (5°C) inside the chamber. The initial temperature was always set to 20°C and, in order to compensate for some variations in room temperature, the dry block was left running at this temperature before sample loading. Once the vial containing straws was introduced into the chamber, an initial period of 5 min at 20°C was always programmed to allow for additional equilibration at this temperature. Based on preliminary trials, three different final temperatures (0°C, −1°C and −2°C) were set in the dry block with the aim of achieving 5°C inside the chamber at the end of the refrigeration period (120 min). Actual temperatures were recorded every 10 min over the 120 min period. At least three runs were performed for each setting and recorded. A final temperature of 5°C was reached inside the chamber when the dry block was set with a target temperature of −1°C. This setting was subsequently used for all experiments into the cryopreservation of Iberian lynx spermatozoa. We found this system more convenient and reliable than refrigeration using a fridge or water-filled containers with temperature lowered by the addition of ice cubes or dry ice.

At the end of refrigeration, straws were removed from the 5-mL cryovial and were frozen using a two-step freezing method (Pukazhenthi *et al*. 2007). Briefly, straws were placed horizontally on a metal rack in a styrofoam container, 7.5 cm above the liquid nitrogen surface, for 1 min and then quickly lowered to a second rack 2.5 cm above the level of the liquid nitrogen for a further 1 min before being plunged into liquid nitrogen. Frozen sperm straws were transferred into a liquid nitrogen tank for transport and storage.

Sperm thawing and assessment

Straws were thawed by exposing them to air for 10 s and then immersing them in a 37°C water bath for 30 s. The contents of the straws were poured into a sterile 1.5-mL microtube prewarmed to 37°C. The sperm suspension was diluted (1 : 3 v/v) by the slow (drop by drop) addition of a modified Tyrode's solution (Gómez *et al*. 2003), as used for IVF (see below). The concentration was adjusted to $0.5–1 \times 10^5$ motile spermatozoa mL^{-1} in 50–100 μL drops covered with mineral oil. Diluted spermatozoa were incubated at 38.5°C under 5% CO_2 in air and sperm subsamples were assessed at various times (0, 1 and 2 h) for motility and acrosomal integrity, as described above.

Oocyte collection and IVM

Ovaries from domestic cats were obtained from veterinary clinics after ovariohysterectomy and were transported refrigerated to the laboratory in 15-mL vials with 5 mL of 0.9% (w/v) NaCl solution supplemented with penicillin and streptomycin ($100\,\mu g\,mL^{-1}$ each). Ovaries that were not processed immediately were stored at 4–8°C for up to 24 h after surgery. Samples were processed as described previously (Gómez *et al*. 2003), with some modifications. The ovaries were sliced and the follicles punctured to release cumulus–oocyte complexes (COCs). Medium TCM-199 (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) bovine serum albumin (BSA; A-8412; Sigma) and penicillin and streptomycin ($100\,\mu g\,mL^{-1}$ each), was used for oocyte collection. After several washes in HEPES-buffered-TCM-199, Grade I (excellent quality), Grade II (good quality) and Grade III (fair quality) COCs were selected and placed in four-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; A-9418; Sigma), $25\,ng\,mL^{-1}$ epidermal growth factor (EGF; Merlo *et al.* 2005), ovine FSH/LH ($10\,\mu g\,mL^{-1}$ each; Sigma), $1\,\mu g\,mL^{-1}$ oestradiol, penicillin and streptomycin ($100\,\mu g\,mL^{-1}$ each). Dishes were cultured at 38.5°C under 5% CO_2 in air and maximum humidity for 24 h.

In vitro *fertilisation*

After 24 h IVM, domestic cat oocytes were coincubated with frozen–thawed Iberian lynx spermatozoa. Oocytes (10–20 per drop) were placed in 50–100 μL drops of Tyrode's solution supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, $100\,\mu g\,mL^{-1}$ penicillin, $100\,\mu g\,mL^{-1}$ streptomycin and 0.6% (w/v) BSA (fatty

Table 1. Phenotypic and seminal parameters of fresh ejaculates obtained from Iberian lynx

Seminal traits for each male are the mean of the different fractions of the ejaculate. Relative testes weight was calculated as the total testes weight divided by body mass. The sperm motility index (SMI) was calculated as (% sperm motility + (quality of motility × 20))/2. The quality of movement was determined using a scale of 0–5, where 0 is no movement and 5 represents rapid linear forward movement

