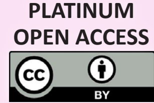

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Communication

Functional sperm assessments of African Lion *Panthera leo* (Mammalia: Carnivora: Felidae) in field conditions

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Functional sperm assessments of African Lion *Panthera leo* (Mammalia: Carnivora: Felidae) in field conditions

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[1–8] Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Orlando Marques de Paiva, 87 – 05508-270, São Paulo, Brazil.
[1] Department of Surgery, Division of Human Reproduction, Federal University of São Paulo, Rua Embaú, 231, 04039-060- Vila Clementino – São Paulo, Brazil.
[1] tbsoler@gmail.com, [2] angrimani@gmail.com, [3] brunorogeriorui@gmail.com, [4] jdalosano@usp.br, [5] luanabicudo@usp.br [6] marcelblank@usp.br, [7] mnichi@usp.br (corresponding author), [8] crissp@usp.br

Abstract*:* Wild African Lion *Panthera leo* populations are decreasing due to inbreeding and reduced genetic variability. Thus, the use of assisted reproduction in the species could one day become essential. Before this is possible, however, studies need to be conducted on the basic reproductive traits of the species, especially those regarding sperm cells. This study aimed to analyze the semen of African Lions in field conditions. We included seven captive African Lions in our study. The animals were chemically restrained and electro-ejaculated. Twenty sperm samples were selected and analyzed for sperm motility and progressive motility, sperm motility index, and sperm morphology. In addition, the samples were analyzed for membrane and acrosome integrity (hypoosmotic swelling test and fast green/rose Bengal dyes, respectively) and assessed for cytochemical activity of the mitochondria. We found that sperm motility rate was 75.25%±2.03, progressive motility rate was 3.25%±0.10, and sperm motility index was 70.12%±1.71. We found morphologic abnormalities roughly at the expected rate with 34.61%±7.22 of the sperm cells having an intact plasma membrane and acrosome integrity of 92.27%±2.73; high mitochondrial activity was 54.26±4.88% and absence of mitochondrial activity was 2.72±0.68% in the sperm cells. These findings show that conventional tests for sperm motility and sperm morphology bring about the expected results for lions according scientific literature. Though a hypoosmotic swelling test may be performed using different concentrations, it might lead to a higher number of sperm cells with membrane damage. Fast green/rose Bengal stain and 3'3 diaminobenzidine assay, however, can be used in sperm analysis of lions in field conditions.

Keywords*:* Mitochondrial activity, plasma membrane, acrosome, sperm analysis.

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Competing interests: The authors declare no competing interests.

Declarations: This experiment and study were as approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science at the University of São Paulo, São Paulo, Brazil (protocol no. 310/2003), and by the Brazilian Institute of Environment and Renewable Natural Resources (process no. 0227.026090/2002-39 IBAMA). Unless otherwise mentioned, all chemicals used were obtained from Sigma-Aldrich (St. Louis, MO, USA).

For **Author Details** & **Author Contribution** see end of this article.

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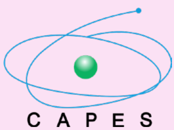

INTRODUCTION

African Lion *Panthera leo* is considered a prolific species and may be used as an experimental model for other endangered large felids (Gilmore et al. 1998; Borrego & Dowling 2016). Large successfully reproducing captive populations provide a satisfactory number of animals for experimental studies aimed at being replicated in wild populations. Lion populations are decreasing in their native countries leading to increased inbreeding and reduced genetic variability (Wildt et al. 1995; McDermid et al. 2017). Other endangered felids such as Jaguar *Panthera onca*, Tiger *P. tigris*, and Snow Leopard *P. uncia* also face similar problems (Caso et al. 2008; Jackson et al. 2008; Chundawat et al. 2011).

The study of the reproductive parameters in lions is fundamental for the successful application of reproductive technologies such as artificial insemination (Goeritz et al. 2012) and cryopreservation (Luther et al. 2017). Although techniques of assisted reproduction in humans and livestock species are well-established, it is important to recognize that these cannot be applied universally without species-specific studies (Howard et al. 1986). Consequently, the knowledge of sperm features in lions is important for the successful application of such techniques in wild felids.

