

PALOMA ROCHA ARAKAKI

**Evaluation of two extenders for cryopreservation of semen from  
golden-headed lion tamarin (*Leontopithecus chrysomelas*)**

São Paulo

2017

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**Evaluation of two extenders for cryopreservation of semen from  
golden-headed lion tamarin (*Leontopithecus chrysomelas*)**

Thesis submitted to the Postgraduate Program  
in Animal Reproduction of the School of  
Veterinary Medicine and Animal Science of the  
University of São Paulo to obtain the Doctor's  
degree in Sciences

**Department:**

Animal Reproduction

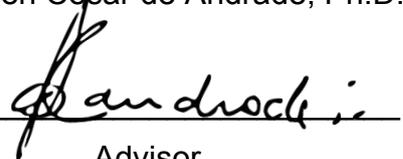
**Area:**

Animal Reproduction

**Advisor:**

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Advisor

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2017

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**CERTIFICADO**

Certificamos que a proposta intitulada "Avaliação de dois diluidores para a criopreservação do sêmen de mico-leão-de-cara-dourada (*Leontopithecus chrysomelas*)", protocolada sob o CEUA nº 1170130317, sob a responsabilidade de **André Furugen César de Andrade e equipe; Paloma Rocha Arakaki** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 06/04/2017.

We certify that the proposal "Evaluation of two extenders for the cryopreservation of semen from golden-headed lion tamarin (*Leontopithecus chrysomelas*)", utilizing 7 Non-human primates (7 males), protocol number CEUA 1170130317, under the responsibility of **André Furugen César de Andrade and team; Paloma Rocha Arakaki** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/06/2017.

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Área: [Reprodução Animal](#)

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Espécie: [Primatas não-humanos](#)

sexo: [Machos](#)

idade: [6 a 14 anos](#)

N: [7](#)

Linhagem: [Leontopithecus chrysomelas](#)

Peso: [605 a 785 g](#)

Resumo: *Leontopithecus chrysomelas*, conhecido como mico-leão-de-cara-dourada, é um primata neotropical pertencente à Família Callitrichidae e é classificado como ameaçado de extinção pela Lista Vermelha de Espécies Ameaçadas da International Union for Conservation of Nature (União Internacional para a Conservação da Natureza). As biotecnologias da reprodução são ferramentas importantes para a conservação desta espécie, sendo que dentre elas a criopreservação se destaca por conservar o patrimônio genético das espécies e auxiliar na preservação da diversidade genética entre populações geograficamente distantes. O sêmen de 7 *L. chrysomelas* adultos será colhido pelo método de vibroestimulação peniana e as amostras serão criopreservadas com dois diluidores comerciais □ Botubov® e TYB®. Amostras do sêmen fresco e do descongelado, serão analisadas para aferição de volume, pH, concentração, motilidades total e progressiva, integridade de membrana plasmática, integridade de acrossomo, atividade citoquímica mitocondrial, fragmentação de DNA, estresse oxidativo e morfologia espermática. As colorações utilizadas para a avaliação de sêmen serão validadas para esta espécie, exceto a coloração de eosina/nigrosina, já validada. Este é o primeiro estudo a respeito da congelação seminal em *Leontopithecus* e acreditamos que os resultados fornecerão informações importantes para o conhecimento da biologia reprodutiva desta espécie e para conservação destes primatas.

Local do experimento: Fundação Parque Zoológico de São Paulo

São Paulo, 09 de maio de 2017

Profa. Dra. Denise Tabacchi Fantoni

Presidente da Comissão de Ética no Uso de Animais  
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## EVALUATION FORM

Author: ARAKAKI, Paloma Rocha

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Thesis submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date: \_\_\_\_ / \_\_\_\_ / \_\_\_\_

### Committee Members

Prof. \_\_\_\_\_

Institution: \_\_\_\_\_ Decision: \_\_\_\_\_

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The Lord, because without Him I am nothing. "For from Him and through Him and for Him are all things. To Him be the glory forever. Amen". Rom. 11.36.

My family, especially my parents Nelson and Raquel, for all support during my whole life, for guiding me and teaching me the values that north who I am. My sisters Kelly and Barbara, for sharing the best moments until now, my brother-in-law Matthew, for friendship, my brother Rafael, his wife Camila and my nephews Joaquim and Heloísa, for bringing joy to my life.

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## RESUMO

ARAKAKI, P. R. **Avaliação de dois diluidores para a criopreservação do sêmen de mico-leão-de-cara-dourada (*Leontopithecus chrysomelas*)**. [Evaluation of two extenders for cryopreservation of semen from golden-headed lion tamarin (*Leontopithecus chrysomelas*)]. 2017. 101 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Muitas espécies de primatas neotropicais são ameaçadas de extinção. As biotecnologias da reprodução podem contribuir consistentemente para a conservação das espécies, estando entre elas os bancos de recurso genético. Entretanto, o sucesso no uso de tais tecnologias depende de avanços no conhecimento da biologia reprodutiva básica de uma certa espécie. Durante esse processo, a avaliação da qualidade espermática é de suprema importância. Nós concentramos nosso trabalho em micos-leões-de-cara-dourada (*Leontopithecus chrysomelas*), mantidos na Fundação Parque Zoológico de São Paulo. *Leontopithecus* sp. é um alvo importante para o desenvolvimento de técnicas reprodutivas com o propósito da conservação, sendo que todas as espécies do gênero são classificadas como ameaçadas ou criticamente ameaçadas de extinção pela IUCN. Para a avaliação da qualidade espermática em *L. chrysomelas*, técnicas como o teste de ligação do espermatozoide, e as colorações não fluorescentes para a avaliação de integridade de acrossomo – fast-green/rosa bengala, integridade de membrana plasmática – eosina-nigrosina e atividade mitocondrial – 3,3' diaminobenzidina – foram validados para sêmen fresco. Nós também validamos uma técnica de avaliação de integridade de acrossomo – corante azul de coomassie, para amostras descongeladas devido a inadequação do método utilizado em sêmen fresco. Algumas espécies de primatas neotropicais apresentam variação na qualidade seminal ao longo do ano. Para avaliar a qualidade do sêmen para essa espécie e para verificar se mico-leão-de-cara-dourada possui essa variação, nós colhemos sêmen nas estações de seca e chuva, durante diferentes períodos da estação reprodutiva – no intervalo dos períodos conceptivos e no final do segundo período conceptivo. As medições dos testículos foram realizadas previamente a cada colheita de sêmen, e uma diferença significativa foi encontrada no volume testicular total entre as estações, com maiores volumes encontrados durante a estação de seca ( $p = 0,0011$ ). As amostras colhidas durante a estação de chuva

mostraram uma maior porcentagem de motilidade total ( $\bar{x} = 93,85$ ) e integridade de membrana plasmática ( $\bar{x} = 95,54$ ) ( $p = 0,0149$  e  $p = 0,0279$ , respectivamente), em comparação com a estação seca (motilidade total  $\bar{x} = 90,96$  e integridade de membrana plasmática  $\bar{x} = 92,46$ ). Mesmo com essas diferenças, sêmen de ambas as estações apresentaram boa qualidade. A taxa de sucesso das colheitas com a técnica de vibroestimulação peniana foi de 100%, e todas as amostras eram constituídas de somente uma fração coagulada. Apesar de não obtermos a dissolução completa do coágulo, nós pudemos acessar um grande número de espermatozoides pela adição do meio Biggers-Whitten-Whittingham (BWW) ao coágulo por 30 minutos a 37°C, e realizamos todas as análises propostas. As mesmas amostras usadas nas avaliações do sêmen fresco foram subsequentemente submetidas à criopreservação usando dois diluidores comerciais diferentes – BotuBOV® (BB) e *Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer*® (TYB). As amostras descongeladas foram analisadas com os mesmos métodos previamente validados e também foram avaliadas para a suscetibilidade ao estresse oxidativo pela quantificação de substâncias reativas ao ácido tiobarbitúrico (TBARS). Para todos os parâmetros seminais avaliados, não houveram diferenças significativas entre BB e TYB, exceto que o BB apresentou porcentagem de acrossomos íntegros ( $p = 0.0101$ ) e concentração de TBARS ( $p = 0.0005$ ) maiores. Apesar dessas diferenças, as amostras descongeladas de ambos diluidores mostraram resultados similares no ensaio de ligação do espermatozoide à membrana perivitelínica do ovo de galinha. Do nosso trabalho, podemos observar que os métodos propostos foram validados com sucesso para *L. chrysomelas*. A qualidade do sêmen fresco foi muito alta e ambos diluidores obtiveram sucesso na criopreservação do sêmen.

Palavras-chave: Conservação de primatas. Primatas neotropicais. Vibroestimulação peniana. Análise seminal. Teste de ligação de espermatozoides.

## ABSTRACT

ARAKAKI, P. R. **Evaluation of two extenders for cryopreservation of semen from golden-headed lion tamarin (*Leontopithecus chrysomelas*)**. [Avaliação de dois diluidores para a criopreservação do sêmen de mico-leão-de-cara-dourada (*Leontopithecus chrysomelas*)]. 2017. 101 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Many Neotropical primate species are endangered of extinction. Reproductive biotechnologies can consistently contribute to species conservation, being among them the genetic resource banks. However, success in the use of such technologies relies on advances in the knowledge of basic reproductive biology of a given species. During these processes, assessment of sperm quality is of supreme importance. We focused our work in captive golden-headed lion tamarins (*Leontopithecus chrysomelas*), housed at São Paulo Zoological Park Foundation. *Leontopithecus* sp. is a very important target for the development of reproductive techniques aiming conservation, as all species from this genus are classified as endangered or critically endangered of extinction by IUCN. For the evaluation of sperm quality in *L. chrysomelas*, techniques like sperm-binding assay, and the non-fluorescent staining for evaluation of acrosome integrity – fast-green/rose-bengal, plasma membrane integrity – eosin-nigrosin and mitochondrial activity – 3.3' diaminobenzidine were validated for fresh semen. We have also validated an acrosome integrity evaluation technique – coomassie blue staining, for thawed samples due to the inadequacy of the method used for fresh semen. Some Neotropical primates species show variation in semen quality over the year. In order to assess semen quality for this species and to verify if golden-headed lion tamarin presents this variation, we collected semen in dry and rainy seasons, during different periods of breeding season – in the interval of conceptive periods and in the end of the second conceptive period. Testicular measurements were taken prior each semen collection, and a significant difference was found in total testicular volume between seasons, with major volumes found during dry season ( $p = 0.0011$ ). Samples collected during rainy season showed higher percentage of total motility ( $\bar{x} = 93.85$ ) and intact plasma membrane ( $\bar{x} = 95.54$ ) ( $p = 0.0149$  and  $p = 0.0279$ , respectively), in comparison with dry season (total motility  $\bar{x} = 90.96$  and intact plasma membrane  $\bar{x} = 92.46$ ). Even with those differences, semen from both seasons presented good quality. The success rate for

collection with penile vibrostimulation technique was of 100%, and all samples obtained were constituted by only a coagulated fraction. Despite not achieving coagulum dissolution, we were able to access a high number of spermatozoa by adding Biggers-Whitten-Whittingham (BWW) medium to the coagulum for 30 min at 37°C, and performed all proposed analyzes. The same samples used of fresh analyzes were subsequently submitted to cryopreservation using two different commercial extenders – BotuBOV® (BB) and Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer® (TYB). Thawed semen samples were analyzed with the same methods previously validated and were also evaluated for the susceptibility to oxidative stress through quantification of induced thiobarbituric acid reactive substances (TBARS). For all seminal parameters assessed, no significant difference was observed between BB and TYB, except that BB presented a higher percentage of intact acrosome ( $p = 0.0101$ ) and concentration of TBARS ( $p = 0.0005$ ). Despite those differences, thawed semen from both extenders performed similarly at sperm-egg binding assay. From our work, we observed that the proposed methods were successfully validated for *L. chrysomelas*. Fresh semen quality was very high and both extenders were successful in cryopreserving semen.

Keywords: Primate conservation. Neotropical primates. Penile vibrostimulation. Semen analysis. Sperm-binding assay.

## LIST OF FIGURES

### Chapter 1

Figure 1 - Golden-headed lion tamarin (*Leontopithecus chrysomelas*).....23

### Chapter 2

Figure 1 - Microscope analysis of the number of spermatozoa bound per mm<sup>2</sup> of egg pervitelline membrane (n=6) for four alive/dead sperm ratios (0%, 25%, 50%, 75%), with linear regression from data shown.....55

Figure 2 - Percentage of intact plasma membrane (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.....56

Figure 3 - Percentage of intact acrosome spermatozoa using fast-green/rose-bengal staining (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.....57

Figure 4 - Percentage of intact acrosome spermatozoa using coomassie blue staining (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.....57

Figure 5 - Percentage of high mitochondrial activity (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.....58

Figure 6 - Smear prepared and stained with fast-green/rose-bengal. A) Smear from a fresh semen sample. B) Smear from a frozen-thawed semen sample. The figure shows clumps all over the field.....60

### Chapter 3

Figure 1 - Filamentous (A) and amorphous (B) seminal coagula from golden-headed lion tamarin, collected by penile vibrostimulation technique.....74

## LIST OF TABLES

### Chapter 1

Table 1. Studies reporting semen analyses in Neotropical primate species.....24

### Chapter 2

Table 1 - Values (mean  $\pm$  SE) of proportions of alive/dead sperm and the variables number of spermatozoa bound to egg perivitelline membrane, plasma membrane integrity, acrosome integrity evaluated by fast-green/rose-bengal staining and by coomassie blue staining and mitochondrial activity class I.....54

### Chapter 3

Table 1 - Mean and standard error ( $\bar{x} \pm$  SE) for the testicular measurements from golden-headed lion tamarin (*Leontopithecus chrysomelas*) and the probability values (P) in the dry and rainy season.....73

Table 2 - Mean, standard error ( $\bar{x} \pm$  SE) and range for animal weight, characteristics of the stimuli and sperm analyses from golden-headed lion tamarin, and the probability values (P) in the dry and rainy season.....75

Table 3 - Mean and standard error ( $\bar{x} \pm$  SE) for sperm morphology (classified into major and minor defects) from golden-headed lion tamarin samples, and the probability values (P) in the dry and rainy season.....76

### Chapter 4

Table 1 - Mean, standard error ( $\bar{x} \pm$  SE) and range for sperm analyses in thawed samples from golden-headed lion tamarin, and the probability values (P) in Botubov and TYB groups .....92

## LIST OF GRAPHS

### Chapter 3

Graph 1 - Concentrations of births of golden-headed lion tamarin over the months at São Paulo Zoological Park Foundation, from 1985 to 2012.....	78
--	----

## LIST OF CHARTS

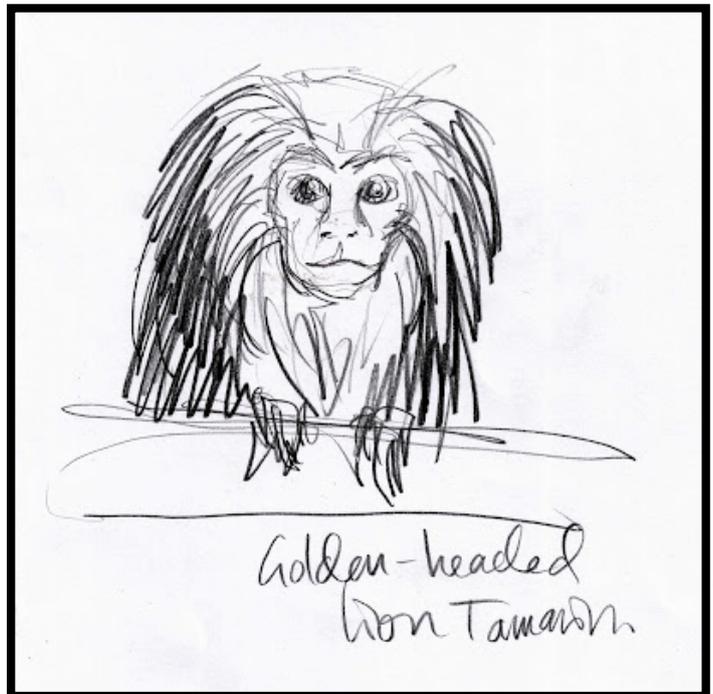
### **Chapter 2**

Chart 1 - Description of the seven golden-headed lion tamarins males studied, from São Paulo Zoological Park Foundation, São Paulo, Brazil.....70

