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## COMMUNICATION

### FIELD FRIENDLY METHOD FOR WILD FELINE SEMEN CRYOPRESERVATION

Gediendson Ribeiro de Araujo, Thyara de Deco-Souza, Letícia Coelho Ferreira Bergo, Leanes Cruz da Silva, Ronaldo Gonçalves Morato, Pedro Nacib Jorge-Neto, Maitê Cardoso Coelho da Silva, Gustavo Guerino Macedo & Tarcízio Antônio Rego De Paula

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## Field friendly method for wild feline semen cryopreservation

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**Abstract:** The aim of this study was to develop a field-friendly method for free-living jaguar and cougar semen cryopreservation. Six captive Jaguars *Panthera onca* and three captive Cougars *Puma concolor* were chemically restrained with a combination of medetomidine (0.08–0.1 mg/kg) and ketamine (5 mg/kg). Semen was collected through a tomcat urinary catheter with an open end, diluted for a final concentration of  $50 \times 10^6$  sperm/mL in a TRIS-egg yolk extender and packaged into 0.25 mL straws. We compared two cooling methods: CoolA - in which straws were placed in a glass tube that was placed in a glass bottle containing water (600mL at 38°C) and transferred to a polystyrene container (12L) containing an 11cm column of ice and water at room temperature; CoolB - where the glass bottle - straws kit was transferred to a 4.26L cooler containing nine blocks (81cm<sup>3</sup>) of Ice Foam recyclable ice, previously frozen in liquid nitrogen. The sperm volume varied from 2 to 720  $\mu$ L for the jaguars and from 80 to 140  $\mu$ L for the cougars. Sperm concentration varied from 224 to  $5,115 \times 10^6$  sperm/mL for the jaguars and from 485.7 to  $562.5 \times 10^6$  sperm/mL for the cougars. Concerning the cooling treatments, there was no difference in frozen-thawed sperm quality between the methods, in both species. Thereby, the cooling method using recyclable ice frozen in liquid nitrogen can be used for semen cryopreservation in wild felines, eliminating the need for electric energy.

**Keywords:** Cryopreservation, free-living, *Panthera onca*, *Puma concolor*, spermatozoa.

**Resumo:** O objetivo deste estudo foi desenvolver um método de campo amigável para a criopreservação de sêmen de onça-pintada e onça-parda de vida livre. Seis onças-pintadas em cativeiro (*Panthera onca*) e três onças-pardas em cativeiro (*Puma concolor*) foram anestesiadas com uma combinação de medetomidina (0,08 a 0,1mg/kg) e cetamina (5mg/kg). O sêmen foi coletado através de um cateter urinário tomcat com extremidade aberta, diluído para uma concentração final de  $50 \times 10^6$  spz / mL em diluente de gema de ovo TRIS e embalado em palhetas de 0,25mL. Comparamos dois métodos de resfriamento: CoolA - em que as palhetas foram colocados em um tubo de vidro que foi colocado em uma garrafa de vidro contendo água (600mL a 38°C) e transferido para um recipiente de poliestireno (12L) contendo uma coluna de 11cm de gelo e água à temperatura ambiente; CoolB - onde as palhetas em garrafa de vidro foram transferidas para uma caixa térmica de 4,26 L contendo nove blocos (81cm<sup>3</sup>) de gelo reciclável, previamente congelado em nitrogênio líquido. O volume espermático variou de 2 a 720 $\mu$ L para as onças-pintadas e de 80 a 140 $\mu$ L para os pumas. A concentração espermática variou de 224 a  $5,115 \times 10^6$  spz/mL para as onças-pintadas e de 485,7 a  $562,5 \times 10^6$  spz/mL para as onças-pardas. Em relação aos tratamentos de resfriamento, não houve diferença na qualidade dos espermatozoides descongelados entre os métodos em ambas as espécies. Desse modo, o método de resfriamento usando gelo reciclável congelado em nitrogênio líquido pode ser usado para a criopreservação de sêmen em felinos selvagens, eliminando a necessidade de energia elétrica.

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For Author details & Author contribution see end of this article.