Previous studies demonstrated the possibility to predict sperm fertility after semen analysis in humans (Nosrati et al. 2016), bovines (Utt 2016), and dogs (Hesser et al. 2017). To our knowledge, however, there is so far no study of this relationship in lions. Moreover, the combination of conventional sperm analysis and sperm functional tests allows a more adequate prediction of the fertility of semen samples (Shen & Ong 2000; Aitken 2006). Today, there are several possibilities to evaluate sperm functionality such as fluorescent probes (Singh et al. 2016), computer sperm analysis (Barranco et al. 2017), and the estimation of lipid peroxidation rates (Nichi et al. 2017).

The evaluation of sperm samples from wild felids should be focused on field conditions since there are limitations in transporting the samples of some of the endangered species to research centres that have the facilities to handle the sperm cells (Hermes et al. 2013). Therefore, the establishment of sperm function rates for lions in field conditions can simplify future fieldwork and help develop reproductive technologies applicable under field conditions.

The aim of this study was to establish the standard rates of sperm evaluation by conventional and functional assessments (i.e., mitochondrial activity, the integrity of acrosome, and sperm plasma membrane) for African Lions under field conditions.

MATERIALS AND METHODS

Animals

We used seven captive adult lions between the ages of four and seven years, which were housed individually at the Fundação Parque Zoológico de São Paulo (São Paulo, Brazil). According to the reproductive records provided by the zoo, all males in this study were proven to be breeders.

Semen collection

Semen collections were made after electro-ejaculation under anaesthesia. The animals were anaesthetized with a combination of Tiletamine and Zolazepam (Zoletil 50, VirbacTM do Brasil, 10mg/kg, IM). Electro-ejaculation was performed using the protocol described by Howard (1993). Semen was collected in sterile plastic tubes (15mL) and immediately evaluated. Each animal was submitted to semen collection at least four times at intervals of five weeks between the handling events. In total, 28 collections were performed, out of which six were interrupted due to problems during the procedure (e.g., anaesthesia or urine contamination) and two samples did not reach the minimum standards.

Conventional sperm analysis

Immediately after semen collection, the motility (0–100%) and progressive motility (0–5) were measured, sperm morphology was examined, and sperm motility index (SMI) was calculated. Motility and progressive motility were assessed using 10µL of semen sample placed on a clean and pre-warmed glass slide at 37°C, covered with a coverslip, and evaluated under a microscope equipped with a hot stage to keep the slides at 37°C (100x and 400x magnification, Nikon® E200, Japan). The sperm motility index was calculated using the formula described by Howard (1993) (motility + 20 x progressive motility). Morphologic alterations were evaluated fixing sperm samples in a 10% formalin buffer solution (V/V) in wet mounts, which were observed under a phase contrast microscope (1000x magnification, Nikon® E200, Japan). Abnormalities were classified according to their locations in the sperm cell (Barth & Oko 1989).

Hypoosmotic swelling test

To evaluate sperm membrane integrity, we used a

hypoosmotic swelling assay. To perform this technique, two media of different osmolarities were prepared, one isoosmotic (300mOsm) and one hypoosmotic (50mOsm). The isoosmotic medium was prepared by mixing sodium citrate (50%) and fructose (50%) in 500ml of distilled water in accordance with the technique described by Jeyendran et al. (1984). One aliquot of 200µl of semen was added to the same volume of isoosmotic and hypoosmotic media. The mixture was homogenized and incubated in a water bath at 37^0C for 30min. The reactions were stopped by adding 10µL of 10% formalin solution (V/V). In the hyposmotic mixture, cells were swelling aiming to establish equilibrium between the intra and extracellular environment. Samples were evaluated in wet mounts under an interference phase microscope (400x magnification, Leitz Dialux 20) by counting the swollen sperm cells showing coiled tails (200 sperm in each medium), which indicate biochemically active cells. As a control group, the isoosmotic medium was used aiming to evaluate tails that were abnormally coiled in the ejaculate. The percentage of sperm cells with intact membranes was calculated by subtracting the percent of cells with coiled tails in the hypoosmotic medium from the percent found in the isoosmotic medium. The results were expressed as percentages (%).