## SUMMARY

<b>1 GENERAL INTRODUCTION.....</b>	<b>19</b>
<b>2 CHAPTER 1: Semen analysis in Neotropical primates: a roadmap for seminal studies in golden-headed lion tamarin (<i>Leontopithecus chrysomelas</i>) .....</b>	<b>22</b>
<b>ORDER PRIMATES .....</b>	<b>22</b>
<b>GENUS <i>LEONTOPITHECUS</i>.....</b>	<b>22</b>
<b>SEMEN ANALYSIS IN NEOTROPICAL PRIMATES .....</b>	<b>24</b>
<b>SEMEN ANALYSIS IN <i>LEONTOPITHECUS CHRYSOMELAS</i>.....</b>	<b>29</b>
<b>TECHNIQUES USED IN THE EVALUATION OF NEOTROPICAL PRIMATES SEMEN.....</b>	<b>30</b>
Plasma membrane integrity.....	30
Acrosome integrity.....	30
Mitochondrial activity .....	31
Oxidative stress .....	32
Sperm-binding assay.....	32
Neotropical primates sperm cryopreservation .....	33
<b>3 CHAPTER 2: Validation of sperm-binding assay and techniques to evaluate plasma membrane integrity, acrosomal integrity and mitochondrial activity in golden-headed lion tamarin (<i>Leontopithecus chrysomelas</i>) semen .....</b>	<b>49</b>
<b>INTRODUCTION.....</b>	<b>50</b>
<b>MATERIAL AND METHODS .....</b>	<b>50</b>
Animals and semen collection.....	51
Semen processing.....	51
Sperm-binding assay.....	52
Plasma membrane integrity.....	52
Acrosome integrity.....	53
Mitochondrial activity .....	53
Statistical analysis .....	54
<b>RESULTS.....</b>	<b>54</b>
Sperm-binding assay.....	56
Plasma membrane integrity.....	56
Acrosome integrity.....	57
Mitochondrial activity .....	58

<b>DISCUSSION .....</b>	<b>59</b>
<b>REFERENCES .....</b>	<b>63</b>
<b>4 CHAPTER 3: Collection and analysis of semen in captive golden-headed lion tamarin (<i>Leontopithecus chrysomelas</i>) in both dry and rainy seasons .....</b>	<b>69</b>
<b>INTRODUCTION .....</b>	<b>70</b>
<b>MATERIAL AND METHODS .....</b>	<b>70</b>
Animals .....	70
Testicular morphometry and semen collection .....	71
Semen processing and analysis .....	72
Statistics .....	73
<b>RESULTS .....</b>	<b>73</b>
<b>DISCUSSION .....</b>	<b>78</b>
<b>REFERENCES .....</b>	<b>83</b>
<b>5 CHAPTER 4: Comparison of two commercial extenders for cryopreservation of sperm from endangered golden-headed lion tamarin (<i>Leontopithecus chrysomelas</i>) .....</b>	<b>87</b>
<b>INTRODUCTION .....</b>	<b>88</b>
<b>MATERIAL AND METHODS .....</b>	<b>89</b>
Animals and semen collection .....	89
Semen handling .....	90
Semen cooling and freezing .....	90
Semen thawing and post analyses .....	91
Statistics .....	92
<b>RESULTS .....</b>	<b>92</b>
<b>DISCUSSION .....</b>	<b>94</b>
<b>REFERENCES .....</b>	<b>96</b>
<b>6 GENERAL CONCLUSION .....</b>	<b>100</b>
<b>REFERENCES .....</b>	<b>101</b>



Rachael Smith

# General Introduction

## 1 GENERAL INTRODUCTION

Biological diversity, or biodiversity, refers to the variety of genes, species, and ecosystems that constitute life on Earth. Its maintenance is essential for the support of balance between all forms of life, including human beings existence (RANDS et al., 2010). However, biodiversity is in danger. The increase of human population and the association of unsustainable consumption in developed nations and longstanding poverty in developing countries lead to an increased anthropogenic action on the environment, resulting in negative impacts for biodiversity (MYERS et al., 2000; MITTERMEIER et al., 2011).

Degradation, fragmentation and destruction of habitats are the factors that cause the greatest impact on biodiversity loss, but these factors also include overexploitation of species, invasive alien species, pollution and climate change (RANDS et al., 2010).

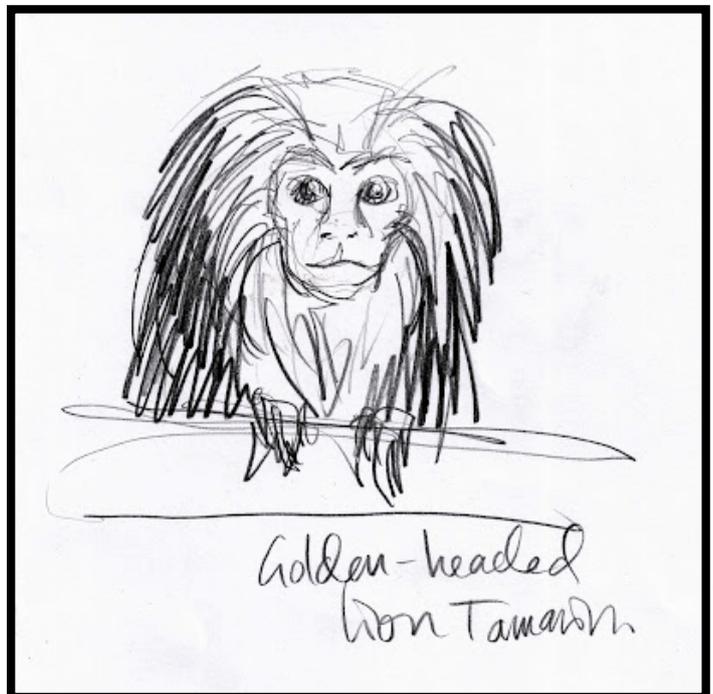
Given the above, thinking about biodiversity conservation increase in importance, and Non-human primates are among the endangered animal species. Of the 25 most endangered species of primates in the world, five occur in the Neotropical region, two of which are endemic to Brazil – kaapori capuchin (*Cebus kaapori*) and northern brown howler monkey (*Alouatta guariba guariba*) (SCHWITZER et al., 2015). The main threats to primates are habitat loss, hunting and illegal trade, factors that lead to declining population size, isolation of populations and extinction of populations and species (ESTRADA, 2009; SCHWITZER et al., 2015).

In this sense, *Leontopithecus chrysomelas*, known as golden-headed lion tamarin, is classified as endangered by the Red List of Threatened Species from International Union for Conservation of Nature (IUCN), due to the decrease of forest remnants where these animals occur in nature (KIERULFF et al., 2008). This species is endemic to the Brazilian Atlantic Forest, an extremely degraded biome that have lost 88.27% of its original area (RIBEIRO et al., 2009). Despite this critical situation, little is known about golden-headed lion tamarin's reproductive biology, and the gaps to be filled are huge, specially regarding andrology.

Assisted reproductive technologies (ART's) are important tools when thinking about conservation, contributing to the maintenance of genetically viable populations. These biotechnologies can support the viability of existing populations, associated

with a suitable genetic management, allowing these animals reproduction over an indefinite period of time or in geographically distant locations. In order to achieve these application with success, knowledge on basic reproductive biology is indispensable (HOLT, 2001).

There are only few studies regarding andrology in golden-headed lion tamarin, which highlights the importance of data presented here. Data about basic reproductive biology and the application of cryopreservation will be described. These results will contribute to the conservation of this and other Neotropical primate species.



Rachael Smith

# Chapter 1

## 2 CHAPTER 1: Semen analysis in Neotropical primates: a roadmap for seminal studies in golden-headed lion tamarin (*Leontopithecus chrysomelas*)

### ORDER PRIMATES

The order Primates is the third largest order of mammals (where nonhuman primates (NHPs) belong) is divided into 16 families, 77 genera, 488 species and 690 subspecies (WILSON; REEDER, 2005; RYLANDS; MITTERMEIER, 2014). Catarrhini is a Parvorder that comprehends the Old World monkeys occurring in Asia and Africa. Another Parvorder is the Platyrrhini that hosts the group of New World monkeys, (also known as Neotropical primates), which occur in Mexico, Central America and South America (GROVES, 2005).

Platyrrhini (Neotropical primates) corresponds to 33% of nonhuman primates, which are classified in 4 or 5 families, 20 genera and 204 species and subspecies (RYLANDS et al., 2012). According to the Primate Specialist Group of IUCN/SSC – Species Survival Commission of International Union for Conservation of Nature – IUCN (2014), of all known nonhuman primates species, about half (50.4%) are endangered of extinction. The percentage for Neotropical primates endangered of extinction is 37.4%.

Neotropical primates are divided in the families Callitrichidae, Cebidae, Aotidae, Pitheciidae e Atelidae. The family Callitrichidae has seven genera (*Callithrix*, *Cebuella*, *Callibella*, *Mico*, *Saguinus*, *Callimico* and *Leontopithecus*). The genus *Leontopithecus*, known as lion tamarins, is formed by the following species: golden lion tamarin (*L. rosalia*), golden-headed lion tamarin (*L. chrysomelas*), black lion tamarin (*L. chrysopygus*) and black-faced lion tamarin (*L. caissara*) (RYLANDS; MITTERMEIER, 2009). It should be emphasized that the taxonomy is not definitive, and changes may happen with the appearance of new studies.

### GENUS *Leontopithecus*

Primates of the genus *Leontopithecus* are small animals, with a body mass of less than 700 grams. They form social groups ranging from 4 to 20 individuals, and present a diet that includes fruits, nectar, exudates and small animals (ESTRADA; GARBER, 2009; FERRARI, 2009; NORCONK et al., 2009).

The lion tamarins consistently produce twin offspring and exhibit a polygynic-polyandric mating system, with reproductive suppression of the subordinated females

and cooperative care of the offspring. In reported cases where two females successfully reared in the same season, the two females were mother and daughter (BAKER et al., 2008).

*Leontopithecus* shows reproductive seasonality in different habitats, both in the wild as in captivity, and different locations as Brazil, North America and Europe. In North America and Europe, there is a decline in the number of births during winter. These populations present a long reproductive season that goes from March to September during which births occur. In Brazil, in captivity, the mating season extends from April to June and most of the births occur between August and October (FRENCH et al., 2008).

All the four species from the genus *Leontopithecus* are endemic to the Brazilian Atlantic Forest. *L. rosalia*, popularly known as golden lion tamarin, is classified as endangered of extinction by IUCN, due to the small number of individuals in the wild and the limited possibilities for population growth in the few fragmented forest areas at their restrict occurrence area. *L. chrysopygus*, known as black lion tamarin, is classified as endangered by IUCN, due to the isolation and small size of the populations in the wild. *L. caissara*, known as black-faced lion tamarin, is classified as critically endangered by IUCN, due to the small size of the population in the wild (400 individuals, with approximately 200 adults), located in three isolated subpopulations (KIERULFF et al., 2008a,b,c).

*L. chrysomelas*, known as golden-headed lion tamarin (Figure 1), is classified as endangered by IUCN, due to a severe reduction in population size, estimated to be over 50% in the last three generations (21 years), due to the high rates of forest coverage loss in the Atlantic Forest. The remaining population are severely fragmented (KIERULFF et al., 2008d).

The golden-headed lion tamarin have an average body mass of 590 grams and form social groups of an average of 6.7 individuals (BAKER et al., 2008; ESTRADA; GARBER, 2009; FERRARI, 2009; NORCONK et al. 2009). They have black fur, with mane, forearms, hands, feet and proximal half of the tail of gold color. Their original distribution ranged from the South of Bahia state to the Jequitinhonha River, in the state of Minas Gerais. Currently, it is much reduced (GROVES, 2001). Mean duration of the gestation in *L. chrysomelas* is 125.3 days. The duration of the ovarian cycle is  $21.5 \pm 2.5$  days, and females give birth once a year (DE VLEESCHOUWER et al., 2000).

Figure 1. Golden-headed lion tamarin (*Leontopithecus chrysomelas*).

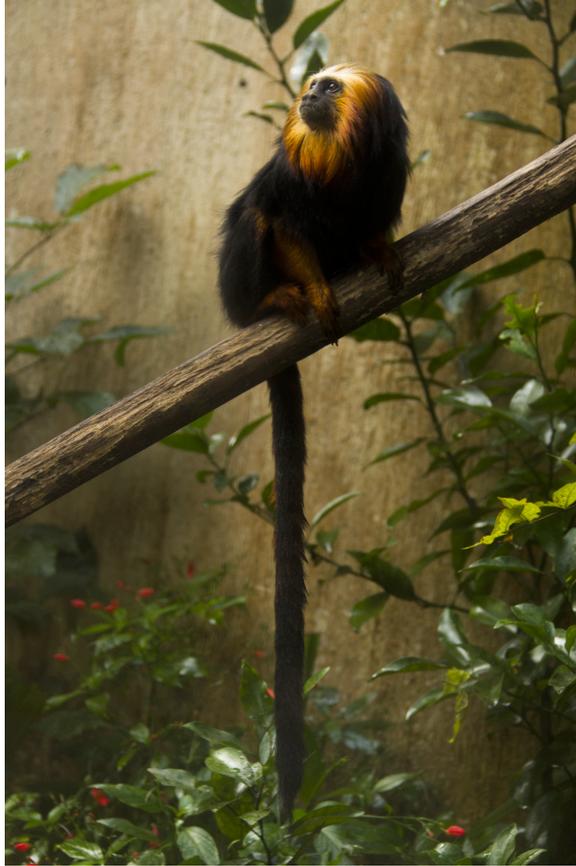


Photo: Paulo Gil/FPZSP

## SEMEN ANALYSIS IN NEOTROPICAL PRIMATES

Known studies that describe semen characteristics of different Neotropical primates, as well as analyzes with fresh semen and the conservation of these gametes are presented in Table 1.

Table 1. Studies reporting semen analyses in Neotropical primate species.

<b>Family</b>	<b>Species</b>	<b>References</b>
Atelidae	<i>Alouatta caraya</i>	CARVALHO, 2012 CARVALHO et al., 2012 CARVALHO et al., 2014 MORELAND et al., 2001 VALLE et al., 2013b VALLE et al., 2004
	<i>Ateles geoffroyi</i>	CERDA-MOLINA et al., 2009 FLORES-HERRERA et al., 2012 HERNÁNDEZ-LÓPEZ et al., 2008 HERNÁNDEZ-LÓPEZ et al., 2002b HERNÁNDEZ-LÓPEZ et al., 2002a
	<i>Ateles marginatus</i>	SILVA, 2005 SILVA et al., 2013
	<i>Ateles paniscus</i>	SILVA, 2005 SILVA et al., 2013
	<i>Brachyteles arachnoides</i>	ARAKAKI et al., 2015b ARAKAKI et al., 2015a
Callithrichidae	<i>Callimico goeldii</i>	ARAKAKI et al., 2017 VALLE et al., 2013a
	<i>Callithrix aurita</i>	GONÇALVES et al., 2017
	<i>Callithrix jacchus</i>	ARAKAKI et al., 2013 CUI, 1996 CUI et al., 1991 DAVIS, 1982 GILCHRIST et al., 1997 GRUPEN et al., 2004 HERNÁNDEZ-LÓPEZ et al., 2005 KUEDERLING et al., 1996 KUEDERLING et al., 2000 MORRELL, 1998 MORRELL et al., 1996 MORRELL et al., 1997 MORRELL et al., 1998 O'BRIEN et al., 2003 O'BRIEN et al., 2005 PUDRITZ, 2000 SCHNEIDERS et al., 2004 TAKAHASHI et al., 2014 TKACHENKO et al., 2010 VALLE, 2007 VALLE et al., 2012

continued

	VALLE et al., 2014
	VALLE et al., 2008
	VALTONEN-ANDRÉ et al., 2005
	VERONA et al., 2009
	WILTON et al., 1993
	YEUNG et al., 1996
<i>Callithrix penicillata</i>	ARAKAKI et al., 2013
	MASSAROTO et al., 2010
<i>Leontopithecus chrysomelas</i>	HENRIQUE et al., 2013
	SANTOS et al., 2014
	VIDAL et al., 2007
<i>Saguinus labiatus</i>	SANKAI et al., 1997
<i>Saguinus leucopus</i>	POCHES, 2012
<i>Saguinus leucopus</i>	POCHES et al., 2013
<i>Saguinus mystax</i>	HARRISON; WOLF, 1985
<i>Saimiri boliviensis</i>	ROUDEBUSH; MATHUR, 1998
	STEINBERG et al., 2009
	YEOMAN et al., 1997a
	YEOMAN et al., 1997b
	YEOMAN et al., 1998
<i>Saimiri collinsi</i>	OLIVEIRA et al., 2015
<i>Saimiri sciureus</i>	ACKERMAN; ROUSSEL, 1968
	BENNET, 1967
	CHEN et al., 1981
	DENIS et al., 1976
	KUGELMEIER, 2011
	LANG, 1967
Cebidae	<i>Saimiri vanzolinii</i>
	OLIVEIRA et al., 2016
	<i>Saimiri cassiquiarensis</i>
	OLIVEIRA et al., 2016
	<i>Saimiri macrodon</i>
	OLIVEIRA et al., 2016
	<i>Cebus apella</i>
	ACKERMAN; ROUSSEL, 1968
	ARAÚJO et al., 2009
	BARNABE et al., 2002
	BUSH et al., 1975
	GUIMARÃES, 1994
	LEÃO et al., 2015
	OLIVEIRA et al., 2010
	OLIVEIRA et al., 2011
	PAZ et al., 2006
	PAZ et al., 2006

In nonhuman primates, several techniques are used for semen collection. Samples can be collected by vaginal washing after intercourse, artificial vagina,

direct puncture of the epididymis, masturbation, rectal probe electrostimulation (RPE) and penile vibrostimulation (PVS) (KRAEMER; CRUZ, 1969; BADER, 1983; LANZENDORF et al., 1992; GOULD; YOUNG, 1996; MORRELL et al., 1996; POPE et al., 1997; YEOMAN et al., 1997; CHYE NG et al., 2002).