	Male (studbook no.)					Mean ± s.e.m.
	SB5	SB6	SB7	SB8	SB11	
Year of birth	2000	2002	2003	2000	2004	
Body mass (kg)	14.3	8.3	10.8	13.4	12.7	11.9 ± 1.1
Total testes weight (g)	2.7	2.7	2.2	2.3	2.7	2.5 ± 0.1
Relative testes weight ($\times 10^{-4}$)	1.8	3.3	2.0	1.7	2.2	2.2 ± 0.3
Ejaculate volume (μL)	590	340	429	675	383	483.4 ± 63.9
Semen pH	8.0	8.3	7.8	8.0	7.5	8.0 ± 0.1
Sperm concentration ($\times 10^6$ spermatozoa mL^{-1})	5.1	15.8	3.4	5.5	8.2	7.6 ± 2.2
Total no. spermatozoa ($\times 10^6$)	3.0	5.4	1.5	3.7	3.1	3.3 ± 0.6
Motility (%)	90.0	68.9	71.1	75.3	62.4	73.5 ± 4.6
Quality of movement (0–5)	3.3	3.0	3.0	2.9	3.1	3.1 ± 0.1
SMI	77.8	64.4	65.1	67.0	62.2	67.3 ± 2.7
Morphologically normal spermatozoa (%)	18.5	28.3	18.2	37.4	15.8	23.7 ± 4.0
Intact acrosomes (%)	38.2	46.9	35.3	45.7	37.4	40.7 ± 2.3

acid free; catalogue no. 1265479; Calbiochem, Madrid, Spain; Gómez *et al.* 2003; Pope *et al.* 2006). Oocytes were coincubated, under mineral oil, with 0.5–1 × 10^5 motile spermatozoa mL^{-1} in an atmosphere of 5% CO_2 in air at 38.5°C for 18–20 h.

In vitro *culture*

After gamete coculture, oocytes were gently pipetted to remove cumulus cells and unattached spermatozoa and washed three times with HEPES-buffered Tyrode's medium. Presumptive zygotes were transferred to four-well culture dishes with 500 μL Tyrode's solution containing 1% (v/v) minimum essential medium (MEM) non-essential amino acids, 0.3% (w/v) BSA (fatty acid free) and supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gómez *et al.* 2003; Pope *et al.* 2006). The culture was performed under 5% CO_2 in air at 38.5°C. Cleavage was evaluated 44–48 h after insemination. Non-cleaved oocytes and cleaving embryos were stained with Hoechst 33342 (10 μg mL^{-1}) in glycerol (1 : 9, v/v), mounted onto a slide in the same medium, covered with a coverslip supported by vaseline : paraffin (10 : 0.7, w/w) and kept refrigerated at 5°C in the dark until examination. Non-cleaved oocytes were examined to assess maturation and fertilisation status, as well as the number of spermatozoa attached to the zona pellucida. Embryos were examined to determine the total number of nuclei.

Statistical analyses

Data were analysed using SPSS ver. 11.5 (SPSS, Chicago, IL, USA). Percentages were arcsine transformed, whereas other variables were $\log_{10}$ transformed. Repeated-measures (RM) ANOVA was used to examine differences between treatments and over time in sperm motility and acrosome integrity. When the results of the ANOVA were significant, multiple comparison tests were performed. To compare fertilisation rates of spermatozoa cryopreserved in TEST or Biladyl, non-parametric tests for two dependent samples were performed. Relationships between fertilisation rates and ejaculate traits were analysed by non-parametric Spearman's rank correlation. Results are presented as the mean ± s.e.m. and $P < 0.05$ was regarded as statistically significant.

Results

Phenotypic traits and semen parameters of male Iberian lynx

Phenotypic traits and semen parameters are given in Table 1. Ejaculates contained a total of 1.5–5.4 × 10^6 spermatozoa (mean 3.3 ± 0.6 × 10^6 spermatozoa). Sperm motility ranged between 62% and 90%, with an average of 73.5 ± 4.6% motile spermatozoa. The SMI was calculated to take into account both the percentage motile spermatozoa and the quality of sperm movement and averaged 67.3 ± 2.7. The Iberian lynx produces a high number of pleiomorphic spermatozoa (Fig. 1*a*), with values of morphologically normal spermatozoa ranging between 19% and 37% (mean number of normal spermatozoa 23.7 ± 4.0%; Table 1). Assessments of acrosome integrity (Fig. 1*b*) revealed low percentages of spermatozoa with intact acrosomes (37–47%), with an average of 40.7 ± 2.3% intact sperm (Table 1).

Incubation in different media and sera

When spermatozoa were incubated in F-10 or TCM-199 (with inactivated FBS), considerable decreases in SMI (percentage motility and quality of motility; $P < 0.05$) were observed in both media over 3 h incubation (Fig. 2*a*). There was also a decrease in the percentage of intact acrosomes over time, but the difference was only significant for sperm incubated in TCM-199 ($P < 0.05$; Fig. 2*b*). There were no significant differences between media with regard to SMI or acrosome integrity ($P = 0.32$; Fig. 2).

