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Influence of cooling rate on the ability of frozen–thawed sperm to bind to heterologous zona pellucida, as assessed by competitive *in vitro* binding assays in the ocelot (*Leopardus pardalis*) and tigrina (*Leopardus tigrinus*)

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Abstract

We evaluated the influence of two cooling rates (from 25 to 5 °C) on post-thaw function of frozen sperm in ocelots (*Leopardus pardalis*; $n = 3$ males) and tigrinas (*Leopardus tigrinus*; $n = 4$ males). Seven normospermic (>70% normal sperm) electroejaculates from each species were diluted with a 4% glycerol freezing medium, divided into two aliquots, and assigned to one of two cooling rates: fast or slow (0.7 or 0.16 °C/min, respectively). Sperm motility index (SMI) and percentage of sperm with an intact acrosome were assessed before freezing and after thawing, and the ability of sperm to bind to the zona pellucida of IVM domestic cat oocytes were assessed in a competitive *in vitro* sperm-binding assay. Regardless of the cooling rate, frozen–thawed sperm from both species exhibited a SMI of 50; ~20 and ~32% of post-thaw sperm had an intact acrosome in ocelots and tigrinas, respectively ($P < 0.05$). The mean (±S.E.M.) number of sperm bound per oocyte was higher for fast-cooled (8.5 ± 1.3) than slow-cooled (2.5 ± 0.3; $P < 0.01$) ocelot sperm. In contrast, more tigrina sperm bound to domestic cat oocytes when cooled slowly versus quickly (5.8 ± 0.9 versus 2.7 ± 0.4, $P < 0.05$). In conclusion, cryopreservation decreased sperm function in both species, and the oocyte-binding assay was the most efficient method to detect functional differences in post-thaw sperm.

Keywords: Ocelot; Tigrina; Sperm cryopreservation; Cooling rate; *In vitro* sperm-binding assay

1. Introduction

Preliminary studies in South American felids demonstrated that current freezing–thawing protocols induced substantial damage to sperm membranes, resulting in a sharp decline (>50%) in the number of motile sperm with intact acrosomes after thawing [1–3]. Based on domestic cat data, this impact may be even

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worse in ejaculates with a high incidence of abnormal sperm, a common finding among felids [4–7]. To improve the function of post-thaw sperm, there is a need for a more systematic assessment of cryobiological properties of felid sperm to optimize factors including cryodiluent composition, type and final concentration of permeating cryoprotectant agent, and rate of cooling to freezing temperatures [5,6].

Although post-thaw sperm viability and acrosome integrity are very important for predicting fertility, sperm have to complete several functional steps before fertilization and embryo development can be achieved; these include transport to the oviduct, capacitation, zona pellucida binding, the acrosome reaction, and oocyte activation. A variety of sperm function tests have been employed in carnivores to study interactions among gametes and to predict the sperm fertilizing potential of raw or post-thaw ejaculates [8–14]. For all wild felid species studied to date, sperm cells were able to bind and to penetrate the (heterologous) domestic cat zona pellucida, providing a valuable tool for evaluating the functionality of feline sperm submitted to various conditions, including new freezing–thawing protocols.

The objective of this study was to evaluate the impact of cryopreservation on post-thaw quality of sperm from normospermic ejaculates of two endangered Brazilian felid species, the ocelot (*Leopardus pardalis*) and the tigrina (*Leopardus tigrinus*). Specifically, we tested the effect of two different cooling rates (to 5 °C) before freezing on post-thaw: (1) sperm motility and acrosome status; and (2) ability of sperm to bind to domestic cat zona pellucida in a competitive, *in vitro* oocyte-binding assay.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all chemicals used were reagent grade (Sigma Chemical Co., St. Louis, MO, USA).