## INTRODUCTION

The Jaguar *Panthera onca* and the Cougar *Puma concolor* are apex predators and play a crucial role in the prey population control, thus both are considered keystone species for the ecosystems conservation (Crawshaw Jr. 1991). Threats such as deforestation and human activity are resulting in a reduced Jaguar and Cougar population in Brazil, and both species are classified as Vulnerable by the Brazilian Red Book of Threatened with Extinction Fauna (ICMBio 2018a). The conservation of such species depends on several actions that can reduce their vulnerability. These actions are defined in the National Action Plan for Big Cats Conservation (NPBigcat) (ICMBio 2018b) produced by the Brazilian Ministry of Environment.

One of the recommended actions by the NPBigcat is to develop assisted reproduction programs, which aim to help increase the genetic variability of the species. Sperm cryopreservation is an assisted reproduction technique that enables keeping viable sperms for an indeterminate period (Silva et al. 2004). In addition, semen cryopreservation allows translocation of genetic material among populations, dispensing the transport of individuals, which reduces the stress caused by the translocation and the risks of transmission of infectious diseases (Wildt 1990).

For cryopreservation, sperm must be cooled from body temperature to 5°C and only then frozen in nitrogen vapor (-70°C) and finally stored in liquid nitrogen at -196°C (Budhan Pukazhenthil et al. 1999; D. Zambelli et al. 2010). Several automatic cooling and freezing equipment are available in the market, however, they are large and require electricity. There are also portable containers for sperm cooling and transportation, which use recyclable ice. Nevertheless, they also need electricity to freeze the ice for 12h before being used. Thus, these devices are not feasible for use in free-living felines, since capture sites are often difficult to access and without electricity. This difficulty is clearly demonstrated when we evaluate the articles published in scientific journals, in which only two papers describe the characteristics of fresh sperm in free-living Jaguars, but they did not cryopreserve the samples (Morato et al. 2001; Araujo et al. 2018). Therefore, one of the challenges in developing assisted reproduction techniques in free-living cats is the lack of portable and electricity-free devices. Thus, this study was aimed to develop a field friendly method for Jaguar and Cougar semen cryopreservation.

## MATERIALS AND METHODS

### Animals

Captive Jaguars (n=6) and cougars (n=3) were used from three different institutions: two Jaguars and two Cougars at Mata Ciliar Association (Jundiá – SP; -23.178°S, -46.941°W), one Jaguar and one Cougar at Paulínia Zoo (Paulínea – SP; -22.764°S, -47.153°W) and three Jaguars at a non-governmental organization NEX - No Extinction (Corumbá de Goiás – GO; -15.859°, -48.476°W). The animals were housed in enclosures with natural lighting, with water ad libitum and fed a meat-based diet. Animal ages were estimated based on medical records of the respective maintainers.

The present study had authorization for scientific activities issued by SISBIO / ICMBio / MMA under no. 46031-4, approved by the Ethic Committee on Animal Use of the School of the Federal University of Viçosa (CEUA-UFV) under protocol no. 79/2015 and was registered in the SISGEN National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Register A327AAC).

### Semen collection

Males were fasted for 12 hours without food and water before chemical restraint, that was performed using anesthetic darts fired with a blowpipe and containing medetomidine (0.08–0.1 mg/kg, Precision Pharmacy, CA, USA) and ketamine (5mg/kg, Dopalen, Vetbrands, SP, Brazil). After semen collection, anesthesia was reversed using Atipamezole (0.25mg/kg, Precision Pharmacy).

The semen was collected by urethral catheterization as described by Araujo et al. (2018). Briefly, 20–40 min after medetomidine administration a semi-rigid tomcat urinary catheter (w/ open end, 3FR, 130mm long) was introduced into the urethra and negative pressure was applied (by a 1mL syringe) to increase suction effect and semen collection. The semen was then placed in a pre-warmed (38°C) 2mL plastic tube and kept in a water bath at 38°C.

### Semen evaluation and processing

Immediately after collection, the semen was diluted (2:1) in maintenance medium (MM; TRIS 24g/L; citric acid 14g/L; glucose 8 g/L; amikacin 2g/L; egg yolk 200g/L; Nutricell, SP, Brazil). Then, subjectively evaluated for forward progressive motility (FPM) on a scale from 0 to 5, where 0 represented no forward movement and 5 represented steady, rapid forward progression; and progressive motility (PM) from 0% to 100%, in

increments of 5% under a 200x magnifying microscope (CBRA 2013). The sperm concentration was measured using a Neubauer chamber.