The most used method of semen collection in Neotropical primates is the RPE, described in species such as *Callithrix jacchus* (CUI et al., 1991), *Alouatta caraya* (VALLE et al., 2004; CARVALHO et al. 2014), *Saimiri sciureus* (BENNETT et al. 1967), *Saimiri boliviensis* (YEOMAN et al. 1997), *Cebus apella* (BARNABE et al., 2002), *Ateles paniscus* e *A. marginatus* (SILVA et al. 2013), *Leontopithecus chrysomelas* (VIDAL et al., 2007) and *Callimico goeldii* (VALLE et al., 2013; ARAKAKI et al. 2017).

However, despite RPE being an effective technique for semen collection, it is an invasive method and the need for anesthesia is a risk factor for the subject. Thus, PVS collection technique is an important alternative to be considered, especially for small species, since they are relatively easy to be physically restrained.

Comparisons between RPE and PVS were performed in *Saimiri boliviensis* and *Callithrix jacchus*. For *S. boliviensis*, semen collection through PVS showed higher sperm concentration and sperm motility when compared to RPE (YEOMAN et al., 1998). The use of PVS in *C. jacchus* also showed better results like higher sperm concentration and motility when compared to RE. In addition, the coagula in the ejaculate obtained by RPE were firmer and more resistant to dissolution compared to ejaculates obtained by PVS (SCHENEIDERS et al., 2004).

The PVS technique has been widely used for semen collection in Neotropical primates, and has been described for other species as *C. penicillata* (MASSAROTTO et al., 2010, ARAKAKI et al., 2013), *Saimiri sciureus* (KUGELMEIER, 2011) and *Leontopithecus chrysomelas* (HENRIQUE et al., 2013; SANTOS et al., 2014).

Most primate species present seminal coagulum (MORRELL; HODGES, 2001), which also occurs in samples from golden-headed lion tamarin (VIDAL et al., 2007). Regarding Neotropical primates, studies using proteolytic enzymes to dissolve coagulum were performed. In *Cebus apella* the enzymes hyaluronidase and trypsin at 0.1% were tested for dissolution of the seminal coagulum. In both cases, solution with the enzymes were added to the semen in the proportion of 25% of the volume of the ejaculate, and incubated at 37°C for 15 min. No enzyme was efficient for the complete dissolution of the coagulum and there was still a reduction of the motility

and sperm vigor in the coagulum fraction when compared to the liquid fraction of the semen. Besides, there was no significant difference in acrosome integrity between liquid and coagulum fractions (PAZ et al., 2006).

Also in *C. apella*, the complete dissolution of the coagulum was obtained by mechanical fragmentation of the coagulum together with the use of a coconut water based diluent (50% coconut water, 25% sodium citrate at 5%, and 25% distilled water), after incubation at 37°C for one or two hours. There was no significant difference in plasmatic membrane integrity of the spermatozoa in their work (ARAÚJO et al. 2009). In another study with *C. apella*, the coagulum was divided into two pieces of equal size; one was diluted with TES-Tris diluent and the other with a coconut water based diluent. In both cases the coagulum was mechanically fragmented until complete dissolution. A higher percentage of motile spermatozoa was obtained in the TES-Tris diluent aliquot, and no significant difference was found in the sperm vigor and plasma membrane integrity (OLIVEIRA et al. 2011).

In *Saimiri sciureus*, Kugelmeier (2011) evaluated trypsin at 5%, 1% and 0.5% in 20 and 40 min of incubation with the coagulum, although no treatment was efficient at the complete dissolution of the coagulum. Semen coagula from *S. collinsi*, *S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon* did not liquefy with dilution in a powdered coconut water (ACP-118) extender with mechanical fragmentation and incubation in water bath at 37°C for 1 to 1.5 hour (OLIVEIRA et al., 2016).

A study with *Ateles geoffroyi* compared two methods for the coagulum dissolution: the manual shaking of the semen immersed in water bath at 37°C for 10 min, and the addition of trypsin at 0.25% in a proportion of 2:3 (trypsin solution: semen) also for 10 min in water bath at 37°C. The sample diluted with trypsin showed higher values of sperm concentration, percentage of motile spermatozoa and integrity of plasmatic membrane (HERNÁNDEZ-LÓPEZ et al., 2002).

Common marmoset (*C. jacchus*) coagulum can be diluted by mechanic impact of a soft pipetting (CUI et al., 1991), as well as by the incubation at 37.5°C for 20-30 minutes (KUEDERLING et al., 2000).

Regarding golden-headed lion tamarin coagulum, completely dissolution was not achieved when incubating coagulum with coconut water *in natura* extender at 37°C for 30 min (HENRIQUE et al., 2013).

However, the enzymatic treatment of the coagulum is not performed in some cryopreservation protocols, since its use may affect sperm quality after thawing (MORREL, HODGES, 2001).

It's worth noting that the characteristics of the coagulum such as color, consistency and viscosity, vary significantly among species already studied (DIXSON; ANDERSON, 2002), which highlights the need for studies directed to each species to better treatment of the whole sample, liquid fraction and coagulum.

#### SEMEN ANALYSIS IN *Lentopithecus chrysomelas*

It is notorious that golden-headed lion tamarin was little investigated with regard to the reproductive characteristics of males of this species. In a first study, Vidal et al. (2007) collected semen from 10 subjects (4 ejaculates from each animal), housed at Rio de Janeiro Primatology Center (CPRJ), using rectal probe electrostimulation (RPE). The volume of the ejaculate was measured of only one sample from each animal, measuring the liquid fraction after removing the coagulum. The semen was diluted in Ham's F-10 solution. Regarding sperm morphology, an average percentage of 32,71% of total pathologies was found, analyzed by the wet chamber preparation method.

In a study from our group, Henrique et al. (2013) obtained 11 ejaculates of six subjects housed at São Paulo Zoological Park Foundation (FPZSP), using penile vibrostimulation technique (PVS). Immediately after collection, the pH of the sample was measured, and semen was diluted in a solution of coconut water *in natura*. Samples were incubated at 37°C for 30 min, in order to allow spermatozoa to be released from the seminal coagulum. The analysis realized were: total and progressive motility, plasma membrane and acrosome integrity. This study revealed that it is possible to collect semen from this species using PVS, as it showed a 90,9% success of the attempts.

In a second study from our group (SANTOS et al., 2014), a validation of an association of eosin and nigrosin stainings for the evaluation of plasma membrane integrity was performed. Four ejaculates of different subjects housed at FPZSP were obtained by PVS method. Samples were diluted into BWW (Biggers-Whitten-Whittingham) medium and then split into two aliquots: one maintained at 37°C and the other subjected to snap-freezing, for induction of lesion in the cell. Then the aliquots were mixed in proportions of 0%, 20%, 40%, 60%, 80% and 100% (live

cells/damaged cells). Plasma membrane integrity of each proportion was evaluated, and the positive correlation of the linear regression analysis with the proportions validated the technique for this species.

## TECHNIQUES USED IN THE EVALUATION OF NEOTROPICAL PRIMATES SEMEN

### *Plasma membrane integrity*

In studies within Neotropical primates, the use of fluorescent dyes is not common, as this technique requires specific conditions to be performed, as specific equipment for the evaluation of the spermatozoa. Given that the availability of such equipment is unlikely in the institutions where studies are usually conducted with these animals – zoos, primatology centers or in the field – alternatives to this technique are necessary. Thus, the evaluation of the membrane integrity of sperm cells from Neotropical primates is carried out, mainly, by means of conventional stainings impermeable to the membrane.

The association of eosin and nigrosin stains is widely used. Cells with lesion on the membrane allow eosin to penetrate the cell, which stain in pink. In spermatozoa with intact plasma membrane, the dye cannot penetrate the cell, and they are visualized in white, in contrast to the dark background, stained by nigrosin. This association of staining has been validated for *Callithrix jacchus* (VALLE et al., 2008) and *Leontopithecus chrysomelas* (SANTOS et al., 2014), and its use is described in *Callithrix penicillata* (MASSAROTTO et al., 2010; ARAKAKI et al., 2013), *Callimico goeldii* (ARAKAKI et al., 2017), *Sapajus apella* (OLIVEIRA et al., 2011), *Saimiri sciureus* (KUGELMEIER, 2011) and *Alouatta caraya* (CARVALHO et al., 2014). The use of eosin B was described in *Ateles geoffroyi* (HERNÁNDEZ-LÓPEZ et al., 2002), and eosin Y in *Sapajus apella* (BUSH et al., 1975), *Callithrix jacchus* (KUEDERLING et al., 2000) and *Alouatta caraya* (VALLE et al., 2004).

### *Acrosome integrity*

The techniques already described for the evaluation of acrosome integrity in Neotropical primates include: the commercial kit Spermac® staining, described for semen of *Callithrix jacchus* (VALLE, 2007), *Callimico goeldii* (ARAKAKI et al., 2017) and *Alouatta caraya* (CARVALHO et al., 2014); the fluorescent staining with

fluorescein-conjugated *Pisum sativum* agglutinin (FITC-PSA) was described for semen of *Callithrix jacchus* (PUDRITZ, 2000; VALLE et al., 2008) and the fast-green/rose-bengal staining by (POPE et al. 1991) validated for semen of *Callithrix jacchus* (VALLE et al., 2008) and described in *Callithrix penicillata* (MASSAROTTO et al., 2010; ARAKAKI et al., 2013), *Callimico goldii* (ARAKAKI et al., 2017), *Saimiri sciureus* (KUGELMEIER, 2011), *Cebus Apella* (PAZ et al., 2006) *Alouatta caraya* (CARVALHO et al., 2014) and *Leontopithecus chrysomelas* (HENRIQUE et al., 2013).

#### *Mitochondrial activity*

One technique that is suitable to evaluate mitochondrial activity and does not require elaborate equipment, is the cytochemical technique of 3,3'-diaminobenzidine (DAB) staining. The analysis of mitochondrial cytochemical activity evaluates cellular respiration and energetic metabolism of the cell. A prerequisite for the osmotic function, motility and maintenance of cell structure, through the enzyme cytochrome c oxidase (CcO). CcO is a closely associated with cytochrome C, a product of the respiratory chain. The cytochemical technique used to evaluate mitochondrial activity is based on the oxidation of 3,3'-diaminobenzidine (DAB) by the cytochrome C complex, including CcO, in a chain reaction in which the reagent is polymerized and deposited at the reaction sites, on the internal mitochondrial membrane of active mitochondria (HRUDKA, 1987). The deposition of the reagent is identified by the visualization of a brown color in the middle part of the sperm where the mitochondria are located. Sperm cells are classified in four classes: Class I – almost 100% of the mid-piece stained, indicating full mitochondrial activity; Class II – more than 50% of the mid-piece stained, indicating medium mitochondrial activity; Class III – less than 50% of the mid-piece stained, indicating low mitochondrial activity; and Class IV – lack of staining in the mid-piece, which indicates no mitochondrial activity

In Neotropical primates, this technique was described in semen of *Callithrix jacchus* (VALLE, 2007; ARAKAKI et al., 2013), *C. penicillata* (MASSAROTTO et al., 2010; ARAKAKI et al., 2013), *Saimiri sciureus* (KUGELMEIER, 2011) and *Alouatta caraya* (CARVALHO et al., 2014).

### *Oxidative stress*

Physiologically, oxygen is the final receptor of electron during the production of ATP by the mechanism of oxidative phosphorylation. However, this energy synthesis produces relatively unstable intermediates, the reactive oxygen species (ROS) (LUZ et al., 2011). ROS are free radicals that play an important role in many physiological processes of the spermatozoa, such as capacitation, hyperactivation and fusion of spermatozoa with oocyte (ALLAMANENI et al., 2004). The accumulation of ROS in the cell occurs when there is an imbalance between the pro-oxidant and antioxidant systems of the cell, which leads to oxidative stress (LUZ et al., 2011). Spermatozoa are sensitive to oxidative stress due to the absence of cytoplasmic defenses. In addition, the plasma membrane of spermatozoa contains lipid in the form of polyunsaturated fatty acids (PUFAs), which are vulnerable to attack by ROS, easily oxidized; in their presence, a chain of chemical reaction called lipid peroxidation is triggered (AGARWALL et al., 2005).

The result of the lipid peroxidation is the accumulation of hydroperoxides and, consequently, the formation of cytotoxic products such as malondialdehyde, which alter the cell membrane and cause permeability disorders. These changes in permeability lead to a loss of selectivity for the entry and/or exit of nutrients and toxic substances into the cell, causing damage to the cellular components (LUZ et al., 2011).

The thiobarbituric acid reactive substances (TBARS) test is used to evaluate the susceptibility of a sperm sample to oxidative stress (OHKAWA et al., 1979). It is based on the measurement of byproduct of the lipid peroxidation, the main one being malondialdehyde (NICHI et al., 2007). The thiobarbituric acid, when reacted with malondialdehyde, forms a complex of pink coloration, which is quantified in a spectrophotometer.

In non-human primates, this technique was described for semen of *Alouatta caraya* (CARVALHO et al., 2012).

### *Sperm-binding assay*

The ability of sperm to interact with the zona pellucida (ZP) is an essential step during the process of in vivo fertilization. Spermatozoa-ZP interaction occurs by the ligation of the spermatozoa with proteins of the zona pellucida 3 (ZP3), performed by a receptor located in the acrosome (CRISCUOLO et al., 2010). This interaction can

be evaluated by spermatozoa-oocyte interaction tests, by the means of in vitro fertilization. However, in some cases, it may be difficult to obtain oocytes of the same species of the semen donor, especially if the species is wild or threatened with extinction.

The chicken egg perivitelline membrane presents glycoproteins that displays homology to the glycoproteins from mammalian ZP (WACLAWEK et al., 1998; BAUSEK et al., 2000; SMITH et al., 2005), which allows the binding of spermatozoa of several mammalian species. Sperm-binding test using chicken egg perivitelline membrane was reported for rooster, mouse, ram, bull, horse, dog, ocelot – *Leopardus pardalis*, oncilla – *Leopardus tigrinus*, collared peccary – *Pecari tajacu*, agouti – *Dasyprocta leporina*, and human semen (BARBATO et al., 1998; CRISCUOLO et al., 2010; CASTELO et al., 2015; LOSANO et al., 2015; ARAÚJO et al., 2015; BRITO et al., 2017; CAMPOS et al., 2017). In Neotropical primates, this technique was performed only in golden-headed lion tamarin semen (results to be published). In vitro assays may aid the evaluation of the fertilizing potential of a male, and predict the capacity of semen samples to fertilize oocytes (BHATTACHARYYA; KANJILAL, 2003).

#### *Neotropical primates sperm cryopreservation*

Attempts to develop a protocol for cryopreservation of nonhuman primates semen, especially from species most commonly found in captivity, capable of preserving gametes satisfactorily, for their subsequent use in assisted reproduction techniques (ARTs) is still limited (MORREL; HODGES, 2001).

The diluent media used for nonhuman primates semen usually contain egg yolk and buffers such as TES, Tris and sodium citrate. The most commonly used cryoprotectant is glycerol, which penetrates the cell and may be toxic for the spermatozoa (MORRELL; HODGES, 2001).

In Neotropical primates, a study with *Saimiri boliviensis* used the diluent TEST (TES+Tris) egg yolk 8%, in which fresh semen motility was 71% and thawed semen presented 41% motile sperm (42,25% less) (YEOMAN et al., 1997). From the same genus, sperm from *S. collinsi*, *S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon* were diluted in ACP-118® with or without egg yolk (during sperm cooling) and were cryopreserved with glycerol 3%, in a two-step semen cryopreservation (OLIVEIRA et al., 2016). These authors concluded that semen from *S. collinsi* can be cooled diluted

in ACP-118® without egg yolk and that sperm from the other three species (free-living animals) can be cryopreserved and presents satisfactory sperm quality in thawed samples.