Acrosome integrity analysis

Acrosome integrity was analyzed using a single-stain solution containing 1% (w/v) rose Bengal, 1% (w/v) fast green FCF, and 40% ethanol in McIlvaine's citrate phosphate buffer (Pope et al. 1991). A mixture of 5µL of stain solution and 5µL of semen was transferred on a pre-warmed slide (37^oC) and, a smear was made using a different slide after 60s. The smears were air-dried and at least 200 cells were counted under a light microscope (Nikon Eclipse E200, Japan) at a 1000× magnification. The results were expressed as percentages (%). The acrosome was considered damaged if the acrosome region remained unstained or brighter than the post-acrosome area. The acrosome was considered intact if the sperm acrosome region was stained in purple or darker than the post-acrosome area.

Evaluation of mitochondrial activity

Semen samples were analyzed for mitochondrial activity using a 3'3 diaminobenzidine (DAB) assay (Hrudka 1987; Angrimani et al. 2017a). Therefore, the semen was diluted (1:1) in 1mg/ml solution of DAB in PBS (Phosphate-buffered saline) and incubated in a water bath at 37^oC for one hour in the dark. Smears were then prepared on glass slides and fixated in 10% formalin for 15min. These were evaluated under the light microscope with oil immersion objective (Nikon Eclipse E200, Japan) at 1000× magnification; 200 sperm cells were evaluated. The results were expressed in percentage (%). Sperm cells were classified into four categories: high mitochondrial activity (100% of the mid-piece stained – DAB Class I), medium mitochondrial activity (more than 50% of the mid-piece stained – DAB Class II), low mitochondrial activity (less than 50% of the mid-piece stained – DAB Class III), and absence of mitochondrial activity (absence of staining in the mid-piece – DAB Class IV).

Statistical analysis

In total, 20 ejaculates exhibiting at least 60% of motility and progressive motility greater than three (scale of 0–5) could be analyzed. All data were analyzed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA). Descriptive analysis was performed using the PROC MEANS. Results are reported as untransformed means ± S.E.M. Spearman correlation was used to calculate the relationship between the variables studied. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Sperm motility rates were 75.25±2.03%. Progressive motility was 3.25±0.10 and sperm motility index averaged 70.12±1.71% (Fig. 1). Mean values of the percentage of morphologic abnormalities observed in the acrosome, head, mid-piece, and tail found in the unstained fixed samples were 2.42±0.95%, 3.89±0.70%, 9.5±2.58%, and 43.07±6.39%, respectively (Table 1).

The percentage of sperm cells with intact membrane evaluated by HOST was 34.61±7.22% and the acrosome integrity rate was 92.27±2.73% in the sperm cells (Fig. 2). High mitochondrial activity (DAB – Class I) was shown by 54.26±4.88% of the sperm cells. Medium mitochondrial activity (DAB – Class II) was shown by 36.7±3.92% and low mitochondrial activity by 6.25±0.88% of the sperm cells. No mitochondrial activity was shown by 2.72±0.68% of the sperm cells (Fig. 3).

Positive correlations were found between the percentage of high mitochondrial activity (DAB – Class I), intact plasma membrane ($r=0.60$, $p=0.049$), and acrosome integrity ($r=0.69$, $p=0.0041$). No other correlations were found in the variables evaluated.

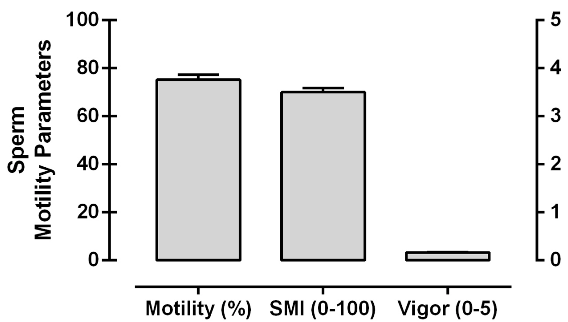

Figure 1. Mean and standard error of the mean (SEM) of motility (Motility in %), sperm motility index (SMI: 0–100), and progressive motility (Vigor, 0–5) in sperm samples from adult African Lions