An aliquot of each diluted semen sample was fixed in Karnovsky fixative (Karnovsky 1965) and later evaluated for sperm morphology (200 cells/ejaculate) under phase-contrast microscopy (1000x magnification). Individual cells were classified as normal, major defects or minor defects in terms of their perceived adverse effects on male fertility (Blom 1973).

The sperm plasma membrane function was accessed by the hypo-osmotic swelling test (HOST), as described by Araujo et al. (2015). Semen was incubated in 100 mOsmol/kg sucrose solution (1:4) at 38 °C for 30 min, and fixed in Karnovisk fixative (Karnovsky 1965). One hundred sperms were evaluated under phase-contrast microscopy (1000x magnification) and those with bent or coiled tail were considered functional – this number was corrected by excluding the sperms with bent and coiled tail in the morphology test.

### Sperm cryopreservation

After evaluation, the semen concentration was standardized for  $100 \times 10^6$  sperm/mL in MM and then diluted (1:1) in cryopreservation media (12% glycerol, 1% de Equex STM Paste in MM). Thus, semen was

cryopreserved in TRIS-egg yolk extender with a final concentration of  $50 \times 10^6$  sperm/mL, 6% glycerol, 0.5% de Equex STM Paste. Samples were package into 0.25mL straws (IMV Technologies, NOR, France).

For each ejaculate, two cooling methods were evaluated: Cooling A (CoolA); we used the previously described method (Deco-Souza et al. 2013; Araujo et al. 2015) in which straws were placed in a glass tube that was placed in a glass bottle containing water (600 mL at 38 °C) and transferred to a polystyrene container (12L) containing an 11cm column of ice and water at room temperature, for 1.5h. The cooling rate was  $-0.53^\circ\text{C}/\text{min}$  (from 38 to 5°C). Cooling B (CoolB); where straws were cooled for 1.5h in a 4.26L cooler container containing nine blocks –  $81\text{cm}^3$  each – of Ice Foam recyclable ice, previously frozen in liquid nitrogen (Image 1). For this the straws were placed in a glass tube that was placed in a glass bottle containing water (600mL at 38°C) and transferred to the cooler. This glass bottle was surrounded by the ice foam blocks. The amount of Ice Foam was previously defined to reach a cooling rate similar to the CoolA group.

Cryopreservation was performed by placing the straws horizontally over a freezing rack inside a Styrofoam container filled with liquid nitrogen and exposed to nitrogen vapor at 10cm above liquid for



Image 1. A & B—Packing the ice foam blocks | C—Collings of semen | D—in the CoolB. © Gediendson Ribeiro de Araujo

15 minutes (Deco-Souza et al. 2013). Afterwards, the straws were immersed in liquid nitrogen ( $-196^{\circ}$ ).

The straws were thawed in a water bath at  $38^{\circ}\text{C}$  for 30s and transferred to a plastic tube where they were maintained during the evaluation. Each frozen-thawed sample was assessed as the fresh semen and for sperm motility, using a computer assisted sperm analysis (CASA) system and staining with fluorescent probes.

#### Frozen-thawed semen evaluation

The plasmatic and acrosomal membranes were assessed using a combination of three fluorescent probes: propidium iodide (PI; Sigma–Aldrich Co. LLC.– P4170), Hoechst 33342 (H342; Molecular Probes–H1399) and Peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA; Sigma–Aldrich Co. LLC. –L7381). The frozen-thawed semen ( $10\mu\text{l}$ ) was incubated with  $10\mu\text{l}$  of H342 ( $25\mu\text{g}/\text{mL}$  in DPBS) and  $60\mu\text{l}$  of FITC-PNA ( $10.3\mu\text{g}/\text{mL}$  in sodium citrate 3% in DPBS) at  $38^{\circ}\text{C}$ . After 8min,  $2\mu\text{l}$  of PI ( $0.5\text{mg}/\text{mL}$  in DPBS) were added and incubated for another 2min. The sperm were evaluated by epifluorescence microscopy (Nikon H550S, excitation: 365nm; emission: 410nm) and were classified based on the fluorescence emitted from each probe as: DI – damaged plasma membrane and intact acrosome (only the nucleus emitting red fluorescence); II – intact plasma membrane and intact acrosome (only the nucleus emitting blue fluorescence); DD – damaged plasma membrane and damaged acrosome (the nucleus emitting red fluorescence and the acrosomal region emitting green fluorescence); and ID – intact plasma membrane and damaged acrosome (the nucleus emitting blue fluorescence in acrosome region and emitting green fluorescence).