2.2. Animals

Adult captive male ocelots (*L. pardalis*; $n = 4$) and tigrinas (*L. tigrinus*; $n = 4$), were housed at one of three institutions in Southern Brazil: Curitiba Zoo (Curitiba, PR; 25 °S: 49 °W), the Hermann Weege Foundation – Pomerode Zoo (Pomerode, SC; 26 °S: 49 °W), or the Klabin Conservation Breeding Center (Telêmaco Borba, PR; 24 °S: 39 °W). Ejaculates were initially collected from 11 males (five ocelots and six tigrinas) and evaluated; only males (four of each species) that consistently produced normospermic ejaculates (>60% morphologically normal sperm) were used for the present studies. Animals were exposed to natural lighting and fed a meat-based diet, with vitamin and mineral supplement and water *ad libitum*. All investigations were approved by the Sector of Biological Sciences Committee for the Care and Use of Laboratory Animals and by the Brazilian Federal Environment Agency (IBAMA).

2.3. Semen collection, analysis, and processing

Each male was subjected to three (ocelots) or two (tigrinas) electroejaculation procedures from September (2004) to March (2005), with at least 60 days between successive procedures. The cats were anaesthetized with a combination of ketamine HCl (20 mg/kg; Ketaset, Fort Dodge Laboratories Inc., Fort Dodge, IA, USA) and xylazine (0.5 mg/kg; Rompun, Mobay Corp., Shawnee, KS, USA), mixed together and injected im (conventional syringe). Semen was collected using an AC, 60 Hz electroejaculator and a rectal probe (1.0 cm × 13 cm or 1.6 cm × 23 cm for tigrinas and ocelots, respectively) with three longitudinal electrodes (P.T. Electronics, Boring, OR, USA) [15]. A consistent sequence of 80 electrical stimuli (2–5 V) was given in three series of 30, 30, and 20 stimuli each, with ~10 min interval between series. Each ejaculate was collected in a warm (37 °C) polyethylene container and semen was evaluated initially for volume (μL), pH (EM- Reagents – 6.0–10 ± 0.2; Merck, Darmstadt, Germany), sperm percent motility (from 0 to 100%, in increments of 10%) and forward progressive motility (scale, 0–5; 5 = best), using subjective analysis under a phase-contrast microscope (100×). To put equal emphasis on both sperm percent motility and progressive motility, a sperm motility index (SMI) was calculated (SMI = [% motility + (20× sperm progressive motility)]/2) [15]. Sperm concentration in 5–10 μL of pooled ejaculate from each series was determined using a hemacytometer [15]. An aliquot (5–10 μL) of raw semen was fixed in 100 μL of 0.3% (v/v) glutaraldehyde in phosphate-buffered saline and later evaluated for sperm morphology (200 cells/ejaculate) under phase-contrast microscopy (1000×) [15]. Ejaculates were immediately diluted 1:1 in Ham's F10 complete medium (Ham's F-10 supplemented with 0.1 mM pyruvate, 2.0 mM glutamine, 5% fetal calf serum, 0.05 g/L streptomycin, 0.05 g/L penicillin, and 1.2 g/L sodium bicarbonate), and centrifuged at 300 × *g* for 10 min. The supernatant was removed,

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and the sperm pellet was resuspended in cryoprotectant medium (PDV, consisting of 11% lactose (w/v), 20% egg yolk (v/v), and 4% glycerol (v/v) [16]) to a target concentration of 50–100 × 10^6 motile sperm/mL. Based on previous data on the total number of sperm/ejaculate in each species [3,17] and to avoid the effect of differences in sample volume on cooling rate, two ~250 μL aliquots of diluted semen were transferred to 1.5 mL microcentrifuge tubes, and cooled from 25 to 5 °C at two rates: a fast cooling rate (30 min in a refrigerator) or a slow cooling rate (in a 60 mL water bath, equilibrated over 3 h to 5 °C) [11,18]. The decrease in sample temperature under field conditions, estimated by thermocouple measurements under laboratory conditions, had a constant temporal (time to achieve 66% drop from initial temperature) of 10 min and a rate of 0.7 °C/min for the fast cooling method, and 53 min and 0.16 °C/min, respectively, for slow cooling. After cooling, samples from both treatments were frozen by pipetting 30 μL drops of diluted semen into indentations made on the surface of dry ice, waiting 3 min and then inverting the block of dry ice to plunge the frozen pellets into liquid nitrogen [15]. Frozen pellets were sealed in cryovials and stored in liquid nitrogen. For thawing, one pellet was immersed into a microcentrifuge tube containing 100 μL of warm (37 °C) Ham's F-10 complete medium, and gently agitated for 30 s in a water bath (37 °C). Thawed sperm were centrifuged a 300 × *g* for 10 min and the supernatant aspirated and discarded to remove cryoprotectant. The resulting pellet was gently resuspended in 50 μL of Ham's F-10 complete medium at room temperature and evaluated for sperm motility, forward progressive motility, and sperm concentration [15].