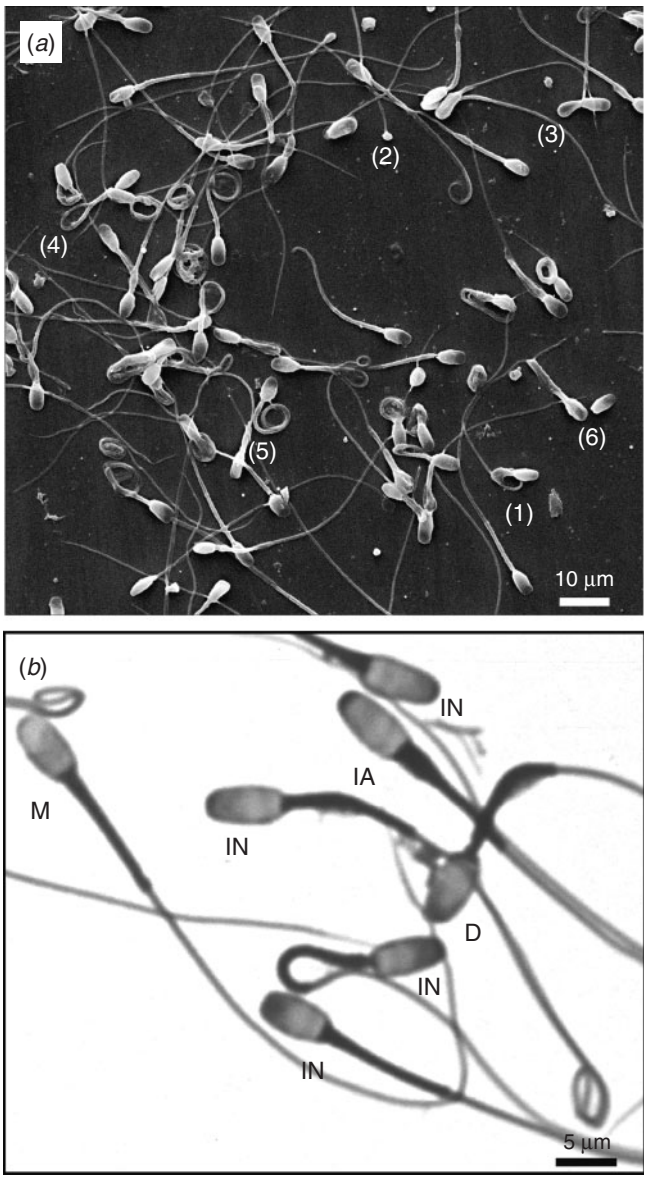

Fig. 1. Sperm abnormalities and acrosome integrity in the Iberian lynx. (*a*) Spermatozoa examined by scanning electron microscopy. (1) normal; (2) head defect (microcephalic); (3) bent midpiece; (4) bent flagellum; (5) coiled tail; (6) detached head. (*b*) Acrosome status of Iberian lynx spermatozoa stained with Coomassie brilliant blue and examined using brightfield optics. IN, intact normal; IA, intact abnormal; D, damaged; M, missing.

When spermatozoa were suspended in F-10 medium with FBS or Iberian lynx serum, a considerable decrease in sperm motility and a less pronounced decrease in acrosome integrity were observed over time with both sera (Fig. 3). No differences were found in motility or acrosome integrity between sera, with the exception of a higher percentage of acrosome integrity in medium containing FBS after 3 h incubation ($P = 0.003$; Fig. 3).

Cryopreservation of Iberian lynx spermatozoa

All male lynx produced semen of sufficient quality for cryopreservation. Mean values of samples prior to centrifugation

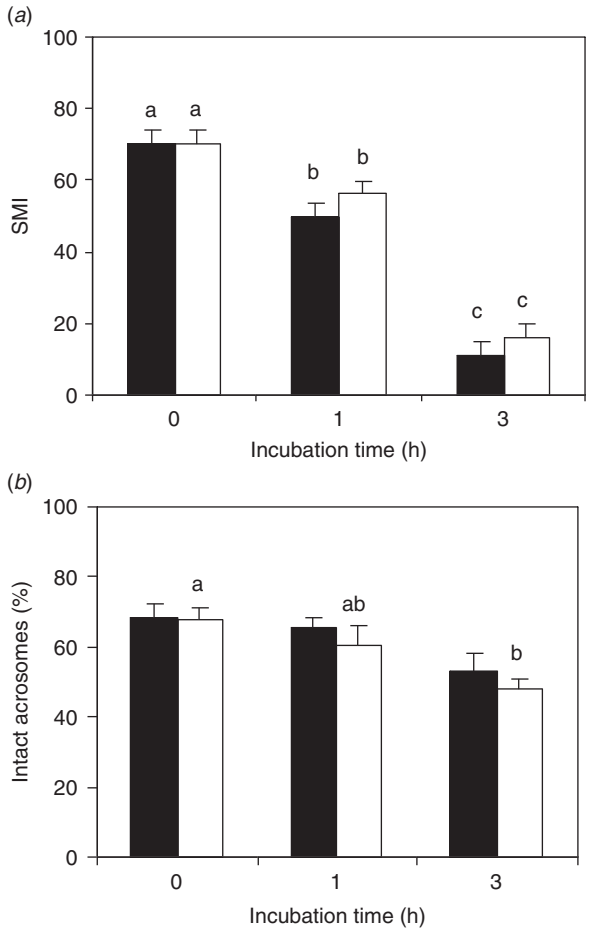

Fig. 2. Incubation of Iberian lynx spermatozoa in HEPES-buffered Ham's F-10 (black bars) or TCM-199 (open bars) media containing fetal bovine serum. (*a*) Sperm motility index (SMI) and (*b*) percentage of intact acrosomes of Iberian lynx spermatozoa after incubation for various times at 37°C under air. Results are the mean ± s.e.m. For each medium, different superscript letters indicated significant differences ($P < 0.05$) between incubation times.

were 77.3 ± 3.7% motile spermatozoa, 70.1 ± 2.7 SMI and 37.6 ± 4.5% intact acrosomes.