In *Cebus apella*, two diluents were compared, the TEST egg yolk with glycerol at 3.5% and a coconut water based diluent with glycerol 2.5%. After semen thawing, the percentage of intact plasma membrane cells was of 26.2% in the first diluent and 13.2% in the second diluent; sperm motility for both diluents was null (OLIVEIRA et al., 2011). In another study from the same group with *Sapajus apella*, sperm was cryopreserved with ACP-118® and glycerol at 3%, 5% or 7% (LEÃO et al., 2015). ACP-118® with 3% glycerol presented better results, maintaining sperm motility and plasma membrane integrity.

The TEST egg yolk with glycerol 4% was used in the freezing of *Callithrix jacchus* semen, in which total sperm motility and progressive motility was 63.93% and 56.07% respectively before freezing, and 7.38% and 4.23% respectively after thawing, a drop of 88.46% at total motility and 92.45% at progressive motility (VALLE, 2007). In a study with *C. jacchus* and *C. penicillata*, the diluent TEST egg yolk was used with two glycerol concentrations, 4% and 6%; both concentrations were not effective in protecting motility of spermatozoa of both species during cryopreservation (ARAKAKI, 2013).

In *Alouatta caraya*, two diluents were compared for cryopreservation of the semen – TEST egg yolk with glycerol 3% and 4%, and TEST soybean lecithin with glycerol 3% and 4%. The diluent based of egg yolk with glycerol 4% presented the best result for semen cryopreservation on this species (CARVALHO, 2012).

In a study with only three samples, semen from *Ateles paniscus* (n=2) and *A. marginatus* (n=3) were cryopreserved with TES and CEBRAN II, a Ringer's lactate based extender, with glycerol 7% in both extenders. TES extender presented better results in thawed samples (SILVA et al., 2013).

Studies that had not performed semen cryopreservation, used a Ringer's lactate solution extender for *Alouatta caraya* (VALLE et al., 2004), Ham's F-10 for *Leontopithecus chrysomelas* (VIDAL et al., 2007), TALP-Hepes for *Callithrix penicillata* (MASSAROTTO, 2010) and a coconut water based extender for *Callimico goeldii* (ARAKAKI et al., 2017).

Neotropical primates are a very diverse group, and as seen in this review, the number of studies that report semen evaluation is very reduced, which are

concentrated in a few species. The use of simpler techniques for semen evaluation needs to be validated for each species due to several differences even within the same genus. Another feature to notice is the small number of cryopreservation studies and, for some species, presenting results with limited efficiency. Our work confirmed the big gap in Neotropical primate andrology knowledge. This fact much change in order to better achieve these animals' conservation.

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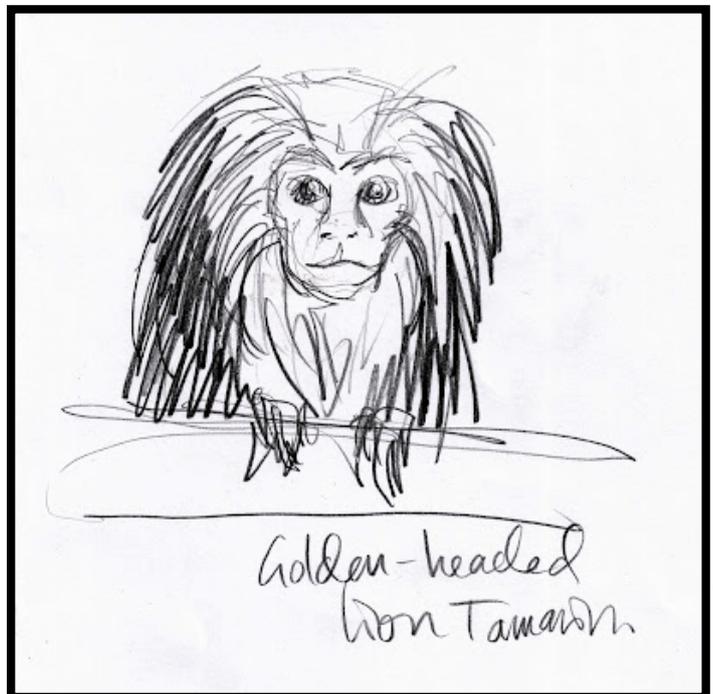
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## Chapter 2

### **3 CHAPTER 2: Validation of sperm-binding assay and techniques to evaluate plasma membrane integrity, acrosomal integrity and mitochondrial activity in golden-headed lion tamarin (*Leontopithecus chrysomelas*) semen**

#### ABSTRACT

Reproductive biotechnologies are essential in conservation programs for endangered species. Gamete cryopreservation, artificial insemination, embryo transfer and in vitro fertilization are some examples of techniques that allow producing offspring in order to ensure biological and genetic diversity. However, a first step required for the application of such techniques is the knowledge of basic aspects of reproductive biology. *Leontopithecus chrysomelas*, the golden-headed lion tamarin, is an endangered Neotropical primate, extremely affected by habitat loss due to anthropic activity. Even though efforts on its conservation have been done, basic research on semen aspects is poorly described. Techniques for semen evaluation must be standardized for any attempt on the use of reproductive technologies. Therefore, we describe the validation for the sperm-egg-binding assay, and non-fluorescent staining, one staining for the evaluation of plasma membrane integrity, two staining for acrosome integrity evaluation and one staining for mitochondrial activity for this Neotropical primate species. Semen was collected by penile vibrostimulation, a non-invasive technique, and either fresh or thawed samples were divided into two aliquots. One aliquot was kept at 37°C (alive) and the other was submitted to snap-freezing to harm sperm cells (referred as dead cells), and subsequent aliquots were mixed into 0%, 25%, 50%, 75% and 100% ratios of alive:dead cells. Analyses of sperm-egg-binding assay and eosin-nigrosin, fast-green/rose-bengal, coomassie blue and 3.3' diaminobenzidine staining were performed. Linear regression showed high positive correlation between alive:dead sperm ratio and: number of spermatozoa bound to the membrane, intact plasma membrane, intact acrosome, and high mitochondrial activity. We considered that the methods described provided accurate results and can be consistently used for sperm evaluation in this species.

Keywords: Primate conservation. Neotropical primates. Semen evaluation. Penile vibrostimulation. Non-fluorescent staining.

## INTRODUCTION

Golden-headed lion tamarin (*Leontopithecus chrysomelas*) is a Neotropical primate considered endangered by International Union for Conservation of Nature (IUCN) Red List of Threatened Species, due to the decrease of forest remnants where these animals occur in nature (KIERULFF et al., 2008). Despite the threats that this species is subjected, little is known about golden-headed lion tamarin's reproductive biology, particularly about andrology. The application of assisted reproductive technologies such as artificial insemination, in vitro fertilization and embryo transfer is an important tool for conservation of endangered species (COMIZZOLI et al., 2000; PUKAZHENTHI et al., 2006; VALLE et al., 2008). Methods developed for semen evaluation in other species may be transferable to primates' semen. Nevertheless, these methods must be optimized to provide reliable data when applied in a new species.

In this article we describe the validation of sperm-egg-binding assay (LOSANO et al., 2015), a stain for plasma membrane integrity (eosin-nigrosin staining) (BARTH; OKO, 1989; VALLE et al., 2008) two acrosome integrity evaluation techniques (fast-green/rose-bengal staining for fresh semen and coomassie blue staining in thawed semen) (POPE et al., 1991; LARSON; MILLER, 1999; VALLE et al., 2008) and 3.3' diaminobenzidine (DAB) staining for mitochondrial activity (HRUDKA, 1987) in golden-headed lion tamarin semen. Sperm functional assays as described herein provide more information on the fertilizing potential of semen samples rather than only their concentration or appearance (AITKEN, 2006). All the staining methods validated are suitable for use under field conditions, an important property in the evaluation of wild animals seminal samples. To our knowledge, the only previous report on such techniques was an abstract from our group (SANTOS et al., 2014). No other work has been published for semen analysis techniques validation for golden-headed lion tamarin.

## MATERIAL AND METHODS

The sperm-binding assay in perivitelline membrane of chicken eggs was performed according to the technique described in bull semen (LOSANO et al.,

2015). For the evaluation on plasma membrane integrity, it was used the eosin-nigrosin staining (VALLE et al., 2008). Acrosomal integrity was assessed using both fast-green/rose-bengal staining (POPE et al., 1991; VALLE et al., 2008), and coomassie blue staining (LARSON; MILLER, 1999). Mitochondrial activity was analysed using 3.3' diaminobenzidine (DAB) staining (HRUDKA, 1987).

Fresh semen of 6 different subjects (n=6) was used to perform the validations of sperm-binding assay and all stainings, except for coomassie blue staining, validated for thawed semen samples from 7 different subjects (n=10).

#### *Animals and semen collection*

All procedures were approved by the Brazilian environmental authorities (Ministério do Meio Ambiente, Instituto Chico Mendes de Conservação da Biodiversidade, Sistema de Autorização e Informação em Biodiversidade – MMA/ICMBio/SISBIO), nº 50859-2, and by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (CEUA/FMVZ), University of São Paulo, nº 1170130317.

Seven mature male golden-headed lion tamarins housed at São Paulo Zoological Park Foundation were used in this study. Semen samples were collected by penile vibrostimulation technique, with a protocol described for *Callithrix jacchus* (VALLE et al., 2008). Stimulation phases lasted 2 min, followed by resting phases of 30 s. Initial stimulation intensity was 75 Hz and 1 mm amplitude for 2 min. The intensity was increased after resting phases if the animal did not show response to the stimulus (erection or pelvic movements) during the previous phase, and was never higher than 85 Hz. Semen was collected into a dry hand made glass tube, maintained at 37°C until the moment of the ejaculation.

#### *Semen processing*

Right after semen collection, samples were diluted into 200 µL of BWW (Biggers-Whitten-Whittingham) medium at 37°C, and were maintained incubated in a microtube at this temperature for 30 min, to allow partial coagulum dissolution. After this period, remaining coagulum was removed from liquid fraction, which was the fraction used for the analyses.

For both fresh and thawed semen, samples were divided into two aliquots: one kept at 37°C (“fresh semen”) and another submitted to snap-freezing (immersed in liquid nitrogen and thawed in a water bath at 37°C for five times) to induce cell injury (“injured cells”). These aliquots were mixed in proportions of 0% (100% injured cells), 25%, 50%, 75% and 100% (100% fresh semen) (injured cells/ fresh semen ratio). From each of these proportions, sperm-binding assay and all stainings were performed (LOSANO et al., 2015).

### *Sperm-binding assay*

For the sperm-binding assay, samples were diluted to a final concentration of  $5 \times 10^6$  spermatozoa per mL using a modified Tyrode’s albumin lactate pyruvate (TALP) medium at 37°C (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO<sub>3</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 21.6 mM lactate, 2 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 10 mM HEPES, 1 mM pyruvate, 6 mg ml<sup>-1</sup> bovine serum albumin and 50 µg mL<sup>-1</sup> gentamycin (PARRISH et al., 1988)), before division into two aliquots to prepare the proportions.

Egg perivitelline membranes of chicken eggs were used in this assay. Membranes were prepared by being isolated from yolks of fresh laid, unfertilized chicken eggs and washed with PBS until the membrane and PBS became translucent, with no visible yolk present. After washing, membranes were transferred to a glass Petri dish containing PBS, where they were gently spread to a single layer and then cut into segments of 0.5 cm<sup>2</sup> with a scalpel blade. Each segment was placed in a plate containing wells and incubated with 25000 spermatozoa diluted in 250 µL of modified TALP medium, at 37°C for 1 hour (LOSANO et al., 2015).

After this period, membranes were washed in 10 mL of PBS at room temperature to remove any unbound cell and then transferred onto a glass slide, gently spread to remove any folds and covered with a cover slip. The number of spermatozoa bound to the membrane was evaluated in five fields, observed in a phase contrast microscope (Nikon® E200, Tokyo, Japan), in a 400x magnification. Results were expressed in number of spermatozoa bound per mm<sup>2</sup> of membrane.

### *Plasma membrane integrity*

Plasma membrane integrity was analysed using eosin-nigrosin staining (BARTH; OKO, 1989; VALLE et al., 2008). In the protocol used, 5 µL of semen were

placed in a 0.5 mL microcentrifuge tube, and mixed with 5  $\mu$ L of eosin-nigrosin stain. After 30 s, the smear was immediately prepared, in a pre-warmed (37°C) glass slide. Two hundred cells were evaluated, and classified as presenting intact or damaged membranes, in a simple microscope (ZEISS® Primo Star, Jena, Germany), in a 1000x magnification, under oil immersion.

#### *Acrosome integrity*

Acrosome integrity was investigated using fast-green/rose-bengal staining (POPE et al., 1991; VALLE et al., 2008) for fresh semen, and by coomassie blue staining (LARSON; MILLER, 1999) for thawed samples. Fast-green/rose-bengal staining was performed by mixing 5  $\mu$ L of semen with 5  $\mu$ L of stain in a 0.5 mL microcentrifuge tube. The solution was incubated for 90 s, and the smear was prepared on a pre-warmed (37°C) glass slide. The slide was evaluated in a light microscope (ZEISS® Primo Star, Jena, Germany), in a 1000x magnification, under oil immersion. Two hundred cells were counted and classified as intact or injured acrosomes.

The protocol for coomassie blue staining was slightly modified, as follows: 5  $\mu$ L of semen were fixed in 100  $\mu$ L of 4% paraformaldehyde solution. Sperm were centrifuged and washed at 2000 G for 8 minutes. After that, 50  $\mu$ L of 100 mM ammonium acetate was added to the pellet, and sample was washed once at 800 G, for 5 minutes. Pellet was resuspended with 10  $\mu$ L of 100 mM ammonium acetate, and a smear was prepared and air-dried. After that, slide was flooded with coomassie blue stain (0.22% Coomassie Blue G-250 in 50% methanol, 10% glacial acetic acid and 40% Milli-Q water) for 2 minutes (LARSON; MILLER, 1999). The slide was washed using distilled water to remove excess stain, and air-dried. Two hundred cells were evaluated in a simple microscope (ZEISS® Primo Star, Jena, Germany), in a 1000x magnification, under oil immersion, and classified as intact or injured acrosomes.

#### *Mitochondrial activity*

Mitochondrial activity was evaluated by 3,3' diaminobenzidine (DAB) staining (HRUDKA, 1987). The protocol with slight modifications used was: 10  $\mu$ L of diluted semen was incubated with 10  $\mu$ L of DAB in an amber microcentrifuge tube at 37 °C

for 1 hour. After this period, the solution was smeared in a pre-warmed (37°C) glass slide, which were air dried in a dark room, at room temperature. Slides were evaluated under a phase contrast microscope (Nikon® E200, Tokyo, Japan), in a 1000x magnification, under oil immersion. Two hundred cells were classified in four classes: Class I – almost 100% of the mid-piece stained, indicating full mitochondrial activity; Class II – more than 50% of the mid-piece stained, indicating medium mitochondrial activity; Class III – less than 50% of the mid-piece stained, indicating low mitochondrial activity; and Class IV – lack of staining in the mid-piece, which indicates no mitochondrial activity.

### *Statistical analysis*

All data was analyzed using SAS System for Windows (SAS Institute Inc., Cary, NC, USA), whereas Guided Data Analysis was used to performed linear regressions of proportion of alive/dead cells (0%, 25%, 50%, 75% and 100%). Linear least square regression was used to determine relationships between expected and measured of the integrity of sperm structures (acrosomal and plasma membranes, mitochondrial activity and sperm binding). A probability value of  $P < 0.05$  was considered statistically significant. Results are reported as untransformed means  $\pm$  SEM.

## RESULTS

The results of each proportion (0%, 25%, 50%, 75%, and 100%) of every method described in this study are presented in Table 1.

Table 1. Values (mean  $\pm$  SE) of proportions of alive/dead sperm and the variables number of spermatozoa bound to egg perivitelline membrane, plasma membrane integrity, acrosome integrity evaluated by fast-green/rose-bengal staining and by coomassie blue staining and mitochondrial activity class I.