2.4. Acrosomal evaluation

The acrosomal status in both raw and frozen–thawed samples was assessed by mixing a sperm aliquot (2 μL) with 10 μL of a rose Bengal/fast green stain [19], maintaining it at 22 °C for 2 min, followed by smearing onto glass slides. The percentages of intact and non-intact acrosomes were determined by evaluating 200 sperm/sample using bright field microscopy. An intact acrosome was indicated by a purplish-blue staining over the anterior portion of sperm head, whereas non-intact sperm appeared colorless to light pink over the same region [2,19]. Acrosomal status evaluations were performed before semen dilution and processing for raw samples, and after washing sperm to remove cryoprotectant for thawed samples.

2.5. Oocyte recovery and in vitro maturation

Oocyte recovery and IVM were performed, with a few modifications, as described in detail previously [20,21]. Ovaries were collected following ovariohysterectomy at four local veterinary clinics and at two university animal hospitals, and stored in PBS supplemented with penicillin (0.05 g/L) and streptomycin (0.05 g/L) at 4 °C. Oocytes were recovered within 6 h post surgery by mincing ovaries in Minimum Essential Medium containing Hank's salts, 2.0 mM glutamine, 0.4% BSA (Fraction V), 25.0 mM HEPES, 0.05 g/L streptomycin, and 0.05 g/L penicillin (HMEM) at 37 °C. Either Grade I oocytes (with a dark and uniform cytoplasm that were completely surrounded by cumulus cells) or Grade II oocytes (with a light–dark cytoplasm, slightly granular, or not completely surrounded by cumulus cells) were selected. Oocytes were washed twice in HMEM and once in the maturation medium: Minimum Essential Medium, containing Earle's salts, 0.1 mM pyruvate, 2.0 mM glutamine, 0.4% BSA (Fraction V), 0.05 g/L streptomycin, 0.05 g/L penicillin, 2 μg/mL FSH (Folltropin V, Bioniche Animal Health, Canada Inc., Belleville, ON, Canada), 2 μg/mL LH (Lutropin V, Bioniche Animal Health Canada Inc.), and 2 μg/mL of β-estradiol. Groups of 10–20 cumulus–oocyte complexes were cultured for 24 h in 100 μL microdrops, under mineral oil (37 °C in air with 5% CO_2). After maturation, oocytes were incubated in HMEM with 0.4% hyaluronidase for 5 min at 37 °C, and cumulus cells were mechanically removed by repeated pipetting. Prior to the binding tests, oocytes were washed in HMEM and Ham's F-10 and transferred in groups of 5–10 to 40 μL Hams-F10 microdrops under oil. Oocytes were processed separately by grade throughout the experimental procedure. After removal of cumulus cells, the incidence of nuclear maturation in each batch of oocytes was analyzed in 10–20% of the oocytes by fixing in 500 μL PBS supplemented with 2% (w/v) paraformaldehyde and 0.04% (v/v) Triton-X for 45 min at 37 °C [20,22]. For assessing meiotic stage, oocytes were stained with 5 μg/mL bisbenzimide H33342 (Hoechst) in 500 μL HMEM for 10 min at 37 °C, mounted on slides, and evaluated under a fluorescence microscope (200×; Axiophot Carl Zeiss microscope, Wetzlar, Germany). Oocytes in metaphase II were considered mature [20].