There was a decline in sperm motility during cryopreservation, with a considerable decrease in motility upon thawing and subsequent incubation (Fig. 4). There were also significant differences ($P < 0.05$) in sperm motility between cryodiluents at various steps in the cryopreservation process (Fig. 4). Significant differences were observed after the addition of cryodiluent, after refrigeration, after thawing and after 1 and 2 h incubation ($P < 0.05$). After thawing, spermatozoa exhibited better motility in TEST (34.0 ± 6.2% motile spermatozoa and 43.0 ± 2.9 SMI) than in Biladyl (7.5 ± 4.8% motile spermatozoa and 11.3 ± 7.2 SMI). Samples from two males (SB5 and SB7) did not have any motile spermatozoa after thawing when Biladyl was used as the cryodiluent.

There were no differences over time in the percentage of acrosome integrity, with the exception of samples incubated for 1 and 2 h after thawing, which showed lower values ($P < 0.05$).

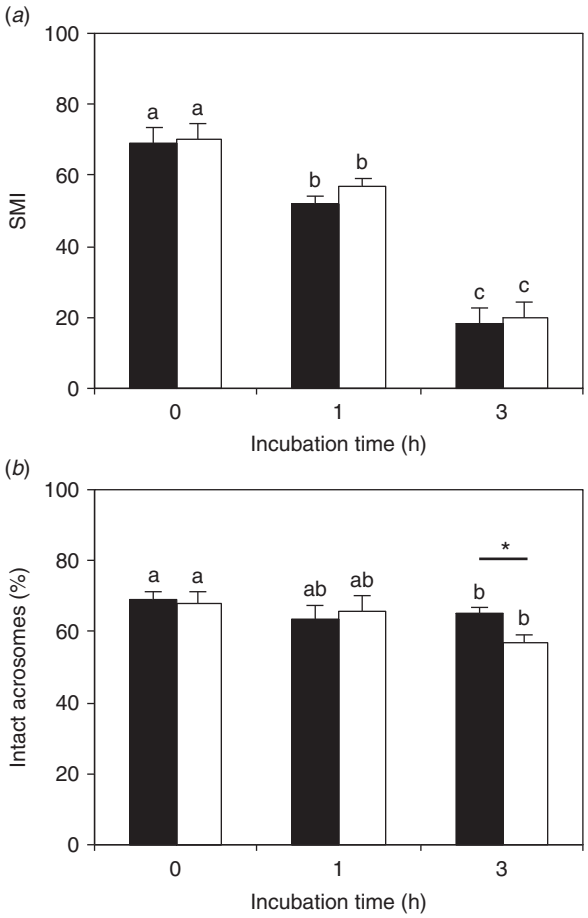

Fig. 3. Incubation of Iberian lynx spermatozoa in HEPES-buffered Ham's F-10 containing either fetal bovine serum (black bars) or Iberian lynx serum (open bars), both inactivated at 56°C for 30 min. (*a*) Sperm motility index (SMI) and (*b*) the percentage of intact acrosomes of Iberian lynx spermatozoa after incubation for various times at 37°C under air. Results are the mean ± s.e.m. For each medium, different superscript letters indicated significant differences ($P < 0.05$) between incubation times. Asterisk indicates significant differences ($P < 0.05$) between sera after 3 h incubation.