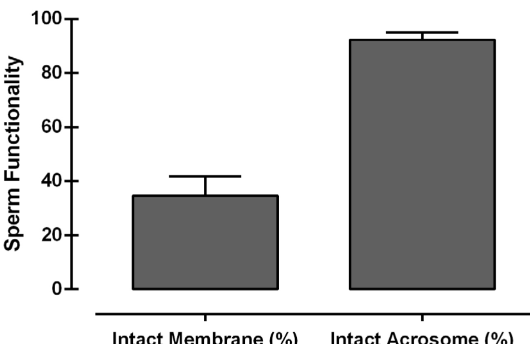

Figure 2. Mean and standard error of the mean (SEM) of plasma membrane integrity (in %) and acrosome integrity (in %) in sperm samples from adult African Lions

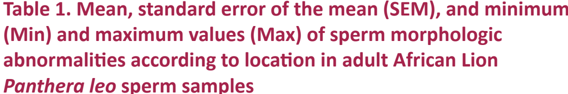

Table 1. Mean, standard error of the mean (SEM), and minimum (Min) and maximum values (Max) of sperm morphologic abnormalities according to location in adult African Lion *Panthera leo* sperm samples

Sperm morphologic abnormalities (%)	Mean	SEM	Min	Max
Sperm head abnormalities	3.89	0.70	1.0	11.0
Sperm mid-piece abnormalities	9.5	2.58	0	46.0
Sperm tail abnormalities	43.07	6.39	8.0	90.0
Sperm acrosome abnormalities	2.42	0.95	0	17.0

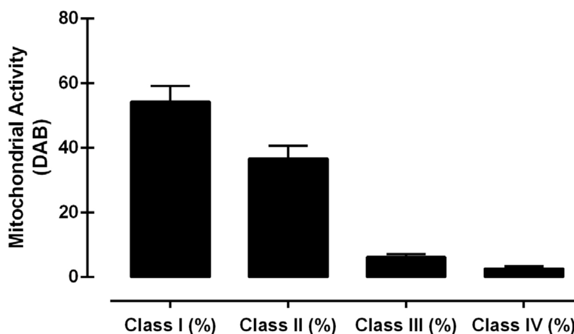

Figure 3. Mean and standard error of the mean (SEM) of mitochondrial activity (A - DAB-Class I: high activity, B - DAB-Class II: medium activity, C - DAB-Class III: low activity, D - DAB-Class IV: absence of activity) in sperm samples from adult African Lions

DISCUSSION

In this study, we evaluated the spermatic features of African Lions by conventional (i.e., motility and morphologic abnormalities) and functional (i.e., mitochondrial activity and plasma membrane and acrosome integrity) tests.

We observed a high motility rate, progressive motility, and SMI values assessed by conventional microscopy. Other authors previously reported similar results (Gilmore et al. 1998; Luther et al. 2017). Our values for sperm morphologic abnormalities were also in accordance with previous studies of lions (Lueders et al. 2012). This shows that sperm parameters were within the expected range for the species in the conventional evaluation. It is important to verify that the sperm is of high quality for the subsequent functional tests. Moreover, it is noteworthy that with this motility and normal morphology rates, the collected semen could be used in cryopreservation protocols (Luther et al. 2017).

Our values for cells with intact membranes (34.61±7.22%), however, were low when compared to other felines such as Tigrina *Leopardus tigrinus* (Angrimani et al. 2017a), Domestic Cat *Felis catus* (Zambelli et al. 2010), and Clouded Leopard *Neofelis nebulosa* (Tipkantha et al. 2017). To our knowledge, this is a pioneer study of the sperm cell membrane integrity in lions using hypoosmotic swelling tests. Lueders et al. (2012) observed 66.3±10.1% of sperm membrane integrity using vitality staining in lions. Thus, we believe that our result is underestimated.

However the sperm cells in this study showed a high motility; if this high percentage of damaged membranes would be correct the efficient transduction of ATP through the cell would be compromised, causing immobility or low motility rates (Amaral et al. 2013; Angrimani et al. 2017b). The relation between a normal mitochondrial function and membrane integrity was demonstrated in this study, when we observed the positive correlation between high mitochondrial activity and plasma membrane and acrosome integrity. In this scenario, we hypothesize that may the hypoosmotic swelling test in the used concentration of fructose and sodium citrate was deleterious for the sperm cells In fact, Comercio et al. (2013) observed modifications in sperm response after different concentrations of

fructose and sodium citrate in the hypoosmotic test in domestic cats. Therefore, further studies with lions are recommended using different concentrations of solutes for the hypoosmotic test, or another method of plasma membrane integrity evaluation, such as eosin/nigrosin stain which can be certainly used in field conditions (Daub et al. 2016).