The sperm motility was accessed using the sperm class analyzer CASA system (Microptic S.L., Spain) with the following settings described by Lueders et al. (2012) in African lions: negative phase (Ph-) with green filter; particle size 5–85; connectivity 14 at of capture of 50fps and 40/50 images; drifting 10; static VCL  $25\mu\text{m}/\text{s}$ ; slow/medium VCL  $65\mu\text{m}/\text{s}$ ; rapid  $100\mu\text{m}/\text{s}$ ; STR 75%; and VAP setting  $7\mu\text{m}/\text{s}$ . Semen sample ( $4\mu\text{L}$ ) at  $25 \times 10^6$  sperm/mL was loaded onto a pre-warmed disposable Leja 4 Chamber Slides (Leja Products BV, The Netherlands) and accessed by total motility (%), progressive motility (%), velocity average pathway – VAP ( $\mu\text{m}/\text{s}$ ), velocity straight line – VSL ( $\mu\text{m}/\text{s}$ ), velocity curved line – VCL ( $\mu\text{m}/\text{s}$ ), amplitude lateral head – ALH (mm), beat cross-frequency – BCF (Hz), straightness – STR (%), and linearity – LIN (%).

#### Statistical analysis

Data on sperm quality from CoolA versus CoolB groups were analyzed using Bayesian t-test with unequal variances (Kery 2010). Data from fresh semen versus CoolA and CoolB groups were analyzed using simple variance (one-way ANOVA) with fixed effect with hierarchical Bayesian modeling. This method of analysis allows inferences about the population and is indicative of the probability that the parameters estimated for each group are derived from the same distribution. According to McCarthy (2007) and Kery (2010) the specification model was:

$$y_{ijk} = \alpha_j(i) + \epsilon_i$$

$$\epsilon_i \sim \text{Normal}(0, \sigma^2)$$

In this model,  $y_{ijk}$  corresponds to the data  $K$  observed from animal  $i$  in the population  $j$ ,  $\alpha_j(i)$  corresponds to the expected value for the data in the population  $j$ , and the residual  $\epsilon_i$  corresponds to the random deviation of the sperm parameter of the animal  $i$  of the mean of its population  $\alpha_j(i)$ .

Observations that did not meet the assumptions of normality were assessed using a Shapiro–Wilk test (Royston 1982) with a significance of  $p < 0.05$  and were log-transformed. Marginal posterior distributions of parameters were estimated using Markov Chain Monte Carlo (MCMC) methods. Analyses were implemented in program R (R Development Core Team 2011) using the rjags package, JAGS version 3.2.0. Each of the MCMC chains was run for 100,000 iterations; the first 20,000 iterations were discarded to allow for burn-in. Convergence was assessed by visually inspecting trace plots to ensure a reasonable exploration of the parameter space, and a potential scale reduction factor of  $< 1.02$  for each variable (Gelman & Rubin 1992). Results were back-transformed, if necessary. At each MCMC step, we calculated the Bayesian equivalent to a  $p$ -value by assessing whether the mean of one group was greater than the other.

#### RESULTS

Semen collection by urethral catheterization was effective in all animals, with good volume and concentration (Table 1), however, one Jaguar and one Cougar only ejaculated seminal fluid and thus were not considered for statistical analysis in frozen-thawed semen.