2.6. In vitro sperm-binding assays

To compare the sperm binding capacity of sperm following either fast or slow cooling, a competition test was used [23], with modifications of the staining

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method. Two pellets of frozen sperm from the same ejaculate (one pellet/treatment) were thawed and evaluated for sperm motility, progressive motility and acrosomal status (as described above). A sperm suspension of 1×10^6 motile sperm/mL (in Ham's F-10) was prepared for each treatment. Subsequently, one of the two sperm suspensions (fast or slow cooling) was randomly assigned to be stained with 0.1 μg/mL of Hoechst stain, leaving the other one unstained. For the binding assays, 5 μL of each sperm suspension were added to 40 μL drops of Ham's F-10, under equilibrated oil, containing 5–10 mature oocytes. Gametes were co-incubated for 18 h at 37 °C and 5% CO_2 in humidified air. After co-culture, the oocytes were washed three times in Ham's F-10 to remove loosely attached sperm, mounted on slides, and evaluated for attached sperm under fluorescence excitation (200×; Axiophot Carl Zeiss microscope, Wetzlar, Germany). All fluorescent sperm bound to the zona were counted. Subsequently, a new staining step was performed, by adding 20 μL of a 5 μg/mL Hoechst underneath the coverslip. The number of fluorescent sperm bound to the zona was again assessed. Total number of sperm of each sperm treatment was determined as follows: number of sperm attached to oocytes for the stained sample was estimated during the initial counting, whereas the number of sperm cells of the unstained sample was calculated by the difference between the first and the second sperm count. All images from each evaluation were saved in a computer system (attached to the microscope) for later analysis. The optimal concentration of Hoechst stain was determined (serial dilutions) in preliminary experiments. The impact of the fluorescent stain on the ability of sperm to bind to the zona pellucida and potential differences in staining methods (within the microcentrifuge tube or underneath the coverslip) were assessed by testing sperm suspensions from both treatments and the same ejaculate either stained or unstained, on different days, with different batches of oocytes.

2.7. Statistical analysis

Differences among species and treatments within species for all sperm evaluations were determined by a Student's *t*-test after angular (arc sine) transformation of variables recorded as percentages (e.g., sperm motility and sperm morphology). For sperm binding tests, differences in the proportion of sperm from each treatment that was bound to the zona between replicates for the same ejaculate (stained versus unstained) were assessed by Chi-square analysis. As no difference was found between replicates, the mean value for the two replicates was used for subsequent analysis. Differences between the two cooling rates, within species, for the sperm-binding tests were detected with a Mann–Whitney test. P values <0.05 were considered significant. Correlation coefficients (r values) were also calculated between post-thaw seminal traits (sperm motility index and % of intact acrosome) and sperm-binding data. All analyses were performed with Statistica software (StatSoft Inc., Tulsa, OK, USA) and data were reported as mean ± S.E.M.

3. Results

3.1. Fresh and frozen–thawed seminal traits

As initially defined, only normospermic ejaculates, with good forward motility and >90% intact acrosomes were used for this study. Although males were normospermic, only 10 ejaculates from ocelots and 7 from tigrinas met the stated criteria. Ejaculates from one tigrina and two ocelots were contaminated with urine, resulting in a substantial decrease in the percent of morphologically normal sperm. These ejaculates were unsuitable for cryopreservation and hence were excluded from this study. Furthermore, all the remaining ocelot ejaculates, despite their high quality, were contaminated with small amounts of urine during electroejaculation. The presence of urine was confirmed by the decrease in pH of the ejaculate pH (normally ~7.5 for electro-ejaculates from these species [17]). Also, data from all ejaculates of one male ocelot was excluded from analysis; all three samples, despite meeting the criteria for inclusion, failed to bind to the zona pellucida (five assays, three ejaculates). Overall mean (± S.E.M.) for fresh seminal characteristics and sperm morphology for each species are shown (Table 1).