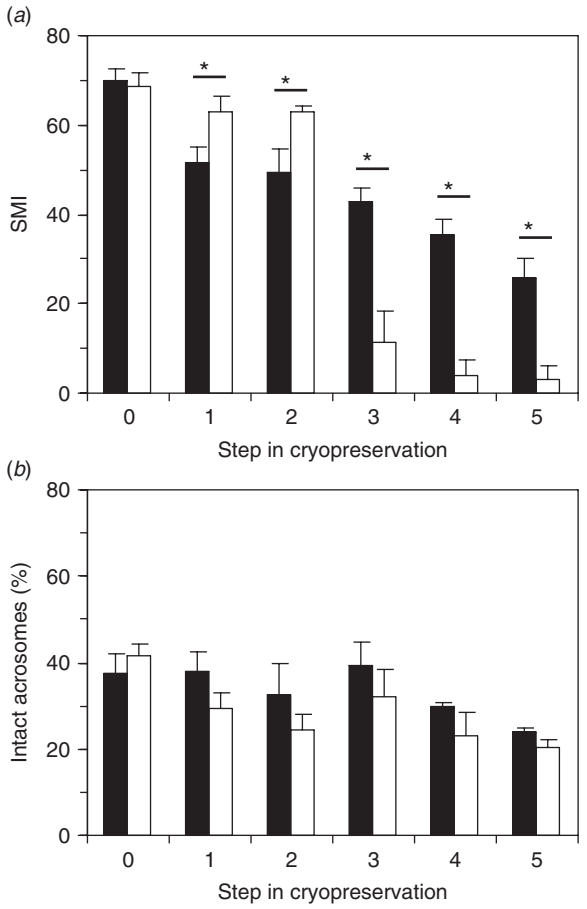

Fig. 4. Cryopreservation of Iberian lynx spermatozoa with two different cryodiluents, namely Tes–Tris (TEST; black bars) and Biladyl (open bars). (*a*) Sperm motility index (SMI) and (*b*) percentage of spermatozoa with intact acrosomes at different times in the cryopreservation process. 0, initial semen pool; 1, after centrifugation and addition of cryodiluent; 2, at the end of refrigeration; 3, after semen thawing (0 h of incubation post-thaw); 4 and 5, after 1 and 2 h of incubation post-thaw, respectively. After thawing, and before evaluation, spermatozoa were diluted with a modified Tyrode's medium and incubated at 38.5°C under 5% CO_2/air. Results are mean ± s.e.m. Within time points, asterisks indicate significant differences ($P < 0.05$) between cryodiluents.

No significant differences were noted in the percentage of intact acrosomes between the TEST and Biladyl cryodiluents (39.4 ± 5.3% and 32.3 ± 6.3% intact acrosomes, respectively).

Heterologous IVF with cryopreserved spermatozoa

Iberian lynx spermatozoa were able to fertilise domestic cat oocytes *in vitro* (Fig. 5). The fertilisation rate obtained varied between 5% and 30% of mature oocytes, depending on the male and the cryodiluent used to preserve the spermatozoa (Table 2). The average fertilisation rate of spermatozoa cryopreserved in TEST (20.5 ± 4.5% of mature oocytes) was approximately twice that of spermatozoa cryopreserved in Biladyl (11.5 ± 6.8% of mature oocytes; Table 2), although the difference did not reach statistical significance, probably due to the low number of males tested.

The percentage of mature and fertilised oocytes that cleaved (embryos at least at the two-cell stage 48 h after insemination) was 11.3 ± 5.3% and 44.7 ± 19.6%, respectively, using spermatozoa cryopreserved in TEST. There was no relationship between the proportion of mature oocytes fertilised and the proportion of oocytes that cleaved.

We explored possible relationships between fertilisation rates of different males and the traits of fresh semen samples from these different individuals. We observed positive significant relationships between the fertilisation rate and both the percentage of normal spermatozoa (Fig. 6*a*) and the percentage of intact acrosomes (Fig. 6*b*).

Discussion

The present study is the first to characterise sperm traits of the critically endangered Iberian lynx, explore the suitability of two

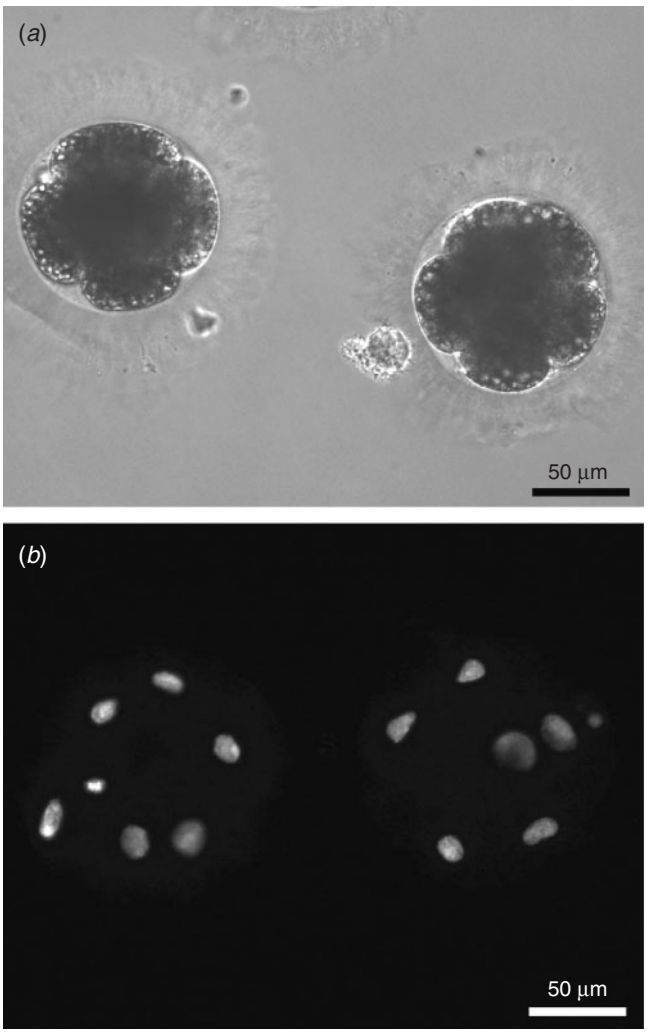

Fig. 5. Hybrid embryos resulting from heterologous IVF using cryopreserved Iberian lynx spermatozoa and *in vitro*-matured domestic cat oocytes. (*a*) Embryos examined by phase contrast 44 h after insemination and (*b*) embryos after staining with Hoechst 33342 and examined under a fluorescence microscope.

diluents for semen cryopreservation and to examine the fertilising ability of cryopreserved spermatozoa using a heterologous *in vitro* fertilisation assay with domestic cat oocytes.