In Jaguars there were differences ( $p < 0.05$ ) in sperm FPM, sperm PM and HOST between fresh and frozen-thawed sperm, there was no difference ( $p < 0.05$ )

**Table 1. Quality of fresh semen collected by urethral catheterization after medetomidine administration in captive Jaguars (*Panthera onca*, N=6) and Cougars (*Puma concolor*, N=3).**

	Jaguar	Cougar
Volume (µl)	292.0 ± 326.6	106.7 ± 30.6
Concentration (x 10 <sup>6</sup> sperm/ mL)	2091.4 ± 1816.2	524.1 ± 54.3
Total number of spermatozoa (x10 <sup>6</sup> )	316.6 ± 399.0	56.5 ± 16.3

**Table 2. Fresh and frozen-thawed Jaguar (*Panthera onca*, N=5) sperm evaluation.**

	Fresh	Frozen-thawed	
		CoolA	CoolB
FPM	3.6 ± 0.4 <sup>a</sup>	2.3 ± 0.3 <sup>b</sup>	2.4 ± 0.3 <sup>b</sup>
PM (%)	73.0 ± 14 <sup>a</sup>	31.0 ± 19 <sup>b</sup>	38.6 ± 17.7 <sup>b</sup>
HOST (%)	55.0 ± 9.5 <sup>a</sup>	26.4 ± 5.8 <sup>b</sup>	24.3 ± 6.5 <sup>b</sup>
Normal sperm (%)	60.7 ± 6.8 <sup>a</sup>	46 ± 11.4 <sup>b</sup>	47.8 ± 5.3 <sup>b</sup>
Major defects (%)	21 ± 6.6 <sup>a</sup>	24.6 ± 12.6 <sup>a</sup>	24.8 ± 6.4 <sup>a</sup>
Minor defects (%)	18.3 ± 12.2 <sup>a</sup>	29.4 ± 7.6 <sup>a</sup>	27.4 ± 3.9 <sup>a</sup>
DI		55 ± 18.7 <sup>a</sup>	39 ± 10.0 <sup>a</sup>
II		21.2 ± 15.7 <sup>a</sup>	23.4 ± 13.8 <sup>a</sup>
DD		23.6 ± 15.3 <sup>a</sup>	37.6 ± 10.5 <sup>a</sup>
ID		0.4 ± 0.9 <sup>a</sup>	0.0 ± 0.0 <sup>a</sup>
Total Motility*		28.4 ± 14 <sup>a</sup>	28.8 ± 5.9 <sup>a</sup>
Progressive motility*		2.0 ± 1.9 <sup>a</sup>	1.8 ± 1.2 <sup>a</sup>
VAP*		10.5 ± 4.5 <sup>a</sup>	10.5 ± 3.4 <sup>a</sup>
VSL*		6.6 ± 4.3 <sup>a</sup>	6.8 ± 3.1 <sup>a</sup>
VCL*		23.5 ± 6.4 <sup>a</sup>	23.5 ± 3.8 <sup>a</sup>
ALH*		2.2 ± 1.8 <sup>a</sup>	2.1 ± 1.4 <sup>a</sup>
BCF*		7.2 ± 6.2 <sup>a</sup>	8.5 ± 6.2 <sup>a</sup>
STR*		58.6 ± 13.4 <sup>a</sup>	62.0 ± 12.5 <sup>a</sup>
LIN*		26.3 ± 11.4 <sup>a</sup>	27.8 ± 9.7 <sup>a</sup>

between CoolA and CoolB parameters (Table 2).

\*Data accessed by sperm class analyzer. Means ± S.D. Means within columns with different letters differ significantly (p<0.05). FPM – Sperm forward progressive motility; PM – sperm progressive motility; HOST – hypo-osmotic swelling test; velocity average pathway – VAP (µm/s); velocity straight line – VSL (µm/s); velocity curved line – VCL (µm/s); amplitude lateral head – ALH (mm); beat cross-frequency – BCF (Hz); straightness – STR (%) and linearity – LIN (%). DI: damaged plasma membrane and intact acrosome; II: intact plasma-membrane and intact acrosome; DD: damaged plasma membrane and damaged acrosome; ID: intact plasma membrane and damaged acrosome.