<b>Ratio (injured cells/ fresh semen)</b>	<b>0%</b>	<b>25%</b>	<b>50%</b>	<b>75%</b>	<b>100%</b>
<sup>1</sup> Spermatozoa per mm <sup>2</sup>	2.24 $\pm$ 0.59	15.48 $\pm$ 2.80	38.30 $\pm$ 3.03	55.26 $\pm$ 7.57	-
<sup>1</sup> Intact membrane (%)	6.33 $\pm$ 3.63	30 $\pm$ 2.64	55 $\pm$ 2.11	72.2 $\pm$ 1.77	91.16 $\pm$ 1.30
<sup>1</sup> Intact acrosome (%) – fast-green/rose-bengal staining	9.83 $\pm$ 3.38	32.5 $\pm$ 2.85	52.66 $\pm$ 2.78	69.6 $\pm$ 0.98	81.66 $\pm$ 1.82
<sup>2*</sup> Intact acrosome (%) – coomassie blue staining	2.2 $\pm$ 0.60	9.2 $\pm$ 1.30	16.1 $\pm$ 1.69	22.9 $\pm$ 2.46	29.9 $\pm$ 3.12
<sup>1</sup> High sperm mitochondrial activity – Class I (%)	10.16 $\pm$ 1.89	25 $\pm$ 2.35	44.33 $\pm$ 3.02	64.6 $\pm$ 4.59	77.66 $\pm$ 2.98

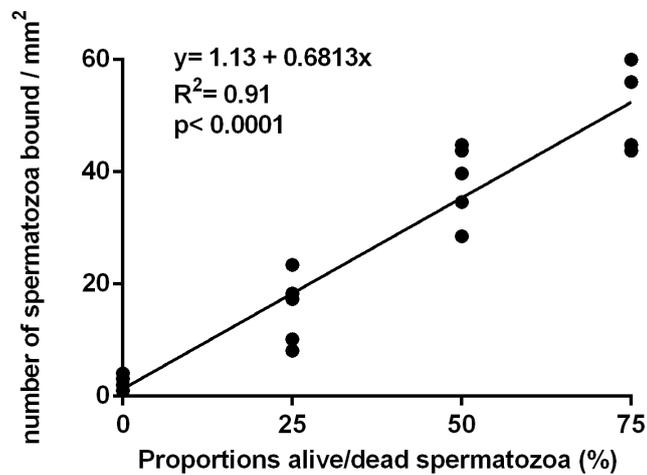
<sup>1</sup>n=6; <sup>2</sup>n=10

\* Analysis with thawed semen. All other analyzes were performed using fresh semen

### *Sperm-binding assay*

Analyses performed in samples from 6 different golden-headed lion tamarin showed that the number of spermatozoa bound per mm<sup>2</sup> of egg perivitelline membrane of chicken eggs increased according to raises in the proportion of alive/dead spermatozoa (Figure 1). Results of the linear regression analysis indicated a consistent relationship between these two parameters ( $R^2 = 0.91$ ,  $p < 0.0001$ ).

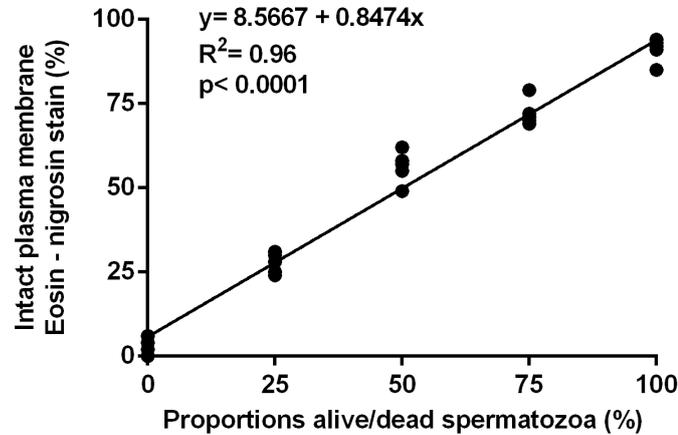
Figure 1 – Microscope analysis of the number of spermatozoa bound per mm<sup>2</sup> of egg perivitelline membrane (n=6) for four alive/dead sperm ratios (0%, 25%, 50%, 75%), with linear regression from data shown.



### *Plasma membrane integrity*

Eosin-nigrosin staining segregates spermatozoa presenting intact and non-intact plasma membrane. Spermatozoa with non-intact plasma membrane stained pink. Contrarily, cells with intact plasma membrane did not stain, and turned white. The percentage of cells presenting intact plasma membrane increased as the proportions of alive/dead spermatozoa increased (Figure 2). Linear regression analysis values were  $R^2 = 0.96$ ,  $p < 0.0001$ .

Figure 2 – Percentage of intact plasma membrane spermatozoa (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.



### *Acrosome integrity*

In the evaluation of acrosome integrity by fast-green/rose-bengal staining, spermatozoa with intact acrosome showed the acrosomal region stained violet, and the post-acrosomal region stained lighter, in blue. Cells with non-intact acrosomes presented the whole head stained in blue. Coomassie blue staining revealed acrosome intact spermatozoa stained dark blue in acrosomal region and spermatozoa with non-intact acrosome reacted showed a patchy staining pattern or no staining in the acrosomal region.

Herein are described results from fast-green/rose-bengal of fresh semen from 6 different golden-headed lion tamarin and coomassie blue staining from 10 frozen-thawed semen samples from 7 different golden-headed lion tamarin (Figures 3 and 4, respectively). For both staining methods, acrosome integrities followed rises in the proportion of alive/dead spermatozoa. Linear regression analysis values were  $R^2 = 0.95$ ,  $p < 0.0001$ , for fast-green/rose-bengal staining and  $R^2 = 0.89$ ,  $p < 0.0001$ , for coomassie blue staining.

Figure 3 – Percentage of intact acrosome spermatozoa using fast green/rose-bengal staining (n=6), for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.

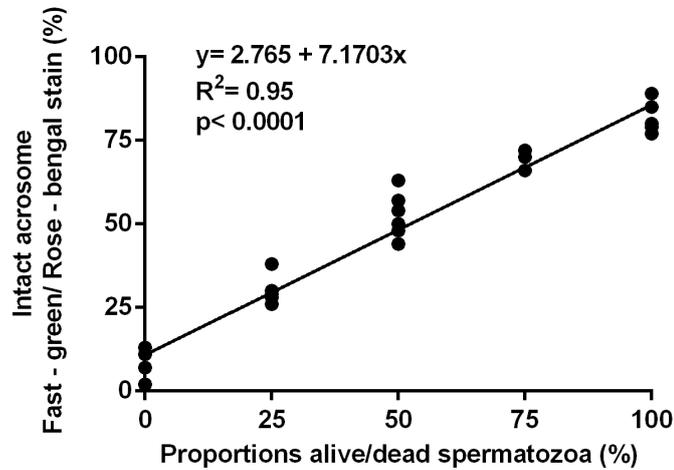
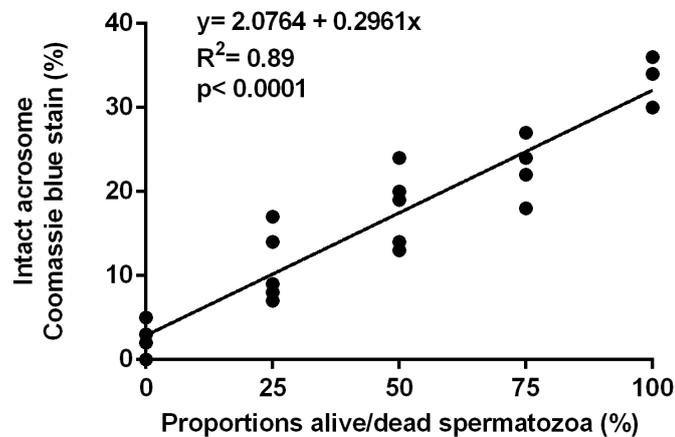


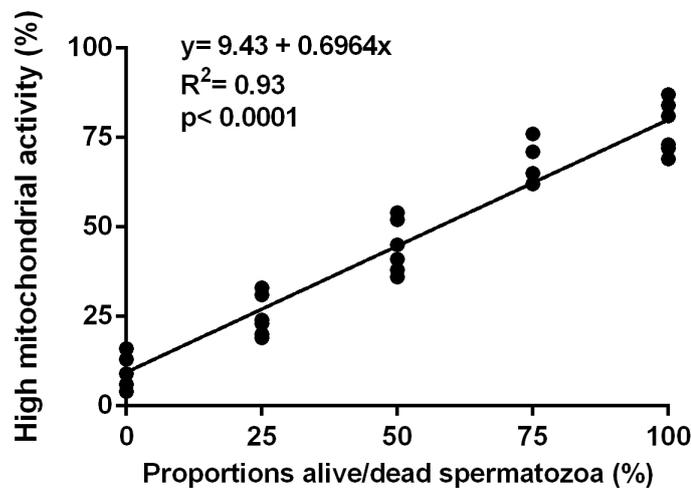
Figure 4 – Percentage of intact acrosome spermatozoa using coomassie blue staining (n=10), for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.



### *Mitochondrial activity*

The percentage of spermatozoa from semen of 6 different golden-headed lion tamarin that presented high mitochondrial activity (Class I) increased according to raises in alive/dead ratio spermatozoa (Figure 5), and the opposite occurred with spermatozoa presenting medium, low and absence of mitochondrial activity (Classes II, III and IV, respectively). Values of the linear regression analysis for Class I spermatozoa were  $R^2 = 0.93$ ,  $p < 0.0001$ .

Figure 5 – Percentage of high mitochondrial activity (n=6) for five alive/dead sperm ratios (0%, 25%, 50%, 75%, 100%), with linear regression from data shown.



## DISCUSSION

The main purpose of semen analysis is to verify the fertilizing potential of a given semen sample (MOCÉ; GRAHAM, 2008). Methods to evaluate this fertilizing capacity in an accurate and affordable manner, under field conditions, are of great value in the analysis of semen from nonhuman Primates. However, it is necessary to adjust the protocols when these methods are applied for any new species.

In order to fertilize an oocyte, mammalian spermatozoa attach and bind with the zona pellucida (ZP) proteins, which surround the mammalian egg (PRASAD et al., 2000). In vitro assays may aid the evaluation of the fertilizing potential of a male, and predict the capacity of semen samples to fertilize oocytes (BHATTACHARYYA; KANJILAL, 2003).

Due to the lack of golden-headed lion tamarin oocytes availability, an alternative method to evaluate no more than sperm binding is the sperm-binding test using chicken egg perivitelline membrane, described for rooster, mouse, ram, bull, horse, dog, ocelot – *Leopardus pardalis*, oncilla – *Leopardus tigrinus*, collared peccary – *Pecari tajacu*, agouti – *Dasyprocta leporina*, and human semen (BARBATO et al., 1998; CRISCUOLO et al., 2010; CASTELO et al., 2015; LOSANO et al., 2015; ARAÚJO et al., 2015; BRITO et al., 2017; CAMPOS et al., 2017). This test could be applied in experiments on semen technologies, or even in the selection of males for reproduction programs.

In general, ZP of mammal oocytes is composed of three glycoproteins, called ZP1, ZP2 and ZP3, although it is known that humans and some primates present a fourth glycoprotein (LEFIÈVRE et al., 2004; WASSARMAN et al., 2004; GANGULY et al., 2008). Glycoproteins from chicken egg perivitelline membrane displays homology to the glycoproteins from mammalian ZP (TAKEUCHI et al., 1999; BAUSEK et al., 2000; SMITH et al., 2005), which allows the binding of spermatozoa from numerous mammalian species. Therefore, the fertility potential of a male could be predicted by spermatozoa binding to this membrane, presuming that it would be related to the binding of spermatozoa to oocytes.

The plasma membrane is a very dynamic structure, it is not simply the border of the sperm cell. From spermatogenesis to the moment of fertilization, sperm plasma membrane is induced to several changes, whether during transit through the epididymis, where maturation occurs (mammalian cells), or in the female genital tract, where spermatozoa goes through numerous events, such as capacitation, ZP binding, acrosome reaction and fusion with the oolemma (FLESCH; GADELLA, 2000). During semen analysis, plasma membrane integrity is a basic parameter to be analyzed.

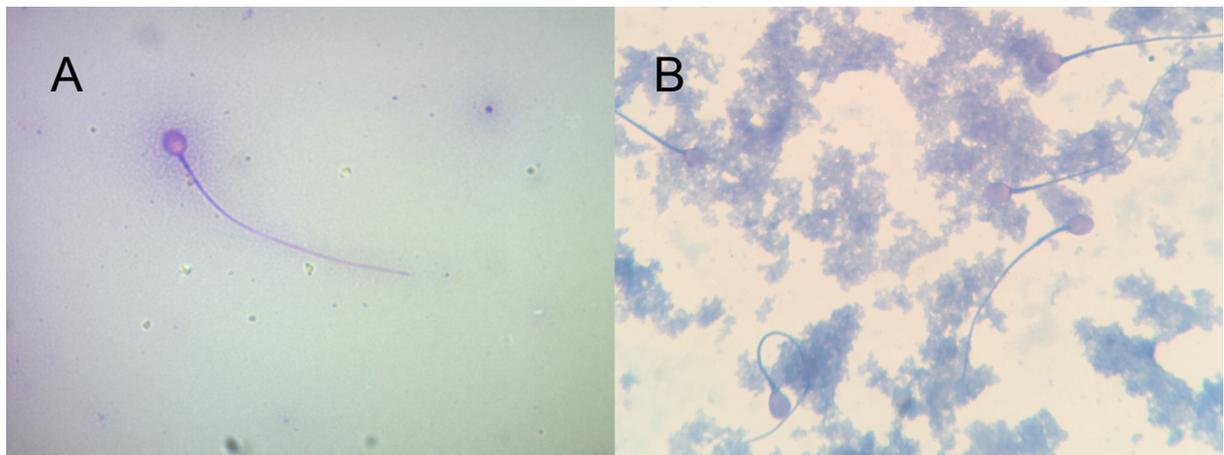
Eosin-nigrosin staining is a method to evaluate plasma membrane integrity widely used in semen from domestic animals. Regarding Neotropical primate species, eosin-nigrosin has been described in black-tufted-ear marmoset – *Callithrix penicillata*, black-and-gold howler monkey – *Alouatta caraya*, squirrel monkey – *Saimiri collinsi*, *S. vanzolinii*, *S. cassiquiarensis*, *S. macrodon*, capuchin monkey – *Cebus apella*, woolly spider monkey – *Brachyteles arachnoides* and Goeldi's monkey – *Callimico goeldii* (VALLE et al., 2014; OLIVEIRA et al., 2011; ARAKAKI, 2013; CARVALHO et al., 2014; ARAKAKI et al., 2015; OLIVEIRA et al., 2016; ARAKAKI et al., 2017), and it has been validated for the common marmoset – *Callithrix jacchus* (VALLE et al., 2008).

The evaluation of acrosome integrity can be performed by several methods, from phase contrast or differential interference contrast (DIC) light microscopy to fluorescent probes (CROSS; MEIZEL, 1989; MOCÉ; GRAHAM, 2008). Among these methods are different stains, which are techniques that can be performed under low technology situations or field conditions.

This study is an extremely important first step of a research project where the primary goal is to cryopreserve semen from golden-headed lion tamarin (results to be

published elsewhere). In this project, all fresh semen samples were analyzed prior to cryopreservation. Evaluation of acrosome integrity was performed with fast-green/rose-bengal staining. However, for frozen-thawed semen from this species, this staining had shown to be inappropriate. Smears were not clear and presented clumps making it difficult to analyze the acrosomes and count the sperm cells (Figure 6). The alternative method chosen was the coomassie blue staining, another consistent and inexpensive technique to evaluate acrosomal status. It is interesting to notice that smears prepared of frozen-thawed semen presented this characteristic only with fast-green/rose-bengal staining.

Figure 6 – Smear prepared and stained with fast-green/rose-bengal. A) Smear from a fresh semen sample. B) Smear from a frozen-thawed semen sample. The figure shows clumps all over the field.



Photos: Paloma Rocha Arakaki

Evaluation of acrosome integrity using fast-green/rose-bengal staining has been reported in a few Neotropical primate species, as common marmoset – *Callithrix jacchus*, black-tufted-ear marmoset – *Callithrix penicillata*, black-and-gold howler monkey – *Alouatta caraya*, woolly spider monkey – *Brachyteles arachnoides* and Goeldi's monkey – *Callimico goeldii* (VALLE et al., 2008; 2014; ARAKAKI, 2013; CARVALHO et al., 2014; ARAKAKI et al., 2015; ARAKAKI et al., 2017). In other study from our group, fast-green/rose-bengal staining was successfully used in frozen-thawed semen samples from *C. jacchus* and *C. penicillata* (unpublished results). For these species, another freezing extender was used. Therefore, a possible reason for the unclear smears would be the reaction of this staining solution composition with the components of the frozen mediums used. For a better comprehension in the application of fast-green/rose-bengal staining with frozen-

thawed semen samples from Neotropical primate species, further studies are necessary.

The use of coomassie blue staining has been described for human, bull, rabbit, boar, horse, buffalo, cat, dog, clouded leopard – *Neofelis nebulosa*, iberian lynx – *Lynx pardinus*, bobcat – *Lynx rufus*, cheetah – *Acinonyx jubatus*, llama – *Lama glama* and Baird's tapir – *Tapirus bairdii* semen, and for guinea pig and mouse sperm (LONG et al., 1996; LARSON; MILLER, 1999; BRUM et al., 2006; CROSIER et al., 2006; PUKAZHENTHI et al., 2006; GAÑÁN et al., 2009a; GAÑÁN et al., 2009b; MEHMOOD et al., 2009; CARRETERO et al., 2015).