The total number of spermatozoa collected in ejaculates of electrostimulated Iberian lynx was relatively low (mean 3.3×10^6 spermatozoa). Total sperm numbers in other medium-sized felids after electroejaculation using a similar protocol have been reported to be much higher, for example 190×10^6 spermatozoa in the ocelot (Stoops *et al*. 2007), 101×10^6 spermatozoa in the serval (Pukazhenthi *et al*. 2002), 56×10^6 spermatozoa in the fishing cat (Thiangtum *et al*. 2006) and 41×10^6 spermatozoa in the caracal (Howard 1993). Conversely, values seen in the Iberian lynx are higher than those reported for other lynx species, such as the Eurasian lynx (1.6×10^6 total spermatozoa per ejaculate during the breeding season; Jewgenow *et al*. 2006), and those

Table 2. Heterologous fertilisation of *in vitro*-matured domestic cat oocytes with frozen–thawed spermatozoa from Iberian lynx

The sperm concentration was 0.5–10^5 motile cells mL^{-1}. Gametes were coincubated for 18–20 h. Cleavage was assessed after 44–48 h in culture after insemination. Non-cleaved and cleaved oocytes were stained with Hoechst 33342 (10 μg mL^{-1}) in glycerol (1 : 10, v/v) to retrospectively assess maturation and fertilisation status. Data are shown for each Iberian lynx male and as the mean ± s.e.m. nd, not done (semen from two males did not survive cryopreservation in Biladyl, with 0% motile spermatozoa after thawing; thus, IVF could not be tested with spermatozoa from these males when this cryodiluent was used)

Male (studbook no.)	TEST				Biladyl			
	No. oocytes fertilised/no. mature oocytes (%)	No. spermatozoa bound per mature oocyte	No. cleaved oocytes/no. fertilised oocytes (%)	No. cleaved oocytes/no. mature oocytes (%)	No. oocytes fertilised/no. mature oocytes (%)	No. spermatozoa bound per mature oocyte	No. cleaved oocytes/no. fertilised oocytes (%)	No. cleaved oocytes/no. mature oocytes (%)
SB5	5/21 (24)	1.7	0/5 (0)	0/21 (0)	nd	nd	nd	nd
SB6	11/37 (30)	0.4	7/11 (64)	7/37 (19)	1/21 (5)	0.8	1/1 (100)	1/21 (5)
SB7	5/29 (17)	2.7	3/5 (60)	3/29 (10)	nd	nd	nd	nd
SB8	9/33 (27)	5.1	9/9 (100)	9/33 (27)	4/22 (18)	3.1	3/4 (75)	3/22 (14)
SB11	1/21 (5)	0.5	0/1 (0)	0/21 (0)	nd	nd	nd	nd
Mean ± s.e.m.	20.5 ± 4.5	2.1 ± 0.9	44.7 ± 19.6	11.3 ± 5.3	11.5 ± 6.8	1.9 ± 1.1	87.5 ± 12.5	9.2 ± 4.5

estimated for the bobcat (approximately 0.8×10^6 spermatozoa per ejaculate; Howard 1993).

The proportion of motile spermatozoa in ejaculates from the Iberian lynx (73.5 ± 4.6%) was lower that reported for the ocelot (83.3 ± 3.0%; Stoops *et al*. 2007) or caracal (85.0 ± 4.0; Howard 1993), but was similar to that reported for the fishing cat (73.0 ± 4.0%; Thiangtum *et al*. 2006), serval (73.0 ± 1.8%; Pukazhenthi *et al*. 2002) and Eurasian lynx (77.0%; Jewgenow *et al*. 2006), and was higher than that reported for the bobcat (48.0 ± 8.6%; Howard 1993).

Compared with the percentage of intact acrosomes in the ejaculate reported for the fishing cat (90%; Thiangtum *et al*. 2006), ocelot (94%; Baudi *et al*. 2008) and Pallas' cat (95%; Swanson *et al*. 2006), values obtained in the present study for the Iberian lynx were relatively low (40.7 ± 2.3%).