**Table 3. Fresh and frozen-thawed Cougar (*Puma concolor*, N=2) sperm evaluation.**

	Fresh	Frozen-thawed	
		CoolA	CoolB
FPM	3.0 ± 0 <sup>a</sup>	2.8 ± 0.3 <sup>a</sup>	2.8 ± 0.3 <sup>a</sup>
PM (%)	70.0 ± 0 <sup>a</sup>	50.0 ± 14.1 <sup>a</sup>	50.0 ± 14.1 <sup>a</sup>
HOST (%)	39.5 ± 6.4 <sup>a</sup>	13.5 ± 3.5 <sup>b</sup>	25.0 ± 1.4 <sup>b</sup>
Normal sperm (%)	40.5 ± 7.8 <sup>d</sup>	23.5 ± 2.1 <sup>d</sup>	31.5 ± 0.7 <sup>d</sup>
Major defects (%)	41.0 ± 12.7 <sup>d</sup>	26.5 ± 2.1 <sup>d</sup>	36 ± 5.6 <sup>d</sup>
Minor defects (%)	18.5 ± 4.9 <sup>d</sup>	44.5 ± 6.4 <sup>e</sup>	32.5 ± 6.4 <sup>de</sup>
DI		38.0 ± 1.4 <sup>a</sup>	50.5 ± 6.4 <sup>a</sup>
II		44.0 ± 9.9 <sup>a</sup>	39.0 ± 12.7 <sup>a</sup>
DD		17.0 ± 7.1 <sup>a</sup>	9.5 ± 4.9 <sup>a</sup>
ID		1.0 ± 1.4 <sup>a</sup>	1.0 ± 1.4 <sup>a</sup>
Total Motility*		40.0 ± 4.7 <sup>a</sup>	36.3 ± 3.2 <sup>a</sup>
Progressive motility*		6.3 ± 1.7 <sup>a</sup>	5.8 ± 4.3 <sup>a</sup>
VAP*		20.8 ± 4.6 <sup>a</sup>	21.3 ± 4.1 <sup>a</sup>
VSL*		13.7 ± 4.3 <sup>a</sup>	14.7 ± 3.9 <sup>a</sup>
VCL*		40.7 ± 6.7 <sup>a</sup>	39.6 ± 9.2 <sup>a</sup>
ALH*		3.6 ± 0.1 <sup>a</sup>	3.1 ± 0.3 <sup>a</sup>
BCF*		12.9 ± 2.8 <sup>a</sup>	14.3 ± 1.7 <sup>a</sup>
STR*		65.3 ± 6.3 <sup>a</sup>	68.4 ± 5.3 <sup>a</sup>
LIN*		33.3 ± 5.1 <sup>a</sup>	36.9 ± 1.3 <sup>a</sup>

As we saw in Jaguars, there were no differences (p>0.05) in sperm quality between the CoolA and CoolB for the cougars (Table 3), however, HOST and minor defects increased in frozen-thawed semen.

\*Data accessed by sperm class analyzer. Means ± S.D. Means within columns with different letters differ significantly (p<0.05). FPM – sperm forward progressive motility; PM – sperm progressive motility; HOST – hypo-osmotic swelling test; velocity average pathway – VAP (µm/s); velocity straight line – VSL (µm/s); velocity curved line – VCL (µm/s); amplitude lateral head – ALH (mm), beat cross-frequency – BCF (Hz); straightness – STR (%) and linearity – LIN (%). DI: damaged plasma membrane and intact acrosome; II: intact plasma-membrane and intact acrosome; DD: damaged plasma membrane and damaged acrosome; ID: intact plasma membrane and damaged acrosome.

## DISCUSSION

The results for fresh semen quality shows that urethral catheterization after medetomidine administration (CT) was effective for semen collection in Jaguars and

Cougars. Thus, this may be an alternative method for electroejaculation.

In Jaguars and in Cougars, the semen volume was lower than previously described (5.3 to 11 mL and 0.45 to 3.4 mL, respectively) (Wildt et al. 1988; Morato et al. 1998, 1999, 2001, 2004; Paz et al. 2000, 2003, 2006, 2007; Swanson et al. 2003; Deco et al. 2010). All those studies, however, used the electroejaculation (EE) for semen collection. It is well known that EE stimulates contractions of the smooth muscles and subsequently the accessory sex glands, which increases the seminal volume (Ball 1986), resulting in more diluted semen samples. On the other hand, with the CT we collected more concentrated semen samples than described in literature in both species. The total number of spermatozoa, however, was smaller than described for Cougars (Wildt et al. 1988; Deco et al. 2010). Because of the small number of Cougars used in this study, we cannot state if this result was related to the collection method or to the animals. The semen volume and concentration were good enough for cryopreservation and the CT was much more practical to be used than EE.