Mitochondria are organelles generally known to be the cell's source of energy, due to their role in the production of adenosine triphosphate (ATP) through oxidative phosphorylation. Besides this, mitochondria are important sources of reactive oxygen species (ROS), metabolic byproduct in both physiological and pathological conditions (FERRAMOSCA; ZARA, 2017). Therefore, mitochondrial activity appears to be an important feature associated with sperm quality and fertilization potential.

Mitochondrial activity analysis using the 3,3'-diaminobenzidine (DAB) staining has been reported in semen from common marmoset – *Callithrix jacchus*, black-tufted-ear marmoset – *Callithrix penicillata*, black-and-gold howler monkey – *Alouatta caraya*, woolly spider monkey – *Brachyteles arachnoides* and Goeldi's monkey – *Callimico goeldii* (ARAKAKI, 2013; CARVALHO et al., 2014; ARAKAKI et al., 2015; ARAKAKI et al., 2017).

During the validation of the methods described in this study, sperm concentrations were always maintained constant while amounts of viable sperm cells were changed (proportions alive/dead spermatozoa), as an internal control. This approach, reinforced by the significant linear regression analysis results, proved to be adequate for these analyses in golden-headed lion tamarin sperm.

Our findings suggest that, for golden-headed lion tamarin semen, sperm-egg-binding assay and the status of plasma membrane, acrosome and mitochondrial activity can be performed and determined accurately for fresh semen using the methods herein described, and that coomassie blue staining is a suitable method to evaluate acrosome integrity for thawed semen. It is known that major differences occur among species, which reinforces the need to validate every method used for a new species.

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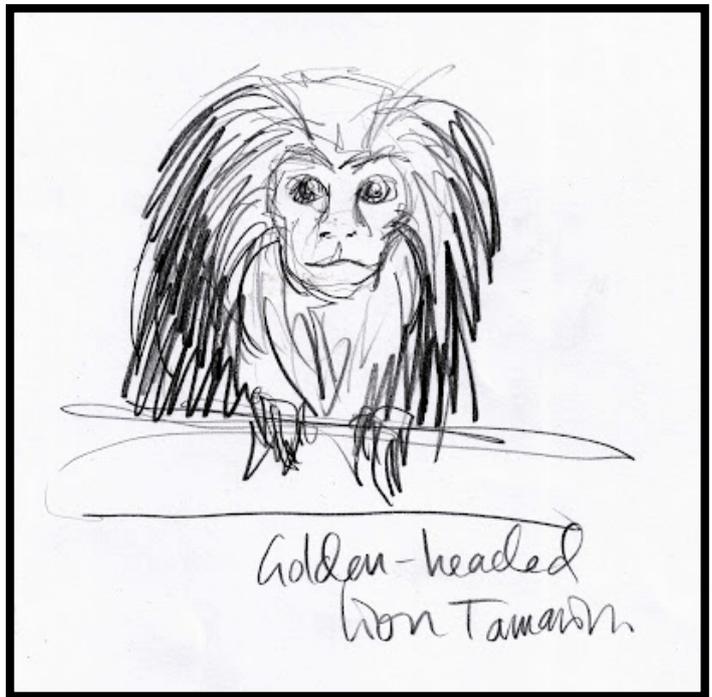
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Rachael Smith

# Chapter 3

#### **4 CHAPTER 3: Collection and analysis of semen in captive golden-headed lion tamarin (*Leontopithecus chrysomelas*) in both dry and rainy seasons**

##### **ABSTRACT**

Knowledge on reproductive biology is crucial for an effective establishment of a breeding program in captivity, including for the development of assisted reproductive techniques in a given species. Golden-headed lion tamarin (*Leontopithecus chrysomelas*) is listed as endangered by the International Union for Conservation of Nature (IUCN), and data on male reproductive characteristics are lacking. We aimed to describe testicular morphometry and seminal evaluation during both dry and rainy seasons. Testicular measures and scrotal circumference were taken prior to each semen collection. Semen was collected by penile vibrostimulation technique from captive males housed at São Paulo Zoological Park Foundation, São Paulo, Brazil, in two different periods of the year: the dry season (July and August), and rainy season (October and November). Semen collection presented a success rate of 100%, and all samples obtained were constituted by only a coagulated fraction. A significant difference was found in total testicular volumes between seasons, with major volumes found during dry season ( $p = 0.0011$ ). Semen was evaluated for pH, volume, concentration, total and progressive motility, plasma membrane and acrosome integrity, and sperm morphology. For these parameters, significant differences between seasons were found for the percentage of total motility and for the percentage of intact plasma membrane sperm ( $p = 0.0149$  and  $p = 0.0279$ , respectively), both presenting higher values during rainy season. In none of the samples semen coagulum liquefied after an incubation period of 30 min with Biggers-Whitten-Whittingham medium. However, we could recover spermatozoa from the coagula in a sufficient number to perform all evaluation described.

**Keywords:** Primate conservation. Neotropical primates. Penile vibrostimulation. Semen coagulum. Sperm morphology.

## INTRODUCTION

Lion tamarins (*Leontopithecus* sp.) are Neotropical primates endemic to the Brazilian Atlantic Forest. There are four species within this genus: black-faced lion tamarin (*Leontopithecus caissara*), golden-headed lion tamarin (*L. chrysomelas*), black lion tamarin (*L. chrysopygus*) and golden lion tamarin (*L. rosalia*); the former is considered critically endangered and the three others are considered endangered by the International Union for Conservation of Nature (IUCN) (KIERULFF et al., 2008a,b,c,d), being the major threats to Neotropical primates habitat loss due to agricultural expansion, followed by livestock farming and ranching (ESTRADA et al., 2017).

Regardless the threats over *Leontopithecus* sp, very few information about male reproductive biology is available. In fact, there is only one study related to semen collection in golden-headed lion tamarin (VIDAL et al., 2007), in which authors have evaluated just semen volume and sperm morphology. It is clear that there is a lack of information regarding male biology of reproduction, in view of the fact that knowledge of semen characteristics and sperm quality in a given species are essential to the development and application of successful assisted reproductive strategies.

Golden-headed lion tamarin presents a highly seasonal reproduction under zoo conditions in Brazil (DE VLEESCHOUWER et al., 2003), however it is not clear if males show low sperm production or worse quality sperm during non-conception periods.

In face of this information, the aim of this study was to provide data on semen characteristics in both dry and rainy seasons, as well as to describe, for the first time, the testicular morphometry in captive golden-headed lion tamarin.

## MATERIAL AND METHODS

### *Animals*

This study was conducted with approval by the Brazilian environmental authorities (Ministério do Meio Ambiente, Instituto Chico Mendes de Conservação da Biodiversidade, Sistema de Autorização e Informação em Biodiversidade – MMA/ICMBio/SISBIO), nº 50859-2, and by the Ethic Committee on Animal Use of the

School of Veterinary Medicine and Animal Science (CEUA/FMVZ), University of São Paulo, nº 1170130317.

Seven mature male golden-headed lion tamarins housed off-exhibit at São Paulo Zoological Park Foundation (23°38'52.6"S 46°37'06.3"W), São Paulo, Brazil were used in this study (Chart 1). Animals received balanced alimentation – in the morning, primate pellets P25 Megazoo® (Megazoo, Betim, MG, Brazil) were offered, and in the afternoon animals were fed with fruits, cooked vegetables and animal protein (cooked meat and insects), according to the day of the week, and water was provided *ad libitum*. Subjects were housed in different group compositions, either with another male, with a female, or with another male and a female. All paired females were under a contraceptive method during the study period.

Chart 1– Description of the seven golden-headed lion tamarins males studied, from São Paulo Zoological Park Foundation, São Paulo, Brazil.

<b>Animal identification</b>	<b>Microchip number</b>	<b>Enclosure</b>	<b>Group composition</b>	<b>Origin</b>	<b>Age (years)</b>
27.690	200001076688	Mic 07	another male and one female	FPZSP	15
29.430	70088312	Mic 16	another male	IBAMA/SP	10
29.559	70025035	Mic 08	another male	FPZSP	10
29.658	70105350	Mic 06	one female	FPZSP	01.25.08
30.164	15880312	Mic 03	one female	FPZSP	7
30.169	16556535	Mic 02	another male	FPZSP	7
30.191	15871781	Mic 16	another male	FPZSP	7

FPZSP = São Paulo Zoological Park Foundation

IBAMA/SP = Brazilian Institute of Environment and Renewable Natural Resources, São Paulo state

Group composition: animals residing the same enclosure of the experimental individual

Origin: individual with IBAMA/SP origin has unknown ancestry, came from the region of municipality of Cotia, São Paulo. Individuals with FPZSP origin were born at the Institution

### *Testicular morphometry and semen collection*

Semen collections were always performed during the mornings, and were scheduled with at least a week of interval between successive collections from the same male. During the procedures, animals were physically restrained by a zoo professional, and animals' genital region were washed with water at 37°C.

Before every semen collection, testicular measures and scrotal circumference were taken, using a pachymeter and a measuring tape, respectively. Testicular measures were used to calculate testicular volume using Lambert's empiric formula: "volume = length x width x height x 0.71" (HSIEH et al., 2009). Total testicular volume was calculated by adding the volumes of right and left testicles.

Semen samples were collected using the penile vibrostimulation technique, with minor modifications of the protocol described for the common marmoset – *Callithrix jacchus* (VALLE et al., 2008). Briefly, stimulation phases lasted 2 min, followed by resting phases of 30 s. Initial stimulation intensity was 75 Hz and 1 mm amplitude for 2 min. The intensity was increased after resting phases if the animal did not show response to the stimulus (erection and/or pelvic movements) during the previous phase, and was never higher than 85 Hz. Semen was collected into a dry hand made glass tube, maintained at 37°C until the moment of the ejaculation.

In this study, semen collections were performed in two different periods of the year, defined as dry season (July and August) and rainy season (October and November).

#### *Semen processing and analysis*

Right after semen collection, pH was evaluated using a pH strip (Merck®, Darmstadt, Germany), placed directly in the tip of the penis. Samples were immediately diluted into 200 µL of BWB (Biggers-Whitten-Whittingham) medium at 37°C. Semen volume was measured by weighing the tube containing the diluted semen using a precision scale (Shimadzu®, AUX220, Tokyo, Japan), and then the tube's weight and the amount of extender used were subtracted, assuming that sperm density in golden-headed lion tamarin is supposed to be equal to that of humans – 1 g/mL, according to WHO (2010).

Samples were incubated in a microtube at 37°C for 30 min, to allow spermatozoa to be released from the seminal coagulum. After this period, remaining coagulum was removed from liquid fraction (basically the extender with sperm cells), which was the fraction used for the analyses. Samples were kept at 37°C during all the period of semen evaluation.

The first characteristic to be evaluated was the percentage of motile sperm and forward progressive sperm motility. In order to perform this, 10 µL of diluted

sample was placed between a slide and coverslip (wet mount), and observed in a simple microscope (ZEISS® Primo Star, Jena, Germany), in a 400 x magnification. In sperm concentration evaluation, 10  $\mu$ L of diluted semen was added to 90  $\mu$ L of formol saline solution (1:10), and sperm concentration was obtained using an improved Neubauer hemocytometer.

The percentage of sperm presenting plasma membrane integrity was determined with eosin-nigrosin staining, validated for this species (results published elsewhere). Acrosome integrity was investigated using fast-green/rose-bengal staining, also validated for golden-headed lion tamarin (results to be published). In both analyses, two hundred sperm cells were evaluated in a simple microscope at a 1000 x magnification, under oil immersion.

Morphological assessment of spermatozoa was performed by the evaluation of sperm cells, through wet mount. For this, 5  $\mu$ L of semen diluted in formol saline were used. Two hundred spermatozoa were observed in a phase contrast microscope (Nikon® E200, Tokyo, Japan), in a 1000x magnification. Sperm defects were classified as either minor or major defects (BLOM, 1973), and their sum is presented as total defects.

### *Statistics*

Data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). In order to evaluate the effect of different seasons (dry x rainy) on spermatic quality, data were tested for normality of residues (normal distribution) and homogeneity of variances using the Guided Data Analyzes application. Thus, Student's T test was used for parametric data as well as the Wilcoxon test was used for non-parametric data. For the results description, untransformed means and their respective standard errors were expressed. The level of significance used to reject H<sub>0</sub> (null hypothesis) was 5%, that is, for a level of significance lower than 0.05 it was considered that statistical differences occurred between the different groups.

## RESULTS

Males evaluated presented testes with normal consistency and mobility during the period of the study. Testicular morphometry showed differences for the volume of

both right and left testes and for scrotal circumference comparing dry and rainy seasons (Table 1), presenting higher values in dry season.

Table 1. Mean and standard error ( $\bar{x} \pm SE$ ) for the testicular measurements from golden-headed lion tamarin (*Leontopithecus chrysomelas*) and the probability values (P) in the dry and rainy season.

		Dry season	Rainy season	P
Right testicle	Length (mm)	11.17 $\pm$ 0.10	11.15 $\pm$ 0.23	0.9396
	Width (mm)	8.46 $\pm$ 0.13	7.61 $\pm$ 0.26	<b>0.0021*</b>
	Height (mm)	8.25 $\pm$ 0.15	7.19 $\pm$ 0.18	<b>&lt;.0001*</b>
	Volume (mm <sup>3</sup> )	557.45 $\pm$ 19.31	438.94 $\pm$ 29.91	<b>0.0015*</b>
Left testicle	Length (mm)	10.93 $\pm$ 0.15	11.11 $\pm$ 0.28	0.5448
	Width (mm)	8.65 $\pm$ 0.16	7.58 $\pm$ 0.19	<b>0.0003*</b>
	Height (mm)	8.19 $\pm$ 0.14	7.27 $\pm$ 0.26	<b>0.0012*</b>
	Volume (mm <sup>3</sup> )	553.21 $\pm$ 18.99	441.08 $\pm$ 31.57	<b>0.0027*</b>
Total volume (mm <sup>3</sup> )		1110.67 $\pm$ 36.10	880.02 $\pm$ 57.24	<b>0.0011*</b>
Scrotal circumference (cm)		4.60 $\pm$ 0.04	4.29 $\pm$ 0.03	<b>&lt;.0001*</b>

\*Significant differences (P<0.05)

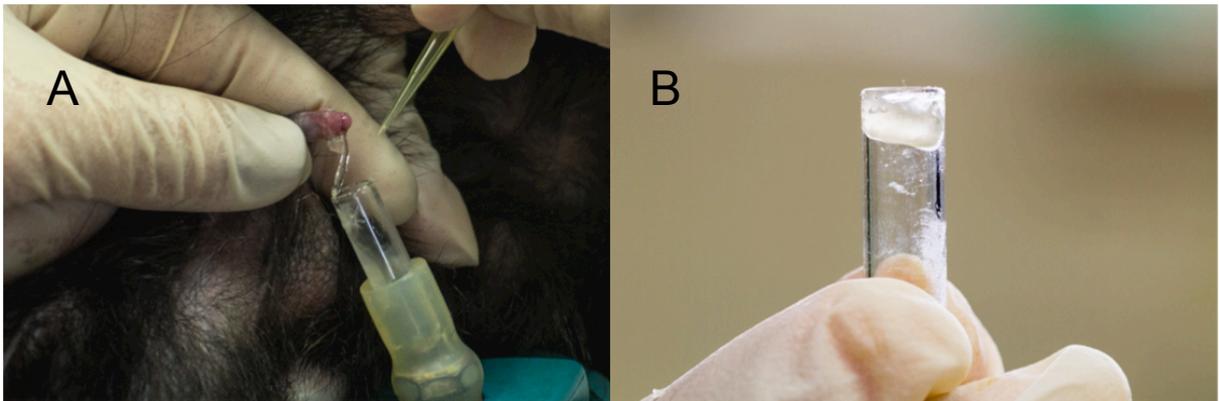
Semen collection was successful in all 39 attempts – 27 performed during dry season and 12 during rainy season. Collection sessions (from cleaning the genital region to the procedures of testicular morphometry and semen collection) lasted, on average, 20 min. Regardless season, all samples obtained were constituted by only a coagulated fraction, with no liquid fraction at the moment of ejaculation. Seminal coagula had filamentous or amorphous presentation (Figure 1), were transparent, whitish or yellowish, and formed a gelatinous mass inside the collection tube or on the tip of the penis.

In none of the samples semen coagulum liquefied after incubation. Therefore, an unknown and variable number of spermatozoa were still trapped in the coagulum. For that reason, it was not possible to make a precise evaluation of sperm

concentration in the total ejaculate/total coagulum. The concentration shown refers to the concentration of free-swimming sperm in the extender.

Total sperm motility and percentage of spermatozoa presenting intact plasma membrane differed significantly among seasons (Table 2), with higher values achieved during rainy season.