Several felids exhibit a high prevalence of teratospermia, with >60% morphologically abnormal spermatozoa in the ejaculate (Pukazhenthi *et al*. 2006*b*). Spermatozoa from teratospermic donors have a reduced ability to undergo capacitation and the acrosome reaction, bind to and penetrate the zona pellucida, enter the periviteline space and fertilise conspecific oocytes. Even normal spermatozoa from teratospermic donors have an impaired ability to penetrate the zona pellucida (Pukazhenthi *et al*. 2006*b*). The number of morphologically normal spermatozoa varies widely in felids (Wildt *et al.* 1983, 1986; for a review, see Pukazhenthi *et al*. 2006*b*), with some species showing high values of normal spermatozoa, such as the ocelot (78%; Stoops *et al*. 2007; Baudi *et al*. 2008), leopard cat (65%; Howard and Wildt 1990) and serval (64%; Pukazhenthi *et al*. 2002), and others with extreme levels of teratospermia, as seen in the cheetah (12% normal spermatozoa; Roth *et al*. 1995) and the Florida panther (6% normal spermatozoa; Barone *et al*. 1994). Compared with other felid species arranged according to the proportion of normal spermatozoa (Pukazhenthi *et al*. 2006*b*), the Iberian lynx was found to be in the lower part of the range. Male Iberian lynx produce a high number of pleiomorphic spermatozoa, with an average low value of morphologically normal spermatozoa (16–37%). These values are similar to those recorded in other lynx species, such as the Eurasian lynx (28.7 ± 13.0% (Howard 1993) and 26.0% (Jewgenow *et al*. 2006)), and in the bobcat (20.3 ± 4.7%; Howard 1993).

It may be argued that sperm parameters in the Iberian lynx based on semen collected approximately 1.5 months before the peak of mating activity (as reported here) may not entirely reflect the true quality of the ejaculate as seen during the breeding season. The Iberian lynx appears to have a restricted seasonal reproduction lasting approximately 2 months. Although this may be true for females, it is not known whether sperm production occurs over a wider time range. We have been able to collect semen in April (5 months later than the timing reported in the present study) and found that the quality of the sperm was similar to that reported herein (N. Gañan, J. Garde, M. Gomendio, E. R. S. Roldan, unpubl. data). Future work will need to address sperm quality in this species at the peak of the mating season. Another factor that may affect sperm quality is inbreeding. Earlier work in various felids has revealed that low genetic variability in different populations is associated with poor sperm quality (for a review, see Roldan and Gomendio 2009). Thus, the low sperm quality identified in the Iberian lynx may be the result of reduced genetic variability and future studies should be designed to examine this possibility.

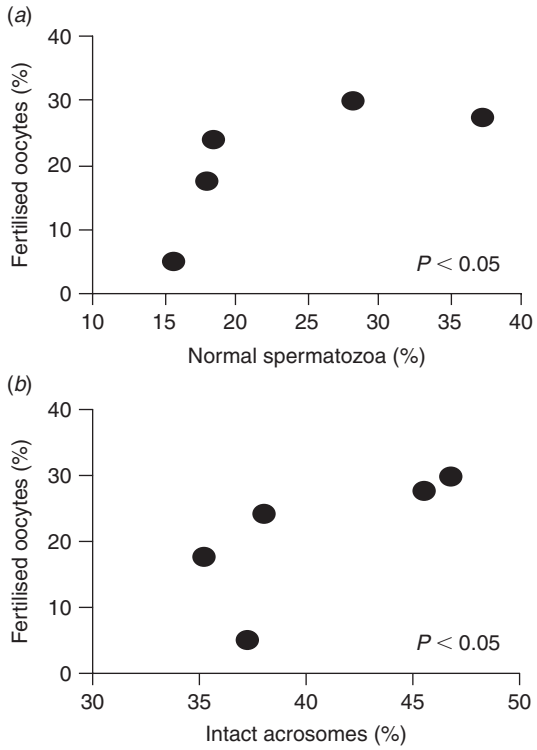

Fig. 6. Relationship between fertilisation rates and sperm traits. Correlation between the percentage of fertilised oocytes and (*a*) the percentage of normal spermatozoa and (*b*) the percentage of intact acrosomes. *P* values in each graph correspond to Spearman's rank correlation coefficients.

Although Ham's F-10 medium has been used for IVF with good results in several felids, including the tiger (Donoghue *et al*. 1990; Johnston *et al*. 1991), puma and lion (Johnston *et al*. 1991) and cheetah (Donoghue *et al*. 1992*b*), we found that F-10 did not support long-term survival of Iberian lynx spermatozoa, with SMI dropping from an initial value of 70 to approximately 15 at the end of a 3-h incubation period. The alternative medium tested in the present study (TCM-199) was no better in sustaining sperm motility during this period of time. When inactivated FBS or Iberian lynx serum were tested in F-10 medium, a decrease in sperm motility and acrosome integrity was observed over time with both sera. There was a much higher decline in SMI than in acrosome integrity in media containing either sera, but no differences were observed between sera. Thus, homologous serum did not improve the survival of spermatozoa during incubation and, regardless of the media or sera used, there was a marked decrease in sperm motility. Further studies are needed to identify alternative media for sperm incubation.

Differences were found between TEST and Biladyl cryodiluents with regard to the proportion of motile spermatozoa recovered after thawing, with significantly better results seen for spermatozoa diluted in TEST. Conversely, no differences were observed between diluents with regards to acrosome integrity.