In contrast to the results on plasma membrane integrity, in the acrosome analysis we found a higher number of cells with intact acrosomes. This membrane endurance is pivotal for the sperm to tolerate post-ejaculation injuries and to be able to bind to the oocyte (Bucci et al. 2017). Thus, this result shows that fast green/rose bengal stain could be an option to field evaluation of semen of African lions or even other wild felids.

Finally the mitochondrial activity test that a high number of sperm cells had the maximum mitochondrial functionality (high and medium activity – DAB Class I and II), which is essential for the production of ATP and consequently for the motility kinetics (Vicente-Carrillo et al. 2015). This was expected in our study since the samples are fresh from animals in reproductive age and with high motility rates (i.e. conventional tests). Besides, the low percentage of DAB Class III and IV (low and absence of mitochondrial activity), was also a predictable result, as high rates of this parameters are associated with mitochondrial dysfunctions due to lesion in axonemal proteins or decreased energy production (de Lamirande and Gagnon 1992a; de Lamirande and Gagnon 1992b; Rui et al. 2017), which were both not found in this study.

In conclusion, the results from conventional tests were as expected for the species. Regarding the functional assessments, the hypoosmotic swelling test did not show to be a good option to analyze plasma membrane integrity in lion. On the other hand, fast green/rose bengal stain and 3’3 diaminobenzidine (DAB) assays appear to be a good optiosn for analyze the sperm from African Lions *P. leo* in filed conditions.

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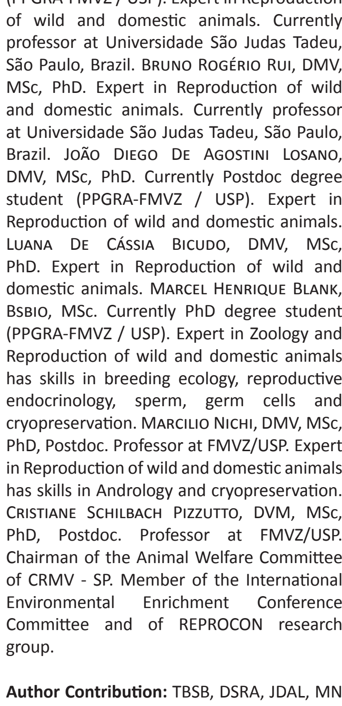

Author Details: Thiesa Butterby Soler Barbosa, DMV, MSc, PhD. Expert in Reproduction of wild and domestic animals. Daniel De Souza Ramos Angrimani, DMV, MSc, PhD. Currently Postdoc degree student (PPGRA-FMVZ / USP). Expert in Reproduction of wild and domestic animals. Currently professor at Universidade São Judas Tadeu, São Paulo, Brazil. Bruno Rogério Rui, DMV, MSc, PhD. Expert in Reproduction of wild and domestic animals. Currently professor at Universidade São Judas Tadeu, São Paulo, Brazil. João Diego De Agostini Losano, DMV, MSc, PhD. Currently Postdoc degree student (PPGRA-FMVZ / USP). Expert in Reproduction of wild and domestic animals. Luana De Cássia Bicudo, DMV, MSc, PhD. Expert in Reproduction of wild and domestic animals. Marcel Henrique Blank, Bsbio, MSc. Currently PhD degree student (PPGRA-FMVZ / USP). Expert in Zoology and Reproduction of wild and domestic animals has skills in breeding ecology, reproductive endocrinology, sperm, germ cells and cryopreservation. Marcilio Nichi, DMV, MSc, PhD, Postdoc. Professor at FMVZ/USP. Expert in Reproduction of wild and domestic animals has skills in Andrology and cryopreservation. Cristiane Schilbach Pizzutto, DVM, MSc, PhD, Postdoc. Professor at FMVZ/USP. Chairman of the Animal Welfare Committee of CRMV - SP. Member of the International Environmental Enrichment Conference Committee and of REPROCON research group.

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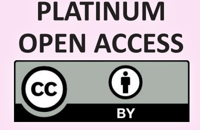

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