Data for post-thaw sperm characteristics are summarized in Table 2. Frozen–thawed sperm from both species exhibited a significant reduction in sperm motility index and percentage of sperm with intact acrosomal membranes in both cooling treatments compared to fresh ejaculates. The slow cooling rate had a greater adverse effect on the percentage of sperm with intact acrosomes of ocelot sperm compared with sperm from tigrinas.

3.2. Oocyte in vitro maturation and in vitro competitive sperm-binding assays

A total of 808 oocytes (390 and 418 Grades I and II, respectively) were collected from 43 pairs of domestic cat ovaries and subjected to IVM; 19% were fixed and

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Table 1
Mean (± S.E.M.) semen characteristics of ejaculates ($n = 7$/species) from ocelots ($n = 3$) and tigrinas ($n = 4$)

	Ocelot ($n = 7$)	Tigrina ($n = 7$)
Ejaculate volume (mL)	0.66 ± 0.1a	0.35 ± 0.1b
Sperm concentration ($\times 10^6$/mL)	190.2 ± 73.2	242.8 ± 85.2
Total sperm/ejaculate ($\times 10^6$)	107.0 ± 42.2a	56.6 ± 14.4b
Sperm motility (%)	81.0 ± 3.2	78.9 ± 1.5
Sperm progressive status (0-5)	3.7 ± 0.2	3.9 ± 0.1
Sperm motility index[a]	76.0 ± 8.5	80.0 ± 2.0
Morphologically normal sperm (%)	78.0 ± 2.7	76.8 ± 0.9
Primary defects (%)	4.3 ± 0.7	5.9 ± 0.7
Secondary defects (%)	18.7 ± 0.7	17.3 ± 6.5
Intact acrosome (%)	94.0 ± 0.7	91.0 ± 5.0

a,b: Within a row, values without a common letters differed ($P < 0.05$).
[a] Sperm motility index = [% motility + (20 × sperm progressive motility)]/2.

stained to assess maturational status. Nuclear maturation rates were similar ($P > 0.05$) for Grade I ($n = 73$; 74 ± 3.4%) and Grade II ($n = 83$; 67 ± 3.3%) oocytes [24]. The remaining oocytes were used for the binding assays, including a total of 21 sperm-binding tests that were performed specifically to evaluate the impact of cooling rate on sperm function. There was no difference between oocyte quality grades in the number of oocytes with at least one sperm cell attached to the zona, nor in the mean number of sperm bound to the zona pellucida per oocyte; therefore, data for both oocyte quality grades were combined for analysis. Cooling rate had a significant effect on the number of sperm attached to oocytes in both species (Table 3). In tigrinas, more sperm subjected to slow cooling attached to the zona pellucida, whereas in ocelots, more fast-cooled sperm were bound. There was no significant correlation between the mean number of sperm bound per oocyte and sperm characteristics, either before or after freezing (Table 3).

4. Discussion

In other mammals, cooling semen from 30 to 0 °C induced an irreparable stress to some cells, which was proportional to the rate of cooling. This phenomenon, known as cold shock, is associated with membrane lipid phase changes resulting in altered function, including decreased sperm motility and loss of membrane integrity. In this study, although cooling rate had minimal impact on sperm motility index, there was a species-specific effect on the ability of cryopreserved sperm to bind to heterologous zona pellucida, although both species belonged to the same genus (*Leopardus*). These data reinforced the need to identify species-specific cryopreservation protocols. We also showed that the competitive oocyte-binding assay was suitable to assess sperm function and to detect differences between treatments that were not possible by conventional sperm assessment techniques (e.g. sperm motility). The advantage of this competitive test over conventional methods is the reduced dependence on large numbers of oocytes typically required to detect significant differences between treatment groups. These findings have considerable potential, especially in species with limited availability of biological materials.