Figure 1. Filamentous (A) and amorphous (B) seminal coagula from golden-headed lion tamarin, collected by penile vibrostimulation technique.



Photos: Paulo Gil/FPZSP

Regarding sperm morphology, Table 3 presents the percentages of spermatozoa with normal morphology, and the percentages of each major and minor defects found during both seasons. The most common sperm morphologic change observed was strongly coiled or folded tail. There was no significant difference between seasons in the percentage of total defects and thus, total normal sperm.

Table 2. Mean, standard error ( $\bar{x} \pm SE$ ) and range for animal weight, characteristics of the stimuli and sperm analyses from golden-headed lion tamarin, and the probability values (P) in the dry and rainy season.

	Dry season	Range	Rainy season	Range	P
Animal weight (g)	688.40 $\pm$ 20.68	605 - 785	693.61 $\pm$ 10.58	654 - 753	0.8125
Duration of the stimuli (minutes)	4.61 $\pm$ 0.80	1 - 15	3.92 $\pm$ 1.26	1 - 16	0.4131
Frequency of the stimuli (Hz)	76.15 $\pm$ 0.50	75 - 85	75.77 $\pm$ 0.52	75 - 80	0.6415
Amplitude of the stimuli (mm)	1.00 $\pm$ 0	1	1.00 $\pm$ 0	1	-
pH	7.40 $\pm$ 0.05	6.8 - 7.9	7.44 $\pm$ 0.07	7.2 - 8.0	0.5653
Volume ( $\mu$ L)	90.73 $\pm$ 5.56	41 - 133	85.08 $\pm$ 8.62	44 - 135	0.5729
Concentration ( $\times 10^6$ spitz/mL)	205.72 $\pm$ 19.43	35.80 - 420.07	251.71 $\pm$ 24.32	133.35 - 392.90	0.1648
Total motility (%)	90.96 $\pm$ 0.73	85 - 95	93.85 $\pm$ 0.61	90 - 95	<b>0.0149*</b>
Forward progressive motility (%)	78.08 $\pm$ 1.04	70 - 85	75.00 $\pm$ 3.05	45 - 85	0.3887
Intact plasma membrane (%)	92.46 $\pm$ 0.81	85 - 99	95.54 $\pm$ 0.97	86 - 99	<b>0.0279*</b>
Intact acrosome (%)	96.30 $\pm$ 0.54	90 - 99	97.31 $\pm$ 0.51	93 - 99	0.2455

Spitz/mL = spermatozoa/mL.

\* Significant differences (P<0.05)

Table 3. Mean and standard error ( $\bar{x} \pm SE$ ) for sperm morphology (classified into major and minor defects) from golden-headed lion tamarin samples, and the probability values (P) in the dry and rainy season.

<b>Morphological sperm classification (%)</b>	<b>Dry season</b>	<b>Rainy season</b>	<b>P</b>
Acrosome defect	0.02 ± 0.02	0.11 ± 0.06	0.0697
Free pathological head	0.06 ± 0.03	0.31 ± 0.16	0.1183
Narrow at base head	0.04 ± 0.03	0.19 ± 0.11	0.1582
Pear-shaped head	0.36 ± 0.20	0.23 ± 0.09	0.6603
Small abnormal head	0.13 ± 0.06	0.08 ± 0.05	0.7544
Abnormal contour head	0.10 ± 0.05	0.19 ± 0.07	0.1431
Coiled tail around head	0.10 ± 0.05	0.08 ± 0.05	0.9810
Strongly coiled or folded tail	11.40 ± 0.80	11.54 ± 1.34	0.9276
Bent tail with droplet	1.31 ± 0.17	1.92 ± 0.35	0.1460
Midpiece defects	0.67 ± 0.17	0.81 ± 0.19	0.3534
Proximal droplet	13.02 ± 1,15	6.54 ± 1.40	<b>0.0006*</b>
Teratological forms	0.02 ± 0.02	0	0.5139
Double forms	0.36 ± 0.09	0.27 ± 0.12	0.5594
<b>Total major defects</b>	<b>27.60 ± 1.48</b>	<b>22.27 ± 2.15</b>	<b>0.0470*</b>
Narrow head	0.04 ± 0.03	0.11 ± 0.06	0.1897
Giant head	0.63 ± 0.17	0.85 ± 0.23	0.2193
Short head	0.13 ± 0.06	0.15 ± 0.06	0.5428
Small normal head	0.75 ± 0.35	0.11 ± 0.06	0.0682
Broad head	0	0.08 ± 0.08	0.1739
Free normal head	4.70 ± 0.74	3.31 ± 0.41	0.5009
Simple bent tail	4.19 ± 0.65	12.70 ± 1.11	<b>&lt;.0001*</b>
Coiled tail	7.94 ± 1.05	9.69 ± 1.56	0.4070
Retro implantation	0.25 ± 0.08	1.31 ± 0.32	<b>0.0007*</b>
Abaxial implantation	1.04 ± 0.26	1.96 ± 0.35	<b>0.0155*</b>
Oblique implantation	0.17 ± 0.05	0.15 ± 0.09	0.7047
Distal droplet	0	0.04 ± 0.04	0.1739
Free normal head + midpiece	0.58 ± 0.11	0.42 ± 0.11	0.5309
<b>Total minor defects</b>	<b>20.42 ± 1.32</b>	<b>30.88 ± 2.26</b>	<b>0.0001*</b>
<b>Total defects</b>	<b>48.00 ± 2.21</b>	<b>53.15 ± 2.92</b>	0.1791
<b>Total normal sperm</b>	<b>52.00 ± 2.20</b>	<b>46.85 ± 2.92</b>	0.1751

\* Significant differences (P<0.05)

## DISCUSSION

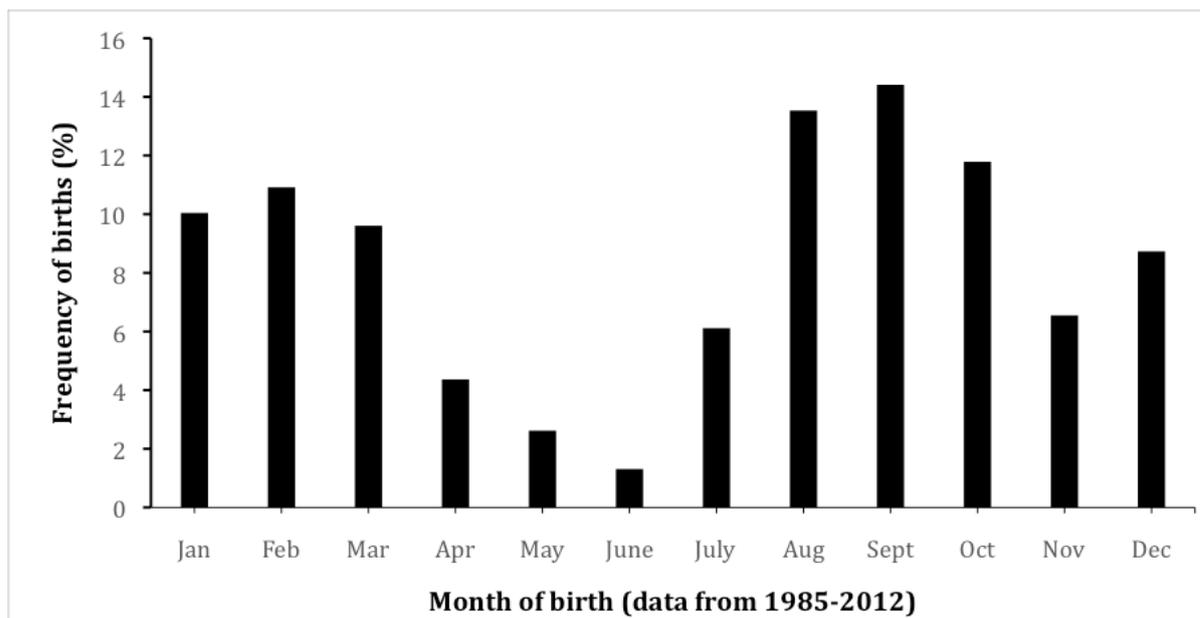
Little information about semen characteristics of golden-headed lion tamarin are related in literature. This is the first report regarding testicular volume and seminal quality for different seasons in this species.

It is known for this species that reproduction in the Brazilian captive population is highly seasonal. De Vleeschouwer et al. (2003), using studbook information on litters born in captivity during the period of 1984-2000, provided an analysis of the reproductive potential of golden-headed lion tamarin under captive conditions. In their study, populations from Rio de Janeiro Primatology Center, Rio de Janeiro Zoo and also São Paulo Zoological Park Foundation (FPZSP) were analyzed. They observed that a distinct birth peak appears in September-October. Given that the mean duration of gestation in this species is 125 days (DE VLEESCHOUWER et al., 2000), there is a concentration of conceptions in the months of May and June.

Another study of French et al. (1996) also reported a birth peak in September and October for golden-headed lion tamarin, golden tamarin (*L. rosalia*) and black-lion tamarin (*L. chrysopygus*) housed at Rio de Janeiro Primatology Center, from 1974 to 1993.

We analyzed golden-headed lion tamarin birth dates in FPZSP from 1985 to 2012 (the last year a birth was recorded at the institution). That data revealed a bimodal distribution of births, with a major peak in August, September and October and a secondary peak in January, February and March (Figure 1, unpublished results). This pattern is very similar to that described for black-lion tamarin, which presents a major birth peak in October and a secondary peak in January and February (FRENCH et al., 1996). Considering the duration of gestation, the concentration of conceptions at FPZSP occurs in two periods of the year, the first one during April, May and June and the second during September, October and November.

Graph 1. Concentrations of births of golden-headed lion tamarin over the months at São Paulo Zoological Park Foundation, from 1985 to 2012.



Based on such data, we can infer that within FPZSP golden-headed lion tamarin population, semen of good quality is produced, at least, twice a year – in the periods when conceptions occurred. Therefore, in this study, semen collections and testicular measurements were performed in two different periods. One in July and August (dry season) during the interval months of the conceptions, and another in October and November (rainy season), when occurred the second concentration of conceptions in the year.

Testicular measures were taken prior to each semen collection, in both seasons. In several adult mammals, testicular volume has been positively related to sperm production rates (MOLLER, 1989). A study with captive capuchin monkey (*Cebus apella*) in Brazil showed that total testicular volume was higher during rainy season, the period when the highest number of copulations occurred (GUIMARÃES et al., 2003). Since this occurs five months before the period of births peak – approximately the gestation period for this species, these authors concluded that pregnancy was achieved during the period of major testicular volumes.

In contrast, we found major testicular volumes in dry season, during the estimated interval months of the conceptions at FPZSP. Major testicular volumes prior to the second concentration of conceptions indicate that males enter this period with higher testicular volume compared to the volumes found in the middle to the end of the second concentration of conceptions (rainy season).

Golden-headed lion tamarins from FPZSP present more sperm-producing tissue, i. e. major testicular volumes, in dry season. This may be due to a preparation for the upcoming conceptions season, when a great number of spermatozoa must be ready to be released in a short period of copulations (HARCOURT et al., 1995). Another possibility is that in the end of the copulations period during rainy season, testicular volumes are supposed to be smaller, since it is known that frequent copulations reduce sperm output (HARCOURT, 1991). Further measurements of testicular parameters year-long should be taken to fully explain this testicular volume variation in this species.

Penile vibrostimulation showed to be an excellent semen collection technique in golden-headed lion tamarin, since it presented a success rate of 100%. This may be due to previously conditioning of the animals, which were familiar with handling and collection procedures. Besides the advantage of not requiring the use of anesthetics, comparisons between rectal electrostimulation and penile vibrostimulation performed in common marmosets (*Callithix jacchus*) and squirrel monkeys (*Saimiri boliviensis*) revealed better semen quality (semen volume, sperm concentration and motile sperm) for both species, when the latter technique was applied (YEOMAN et al., 1998; SCHNEIDERS et al., 2004; ARAKAKI et al., 2017).

Semen from most primates coagulates during or after ejaculation, including Neotropical primate species (YEOMAN et al., 1998; DIXSON; ANDERSON, 2002; PAZ et al., 2006; VIDAL et al., 2007; HERNÁNDEZ-LÓPEZ et al., 2008; ARAÚJO et al., 2009; SILVA et al., 2013; OLIVEIRA et al., 2016; ARAKAKI et al., 2017), one described exception being black howler monkeys – *Alouatta caraya* (MORELAND et al., 2001; VALLE et al., 2004).

It has been proposed that sexual selection has affected several features of males' reproductive anatomy and physiology during primate evolution, including seminal coagulation (DIXSON; ANDERSON, 2002). According to Dixson and Anderson (2002) coagulum formation is more prevalent in those genera where females mate with multiple partners, and sperm competition (when the gametes of two or more males compete to fertilize a given set of ova) pressure is higher.

Golden-headed lion tamarin belongs to the Callitrichidae Family, which has been considered to present a monogamous mating system. Despite this, marmosets, tamarins and lion tamarins exhibit both monogamous and facultative polyandrous mating strategies, showed by studies in the field (DE VLEESCHOUWER et al., 2000;

FRENCH et al., 2017). According to De Vleeschouwer et al. (2000), in wild populations of golden and golden-headed lion tamarins, the dominant male monopolizes copulations during the breeding females' estrus, through dominance hierarchies and mate guarding, which supply evidence for the existence of male-male competition. Such data could explain the presence of the seminal coagulum in all samples from golden-headed lion tamarin.

Given that in none of the samples semen coagulum liquefied after the period of incubation, coagulum was removed from the aggregate liquid fraction and extender in order to avoid absorption of the aggregate and even sperm cells after 30 min of incubation, as occurs in rhesus monkeys – *Macaca mulatta* (VANDEVOORT, 2004).

Despite the differences found between seasons for testicular measurements, no significant difference was found neither for volume nor for sperm concentration comparing dry and rainy seasons. Sperm cells and seminal plasma compose semen, and about 95% of ejaculated semen volume is supplied by seminal vesicles and prostate, in the common marmoset, *Callithrix jacchus* (VALTONEN-ANDRÉ et al., 2007). Thus, an alteration in semen volume related to the major testicular volume found in dry season should not be expected. Regarding sperm concentration, as an unknown number of sperm cell remained trapped in the coagulum even after the addition of the extender and incubation period, and since there were no liquid fractions in the ejaculates, confirmation on real ejaculate concentration cannot be done. Although, we could assess sperm concentration from the aggregate of dissolved sperm cells and extender in all samples, having a reliable concentration measure of the portion that was effectively used in our experiments.

It has been reported that there is a correlation between motility and plasma membrane integrity in human semen (DENIL et al., 1992; AGHA-RAHIMI et al., 2016). These two parameters were the only ones who showed a significant difference, with higher values in rainy season. Nevertheless, rainy season samples were of somewhat better quality than dry season samples. The months of October and November are the period when occurred the second concentration of conceptions in FPZSP, and this may suggest that identifying a period in the year more suitable for semen collection is an interesting approach prior to the establishment of a new project such as a subsequent application in assisted reproductive techniques like artificial insemination.

In the present study, there was no difference in the percentage of sperm presenting normal morphology between both seasons. A study with golden-headed lion tamarin housed at Rio de Janeiro Primatology Center reported a higher mean of normal morphology sperm in collections performed during the months of November and December, with another semen collection technique, the rectal electrostimulation (VIDAL et al., 2007). It has been reported that penile vibrostimulation technique improves semen quality compared to rectal electrostimulation in other Neotropical primate species (YEOMAN et al., 1998; SCHNEIDERS et al., 2004). However, Schneiders et al. (2004) found no difference regarding sperm morphology comparing both collection techniques in common marmoset. The difference between our results and those reported by Vidal et al. (2007) may be due to several variables, like differences in groups composition, subjects management, extender used, among others, but as in common marmoset, not due to semen collection technique. However, an important factor to be considered is that in the study performed at Rio de Janeiro Primatology Center, semen presented a liquid fraction which was the fraction analyzed, which may present sperm with different morphology than those sperm from coagulum.

In conclusion, our results indicate that spontaneous in vitro dissolution of the seminal coagulum does not occur in this species. Semen appears to present better quality during rainy season, as shown by the better motility and percentage of membrane integrity found, although more research is required, including year-long semen collection and analysis. Even without the dissolution of the coagulum, we obtained samples with sufficient number of spermatozoa to perform the evaluation techniques and for latter application in reproductive biotechnologies, including gamete cryopreservation.