The percentage of motile spermatozoa after cryopreservation in TEST (34% motile spermatozoa; SMI 43) was lower than the SMI of 50–55 reported for the fishing cat (Thiangtum *et al*. 2006), but better than values seen after cryopreservation of ocelot spermatozoa (25% motile sperm; Stoops *et al*. 2007); in all cases, a TEST–20% egg yolk–4% glycerol diluent was used and the refrigeration and freezing procedures were similar between studies. The proportion of intact acrosomes after cryopreservation of Iberian lynx spermatozoa in TEST was 39%. Although low, these values are better than those reported for other medium-sized felids, such as the fishing cat (20%; Thiangtum *et al*. 2006) and ocelot (25%; Stoops *et al*. 2007), after similar cryopreservation procedures.

We found that Iberian lynx spermatozoa were capable of fertilising viable IVM domestic cat oocytes, thus opening up the possibility of examining the functional capacity of spermatozoa from this species under laboratory conditions. There were differences between the fertilisation rates achieved by the various Iberian lynx males, which agrees with previous reports indicating that fertilisation rates show a high degree of inter-male variation (Howard and Wildt 1990; Donoghue *et al*. 1992*b*). For spermatozoa cryopreserved in TEST, the lowest fertilisation rate (5%) was seen with spermatozoa from the youngest male used in the present study (approximately 2.5 years old at time of semen collection). The best results were obtained with spermatozoa from a small male (8.3 kg) that was approximately 3.5 years old. Fertilisation rates were slightly lower for the other males examined, both of which were slightly older at approximately 5.5 years of age at the time of semen collection. Fertilisation rates were assessed based on cleavage to at least the two-cell stage plus the presence of pronuclei and polar bodies in non-cleaved oocytes 48 h after insemination. Fertilisation by spermatozoa cryopreserved in TEST (20% of mature oocytes) was approximately twice that observed with spermatozoa cryopreserved in Biladyl (11% of mature oocytes), although the difference did not reach statistical significance, probably due to the low number of males available for the study.

The fertilisation rates obtained for Iberian lynx spermatozoa (20% for spermatozoa cryopreserved in TEST) are lower than those obtained with ejaculated and cryopreserved spermatozoa from other medium-sized felids using similar conditions for coincubation with viable domestic cat oocytes (and assessed on the basis of cleavage to at least the two-cell stage 48 h after insemination). Howard and Wildt (1990) reported that leopard cat spermatozoa fertilised approximately 55% of cat oocytes, compared with 62%, 64% and 46% for the fishing cat (Thiangtum *et al*. 2006), ocelot (Stoops *et al*. 2007) and Pallas' cat (Swanson *et al*. 2006), respectively. There may be several reasons for the lower fertilisation rates seen with Iberian lynx spermatozoa. First, Iberian lynx sperm were collected just before the breeding season, which may have resulted in suboptimal sperm function (i.e. the ability to undergo changes required for fertilisation). Second, low fertilisation success may be related to poor sperm quality in the Iberian lynx (on average 24% normal spermatozoa and 41% intact acrosomes) compared with higher sperm quality in other felid species (45–78% normal spermatozoa, 90–95% intact acrosomes; see above) with higher fertilisation rates. Sperm morphology and acrosome integrity are important determinants of fertilisation success (Gomendio *et al*. 2007) and abnormal spermatozoa cannot participate in fertilisation (Howard *et al*. 1991).

Thus, we explored possible relationships between the fertilisation rates of different males and sperm traits of fresh ejaculates. We found positive significant relationships between the percentage of heterologous fertilisation and both the percentage of normal spermatozoa and the percentage of intact acrosomes, in agreement with relationships found using IVF assays in other species (Amann and Hammerstedt 1993; Gadea 2005; Aitken 2006). These results are important because they allow us to explore further the functional capacity of spermatozoa from Iberian lynx. There may be limited opportunities to test sperm fertility using AI or IVF with homologous oocytes, so the possibility of using domestic cat oocytes will have advantages for the assessment of either males before the breeding season or of cryopreserved spermatozoa stored in a genome resource bank.

In conclusion, the present study is the first detailed investigation of basal semen traits and sperm cryopreservation in the Iberian lynx. The results indicate that: (*1*) it is possible to collect and cryopreserve semen from captive animals; (*2*) various sperm traits of the Iberian lynx are similar to those of other small-sized felids and better than those recorded in other lynx species; (*3*) inactivated homologous serum does not result in better sperm survival than inactivated FBS when added to media used to wash and incubate spermatozoa; (*4*) there were no major differences between two complex media (F-10 *v.* TCM-199) with regard to sperm survival and acrosome integrity; (*5*) spermatozoa from the Iberian lynx survive cryopreservation with a protocol generally used for felid spermatozoa, with better results obtained using TEST as the cryodiluent than Biladyl; and (*6*) frozen–thawed spermatozoa from the Iberian lynx are capable of fertilising heterologous oocytes from domestic cats *in vitro*, thus demonstrating that this is a useful method to evaluate sperm function in the laboratory.

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