The ejaculates used in this study were all of high quality, based on both quantitative and qualitative traits, and were representative of seminal traits of the species studied [1,17]. However, freezing–thawing procedures, irrespective of the cooling rate, had detrimental effects on sperm characteristics. Decrease in sperm motility index and percentage of intact acrosome, in comparison with fresh samples in the present study seemed higher than those previously reported for these felids [2,3], particularly for ocelot. This difference might be a consequence of the relatively small number of males and ejaculates used in this study, a limiting factor when studying small cats [25]. A recent report on boar sperm

Table 2
Mean (± S.E.M.) sperm motility index and percentage of sperm with an intact acrosome in fresh and frozen–thawed ocelot and tigrina sperm ($n = 7$ ejaculates/species), subjected to either fast or slow cooling

	Species	Fresh semen	Frozen–thawed semen	
			Fast cooling	Slow cooling
Sperm motility index[a]	Ocelot	76.0 ± 8.5a	50.0 ± 1.8b	49.2 ± 3.0b
	Tigrina	80.0 ± 2.0a	51.0 ± 4.0b	49.0 ± 2.9b
Intact acrosome (%)	Ocelot	94.0 ± 0.7a	21.6 ± 3.5b	19.8 ± 3.2b[*]
	Tigrina	91.0 ± 5.0a	30.8 ± 5.6b	33.4 ± 2.2b

a,b: Within a species, values without a common letters differed ($P < 0.05$).
[a] Sperm Motility Index = [% motility + (20 × sperm progressive motility)]/2.
[*] Difference ($P < 0.05$) between species in intact acrosomes following slow cooling.

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Table 3
Mean (± S.E.M.) competitive binding of frozen-thawed ocelot and tigrina sperm ($n = 7$ ejaculates/species) to *in vitro*-matured domestic cat oocytes

Species	No. of oocytes	Oocytes binding sperm (%)[a]	No. of sperm/oocyte	No. of differentially stained sperm/oocyte	
				Fast cooling	Slow cooling
Ocelot	74	96.6 ± 2.2	11.0 ± 1.0	8.5 ± 1.3 **	2.5 ± 0.3
Tigrina	70	97.2 ± 1.8	8.5 ± 1.1	2.7 ± 0.4 *	5.8 ± 0.9

Two sperm populations, exposed to two different cooling protocols (fast and slow) were simultaneously used for *in vitro* fertilization; the number of sperm (from each cooling protocol) that bound to each oocyte was determined by sequential staining.
[a] Percentage of oocytes with at least one sperm cell attached to the zona pelucida.
* Difference between cooling protocols ($P < 0.05$).
** Difference between cooling protocols ($P < 0.01$).

cryopreservation using a cryomicroscope concluded that post-thaw membrane integrity in some animals tended to be superior to others, irrespective of the cooling rates used [26]. Therefore, data herein presented should be interpreted with caution, as it may represent individual variability rather than a pattern for the species. In that regard, the sperm of the particular ocelot that failed to bind to the zona pellucida may be a result of such an individual effect. In the ocelot, although electroejaculates were normospermic, minor urine contamination of all samples may have accounted for the reduced resistance of sperm membranes. High levels of morphologically abnormal sperm, primarily bent and coiled flagella, suggested membrane dysfunction induced by urine contamination, and was reported previously for the ocelot [2]. The same was found for those ejaculates with substantial urine contamination that were not used in our study ($n = 4$), which exhibited ~40% of sperm with tail defects. If teratospermic (>60% abnormal sperm) ejaculates had been included in the present study, we would have observed a severe impact of cooling and cryopreservation on sperm motility and acrosomal integrity [3]. Ongoing experiments in our laboratory are assessing new protocols for teratospermic ejaculates from ocelots and tigrinas. Furthermore, species-specificity and differences in sperm membrane resistance to the osmotic and temperature stress also warrant additional studies.