The SPM and PM (3.6 and 76%, respectively) in Jaguars were superior than previously described (2.2 to 3.3 and 50.6 to 64%, respectively) (Morato et al. 1998, 1999; Swanson et al. 2003; Silva et al. 2004; Paz et al. 2006). On the other hand, in Cougars the SPM and PM were superior to the 2.5–3 and 40–50 % described by Miller et al. (1990) and similar to the 3.5 and 75% described by Deco et al. (2010). Both parameters were considered good quality for cryopreservation.

In the present study, Jaguars had more normal sperm (60.7%) than Cougars (40.5%); as well as more normal sperm than described in literature (46.7% (Morato et al. 1998); 49% (Morato et al. 1999); 31.7% (Paz et al. 2000); 50% (Morato et al. 2001); 57.3% (Swanson et al. 2003); 48.7% (Paz et al. 2003)). Cougars had higher or even similar normal sperm than described for the species (26% (Wildt et al. 1988); 1–18 % (Miller et al. 1990); 8.6% (B. Pukazhenthil et al. 2001); 46.13% (Deco et al. 2010)). Felines usually have high proportion of pathologic sperm in the ejaculate, however, the etiology and impact of those in fertility is controversy (Howard et al. 1986). Several factors may affect sperm morphology; although, nutrition and stress are the main factors in captive animals.

After cryopreservation sperm quality reduced in both species. This is expected for any species as cryopreservation damages sperm, impairing their ability to fertilize oocyte. Despite the reduction in the quality of frozen-thawed sperm, SPM and PM values were similar

to those described for Jaguars (SPM: 2.7 and PM 30% (Paz et al. 2000); SPM 3.1 and PM 26.7% (Paz et al. 2007)) and for Cougars (SPM 2.5 and PM 42% (Deco-Souza et al. 2013)). To obtain semen samples from wild animals is always a challenge, because of the reduced number of captive animals (several of them are vasectomized) and the difficulty of accessing free-living animals. Therefore, frozen-thawed semen must be used, even if they are of poor-quality. For this, we can use artificial insemination via laparoscopy – depositing sperm closer to the site of fertilization – or even the intracytoplasmic sperm injection – ICSI. In addition, studies should be done to increase sperm quality after thawing, thus increasing the efficacy of its use for assisted reproduction programs.

For sperm cryopreservation sample must be cooled (from body temperature to 5°C), frozen (in liquid nitrogen vapor at -70°C) and stored (in liquid nitrogen at -196°C). Sperm cell also may be stored at the cooling temperature, however, it remains viable only for a few days. Several protocols and equipment are evaluable for carnivore semen cryopreservation (and cooling) (Zambelli et al. 2002; Luvoni et al. 2003; Tsuitsui et al. 2003; Macente et al. 2012). Some of those are also used for wild felids (Paz et al. 2007; Deco-Souza et al. 2013; Araujo et al. 2015; Jorge Neto et al. 2019). In these cases, cooling was performed using refrigerators, automatic cooling and / or freezing equipment, or even in portable containers using previously frozen recyclable ice. All these methods depend on electricity and cannot be used in the field, as in several places there is no electricity available.

The CoolA method was successfully used for cougar and ocelot semen cryopreservation (Deco-Souza et al. 2013; Araujo et al. 2015), however, it still needs electricity to store ice. Thus, we used nontoxic recyclable ice to reach the same cooling rate (CoolB), with the advantage of being frozen and kept in liquid nitrogen – which is necessary for the later stages of semen freezing. This enables this method to be used in fields where there is no energy available. There was no difference in sperm quality in both cooling methods, demonstrating that the CoolB may be used for semen cryopreservation from the felines. This makes it feasible for sperm banks to use semen from free-living animals, increasing the genetic resources of these species.

## CONCLUSION

The cooling method using recyclable ice frozen in liquid nitrogen offers good semen quality and may be



used for feline semen cryopreservation, eliminating the need of electricity. Thus, this is a more practical method to be used in the field.

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## Article

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