Simple cooling of domestic cat semen from ambient temperature to 5 °C caused massive acrosomal membrane damage, without a concomitant loss in motility characteristics [4]. The authors highlighted the novelty of these findings, since in previous studies in felids, the damage to the acrosomal membranes was always assumed to have been due to cryopreservation. These previously unsuspected aspects of cold-shock were also elucidated by studies of cryomicroscopy during simple cooling of ram sperm; the apparent ability of sperm to maintain membrane integrity at low temperature but then lose both membrane integrity and motility during rewarming to temperatures above 20 °C [26]. As expected, the proportion of acrosomal damage observed in the present study was higher than that reported for cooling experiments in domestic cats [4], since freezing–thawing procedures induce higher levels of damage to the sperm. During cryopreservation, besides cold shock, several factors including ice formation and exposure to severe osmotic fluctuations, also influence sperm cryosurvival. It was previously reported that felid sperm are resistant to hyperosmotic conditions, but undergo extensive damage upon return to isosmotic conditions [5]. In the ram, sperm plasma membrane integrity was maintained throughout cooling and freezing, even in the absence of cryoprotectants. However, membrane damage was observed only during post-thaw rewarming [27,28]. Altogether, the stress associated with each step of sperm cryopreservation was likely to induce structural rearrangements involving lipids and proteins, which in turn may modify sperm membrane permeability and function, despite an apparent lack of visible structural damage. In the present study, we did not attempt to identify the actual steps where acrosomal damage was incurred; rather, we assessed the interaction between the initial cooling rate and cryosurvival. Clearly, simply changing the cooling rate was not adequate to mitigate the damage observed during cryopreservation of ocelot or tigrina sperm.

Sperm cryopreservation induces sublethal damages, including injuries to surface proteins, inactivation of membrane-bound enzymes, and decreased lateral membrane protein diffusion. For example, P25b, a protein marker for fertilizing ability in bovine, expressed a threefold decrease after 28 days of cryopreservation compared to fresh sperm [29]. Another important cryoinjury is the induction of premature capacitation-like changes in sperm, which may compromise sperm function after thawing [11,19]. Cryopreserved feline sperm are capacitated and acrosome-reacted at higher rates after *in vitro* incubation than fresh sperm [30,31]. Experiments

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with cryopreserved canine sperm demonstrated that capacitated, viable and motile sperm lost their ability to bind to the zona pellucida [32]. Perhaps premature capacitation was involved in the ejaculates of the particular ocelot male with failure of zona binding. Although the specific mechanisms remain yet to be determined, slow cooling reduced membrane modifications in tigrina sperm, whereas for the ocelot samples it was detrimental. In domestic cats, it was assumed that a slower cooling rate minimized the detrimental effects of cold shock [4]. Conversely, a slow cooling rate increases the interval that sperm remains in contact with glycerol at temperatures above zero, which would increase its potential for cell toxicity. However, in a recent study focused on free-living cheetahs, exposure of sperm to 4% glycerol for up to 60 min at ambient temperature had minimal effect on sperm motility and acrosomal integrity after cryopreservation [33]. Presumably, differences in sperm membrane composition among species may play a role in overall sperm cryosurvival.

Considering only data for sperm motility index and proportion of cells with intact acrosome, irrespective of when cell damage occurred, it might be concluded that changing the cooling rate did not improve sperm function after thawing in either species. However, the *in-vitro* binding assay revealed a significant effect of cooling rate on sperm function. The sperm–zona pellucida interaction is a crucial event during mammalian fertilization; *in-vitro* zona-bindings assays are highly predictive of the IVF outcome in humans [34]. Thus, zona-binding assays provided additional information that cannot be obtained by conventional methods of sperm trait assessments. The prediction of fertility *in vivo* by binding assays, however, was questionable, due to controversial data regarding the correlation of sperm binding ability to fertility outcome in domestic animals [35].

In conclusion, irrespective of cooling rate, the freezing and thawing protocols used in the present study were unable to improve sperm cryosurvival. These findings warrant a systematic assessment of other factors including freezing rate, thawing rate and cryoprotectant removal on sperm cryosurvival [18]. For example, protocols for a more gradual return to isosmotic conditions should be tested for the genus *Leopardus*. Although future studies may allow a better understanding of the physiological basis for these differences, the present study clearly demonstrated the need to thoroughly examine the cryobiological properties of sperm from each species to be cryopreserved. Furthermore, the ability of zona binding assays to detect differences in sperm function after cryopreservation supported a broader utilization of this assay, especially in species where limited numbers of ejaculates and oocytes are available for assessing sperm–oocyte interaction.

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