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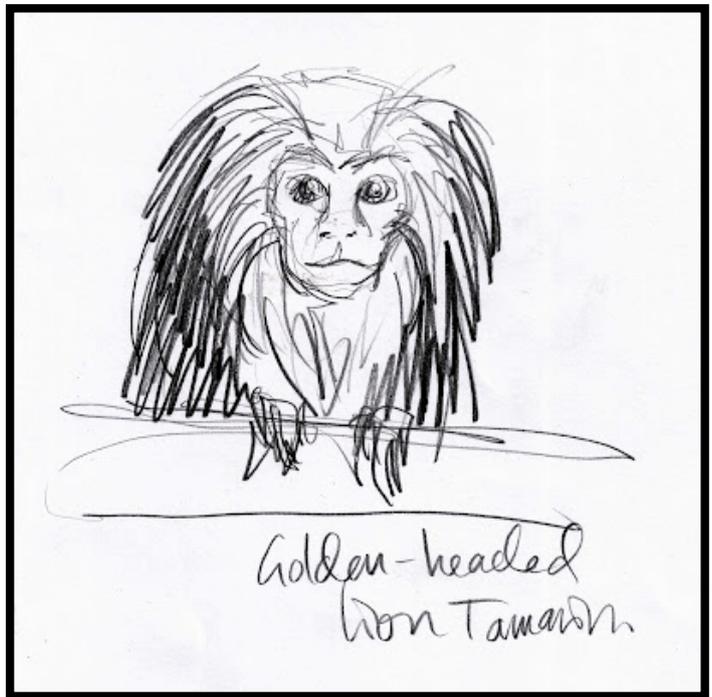
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Rachael Smith

# Chapter 4

## **5 CHAPTER 4: Comparison of two commercial extenders for cryopreservation of sperm from endangered golden-headed lion tamarin (*Leontopithecus chrysomelas*)**

### ABSTRACT

Wild animal genetic resource banking (GRB) – organized collections of biological materials – has an immeasurable value as a tool in conservation reproductive programs. Cryopreserving spermatozoa provides an open range of possibilities especially in endangered species like golden-headed lion tamarin, *Leontopithecus chrysomelas*. Therefore, in the present study, we aimed to establish a sperm cryopreservation protocol comparing BotuBOV® (BB) and Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer® (TYB) extenders, evaluating thawed semen using sperm functional assays. Samples were collected by penile vibrostimulation technique from animals housed at São Paulo Zoological Park Foundation, São Paulo, Brazil. After dilution into Biggers-Whitten-Whittingham medium and initial analysis, remaining samples were equally divided into two aliquots and diluted using BB and TYB. After thawing, samples were evaluated for motile sperm and forward progressive sperm motility, plasma membrane and acrosome integrity, mitochondrial activity, susceptibility to oxidative stress through quantification of induced thiobarbituric acid reactive substances (TBARS), and were additionally submitted to a functional sperm test, the sperm-egg-binding assay. For all seminal parameters assessed, no significant difference was observed between BB and TYB, except for percentage of intact acrosome ( $p = 0.0101$ ) and concentration of TBARS ( $p = 0.0005$ ), both presenting higher values in BB. Despite these differences and regardless of the extended used, it was possible to cryopreserve sperm from the endangered golden-headed lion tamarin, achieving high values for total motility for a Neotropical primate species.

Keywords: Primate conservation. Neotropical primates. Botubov. TYB. Glycerol.

## INTRODUCTION

The creation of genome or genetic resource banks (GRBs), which consists of collecting, storing and using, in an organized way, biological materials like spermatozoa, oocytes, embryos, among others, from subjects of threatened or not species to be used in the future, is among the tools that wildlife institutions can implement to contribute to species conservation (COMIZZOLI et al., 2000; WILDT, 2000; GUAL-SILL; RÉNDON-FRANCO, 2011).

There are several advantages in cryopreserving spermatozoa, such as the transfer of sperm between zoos rather than transporting stress-susceptible males, infusion of new genetic material across populations by artificial insemination, genetic maintenance from genetically valuable males in GRBs, increase of generation interval indefinitely, allowance of maintenance of fewer males in captivity since genetic diversity is maintained frozen, and the possibility to introduce stored sperm into contemporary population immediately or even centuries in the future (PUKAZHENTHI; WILDT, 2004; ANDRABI; MAXWELL, 2007).

Neotropical primates present a huge field to the application of sperm cryopreservation aiming species conservation. The Neotropical region – Mexico, Central and South America, holds 171 primate species, from which 36% are threatened of extinction (ESTRADA et al., 2017). Besides this, regarding the 25 most endangered species of primates in the world, five occur in the Neotropics (SCHWITZER et al., 2015). Hence, the development of sperm cryopreservation methods among these animals is an urgent task.

In fact, cryopreservation of sperm from a little number of Neotropical primate species has been performed: *Saimiri boliviensis*, *S. collinsi*, *S. vanzolinii*, *S. cassiquiarensis*, *S. macrodon*, *Alouatta caraya*, *Callithrix jacchus*, *C. penicillata*, *Ateles paniscus*, *A. marginatus* and *Sapajus apella* (YEOMAN et al., 1997; OLIVEIRA et al., 2011; CARVALHO, 2012,2016; ARAKAKI, 2013; SILVA et al., 2013; LEÃO et al., 2015; OLIVEIRA et al., 2016). Although, one sperm cryopreservation protocol that is likely to be effective in various species is not available, due to extensive protocols and results achieved described.

Egg yolk-based extenders have been widely used in Neotropical primate sperm cryopreservation, as well as glycerol is the cryoprotectant of choice. Some

studies used own production extenders, as other used commercial ones. BotuBOV® (BB) is an extender developed for use in bovine sperm and Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer® (TYB) is designed for human sperm cryopreservation (CAMPANHOLI et al., 2017; PARAS et al., 2008). Both extenders contain egg yolk and glycerol in composition.

*Leontopithecus chrysomelas*, known as golden-headed lion tamarin, is a Neotropical primate species endemic to the Brazilian Atlantic Forest considered endangered by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, due to the decrease of forest remnants where these animals occur in nature (KIERULFF et al., 2008), and the subject of this study. We aimed to compare the use of BB and TYB in this species sperm cryopreservation through evaluation of thawed samples with sperm functional assays, including a sperm-egg-binding assay.

## MATERIAL AND METHODS

### *Animals and semen collection*

The procedures performed in this study were approved by the Brazilian environmental authorities (Ministério do Meio Ambiente, Instituto Chico Mendes de Conservação da Biodiversidade, Sistema de Autorização e Informação em Biodiversidade – MMA/ICMBio/SISBIO), nº 50859-2, and by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (CEUA/FMVZ), University of São Paulo, nº 1170130317.

The males were housed at São Paulo Zoological Park Foundation, São Paulo, Brazil. The experimental group (n= 7 males, with an average of 9 years old) was selected due to their conditioning to the technique of semen collection used.

Semen collections occurred during the mornings. In order to perform the procedures, animals were physically restrained by a zoo professional, and had their genital region washed with water at 37°C. After inspection and palpation of the testicles, semen samples were collected using the penile vibrostimulation technique, with minor modifications of the protocol described for the common marmoset – *Callithrix jacchus* (VALLE et al., 2008). Semen was collected into a dry hand made glass tube, maintained at 37°C until the moment of the ejaculation.

### *Semen handling*

Immediately after collection, semen was diluted into 200  $\mu\text{L}$  of BWW (Biggers-Whitten-Whittingham) medium at 37°C. Semen volume was assessed by weighing the tube containing the diluted semen using a precision scale (Shimadzu®, AUX220, Tokyo, Japan), and then the tube's weight and the amount of extender used were subtracted, assuming that sperm density in golden-headed lion tamarin is supposed to be equal to that of humans – 1 g/mL, according to WHO (2010).

The diluted semen was incubated at 37°C for 30 min, to allow coagulum dissolution. After this period, remaining coagulum was removed from liquid fraction, which was the fraction used for the analyses. Samples were kept at the same temperature during all the period of semen evaluation.

The variables analyzed with fresh semen were motile sperm and forward progressive sperm motility, plasma membrane integrity, acrosome integrity, sperm concentration and sperm morphology (results published elsewhere). After these analyses, remaining samples were prepared for cooling and freezing.

### *Semen cooling and freezing*

Remaining samples were equally divided into two aliquots and processed in parallel using two commercially available extenders, BotuBOV® (BB) (Botupharma Biotecnologia Animal, Botucatu, Brazil) and Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer® (TYB) (Irvine Scientific, Santa Ana, USA).

Based on the sperm concentration values, the volume of BB added to one of the aliquots was calculated to achieve a final concentration of  $25 \times 10^6$  spermatozoa/mL, with the dilution of one part of sample to, at least, three parts of BB (M. NICHI, personal communication, 2016). The aliquots diluted in TYB were previously diluted in BWW to achieve a concentration of  $50 \times 10^6$  spermatozoa/mL, and TYB was added in the proportion of 1:1, in order to obtain a final concentration of  $25 \times 10^6$  spermatozoa/mL and 6% of glycerol. In both cases, extenders were at 37°C and were added dropwise until the final extender amount was achieved.

Samples were aspirated into French straws, which had been previously cut to reach a final capacity of 100  $\mu\text{L}$ . Straws were placed into a refrigerator at 5°C on a horizontal rack inside an acrylic box containing water at 25°C (150 mL), for 2 hours, until the samples reached 5°C. After this period, straws were transferred to nitrogen

vapor (5 cm above the liquid nitrogen column), where they remained for 10 min. Sequentially they were immersed and stored in liquid nitrogen, as a protocol described for *Callithrix jacchus* (VALLE, 2007).

Semen was cryopreserved from six different ejaculates from each male, except for one individual, which died during the period of study (due to liver and kidney problems/advanced age), and had semen from 3 ejaculates cryopreserved.

#### *Semen thawing and post analyses*

Samples remained cryopreserved for at least 6 months and were thawed in immersion in water at 37°C for 15 seconds. Semen was transferred to a microtube and remained at the same temperature during all the period of semen evaluation.

Immediately after thawing, semen was analyzed for the percentage of motile sperm and forward progressive sperm motility, which were assessed subjectively using a simple microscope (ZEISS® Primo Star, Jena, Germany), in a 400 x magnification.

The percentage of sperm presenting plasma membrane integrity, acrosome integrity and mitochondrial activity was determined through eosin-nigrosin staining, coomassie blue staining and 3.3' diaminobenzidine (DAB) staining, respectively. All these techniques were validated for this species (results published elsewhere).

The evaluation of samples' susceptibility to oxidative stress was based on a protocol described by Nichi et al. (2007), in which malondialdehyde, a byproduct of lipid peroxidation, reacts with thiobarbituric acid producing a pink complex, quantified in a spectrophotometer. For quantification of induced thiobarbituric acid reactive substances (TBARS), 30 µL of thawed semen was diluted in 970 µL of PBS and centrifuged (3.350 g, for 10 min). Supernatant was discarded (800 µL) and the remaining 200 µL were incubated with sodium ascorbate (50 µL, 20mM) and ferrous sulfate (50 µL, 4 mM) at 37 °C for 90 min, with the lid of microtube open. Subsequently, 600 µL of 10% trichloroacetic acid at 4°C was added to the solution, and samples were centrifuged at 5 °C (3.350 g, for 10 min) in order to precipitate proteins. Supernatant was recovered (800 µL) and stored at -20°C. TBARS samples were thawed and incubated with 800 µL of 1% thiobarbituric acid at 100°C, for 15 min. After incubation, samples tubes were refrigerated into an ice bath (0°C) to interrupt the chemical reaction. Levels of TBARS were quantified with a

spectrophotometer (Ultrospec® 3300 *pro*, Amersham Biosciences, Buckinghamshire, England) at 532 nm. Lipid peroxidation in semen was expressed as nanograms of TBARS/ $10^6$  sperm.

Frozen-thawed samples were additionally submitted to a functional sperm test, the sperm-egg-binding assay, previously validated for golden-headed lion tamarin (results published elsewhere).

### *Statistics*

Data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). In order to compare the effect of two different extenders (Botubov x TYB) on spermatid quality, data were tested for normality of residues (normal distribution) and homogeneity of variances using the Guided Data Analyzes application. Thus, Student's T test was used for parametric data as well as the Wilcoxon test was used for non-parametric data. For the results description, untransformed means and their respective standard errors were expressed. The level of significance used to reject  $H_0$  (null hypothesis) was 5%, that is, for a level of significance lower than 0.05 it was considered that statistical differences occurred between the different groups.

## RESULTS

A total of 78 semen straws were thawed – 39 for each extender. For all seminal parameters assessed, no significant difference was observed between BB and TYB, except for percentage of intact acrosome ( $p = 0.0101$ ) and concentration of TBARS ( $p = 0.0005$ ), both presenting higher values in BB. Total motility tended to be higher in sperm from BB ( $p = 0.0571$ ), however this difference was not significant (Table 1).

Table 1. Mean, standard error ( $\bar{x} \pm SE$ ) and range for sperm analyses in thawed samples from golden-headed lion tamarin, and the probability values (P) in Botubov and TYB groups.

	<b>Botubov</b>	<b>Range</b>	<b>TYB</b>	<b>Range</b>	<b>P</b>
Total motility (%)	60.00 $\pm$ 2.32	30 - 80	53.08 $\pm$ 2.73	10 - 80	0.0571
Forward progressive motility (%)	49.49 $\pm$ 2.32	20 - 70	43.15 $\pm$ 2.63	8 - 70	0.0751
Intact plasma membrane (%)	85.28 $\pm$ 1.60	47 - 98	84.43 $\pm$ 2.45	9 - 97	0.5090
Intact acrosome (%)	27.33 $\pm$ 1.11	12 - 42	23.13 $\pm$ 1.15	7 - 38	<b>0.0101*</b>
High mitochondrial activity (%)	38.46 $\pm$ 2.31	8 - 68	37.90 $\pm$ 2.23	5 - 61	0.8612
Medium mitochondrial activity (%)	32.54 $\pm$ 1.15	19 - 45	33.82 $\pm$ 1.35	14 - 49	0.4712
Low mitochondrial activity (%)	11.70 $\pm$ 1.14	1 - 33	11.05 $\pm$ 1.09	2 - 41	0.7942
Absence of mitochondrial activity (%)	17.31 $\pm$ 2.57	2 - 65	17.23 $\pm$ 2.52	2 - 63	0.8950
Concentration of TBARS (ng TBARS/10 <sup>6</sup> sperm)	288.06 $\pm$ 28.62	122.01 - 1402.75	190.05 $\pm$ 20.53	66.64 - 678.04	<b>0.0005*</b>
Sperm bound to perivitelline membrane (sperm/mm <sup>2</sup> )	19.29 $\pm$ 2.72	2.04 - 87.60	20.12 $\pm$ 2.52	2.04 - 71.30	0.5732

\* Significant differences (P<0.05)

## DISCUSSION

This is the first report describing sperm cryopreservation in golden-headed lion tamarin (*Leontopithecus chrysomelas*). In this study, the main objective was to test and to establish an effective cryopreservation protocol for this endangered species, by simultaneously assess cryopreservation with BB and TYB. Our results did not show significant difference among extenders for the greater part of the variables evaluated.

Results obtained in thawed semen analyzes for both treatments exhibited extremely high values for Neotropical primates. Comparisons between BB and TYB showed that BB presented a higher percentage of intact acrosomes than TYB. In turn, TYB has been shown to be less susceptible to oxidative stress during cryopreservation. Another characteristic that worth mentioning is the higher percentage of total motility found in BB, which did not present a significant difference, but showed a tendency to, with  $p = 0.0571$ .

The accumulation of cytotoxic products, derived from oxidative stress affect cell membrane permeability, which results in loss of membrane fluidity and integrity (LUZ et al., 2011). Although TBARS concentration found in BB thawed semen were significantly superior, the percentage of intact plasma membrane and the mean of sperm bound to perivitelline membrane was similar in both extenders, demonstrating that the differences we found did not represent functional impairment for any of the diluents, and emphasizes the importance of conducting tests that predict fertilizing capacity.

In a study with another Neotropical primate species, *Alouatta caraya*, semen was cryopreserved with the same extenders from our work (CARVALHO, 2016). This author reported that, in general, samples cryopreserved with TYB showed superior quality. In her study, the concentrations of glycerol used were 4% and 5%. In our study, the concentration used was 6%. Regardless of the extender, our results showed much higher values for the percentages of sperm motility and membrane integrity. These differences found may be due to the medium used in fresh semen, Ringer's lactate for *A. caraya* and BWW in our study, or it may be due to intrinsic differences of the species, since the freezing protocol used was the same in both studies (VALLE, 2007).

In conclusion, it was possible to cryopreserve sperm from golden-headed lion tamarin using either BB or TYB, making it viable to establish a sperm genome resource bank aiming the conservation of an already endangered species. The next steps should be 1) to expand the application and evaluation of the protocols described here to the other species from the genus *Leontopithecus*, which all are classified as either endangered or critically endangered, making this group an important focus of Neotropical primates conservation studies, and 2) to develop assisted reproductive technologies using thawed sperm such as artificial insemination, for these endangered species.

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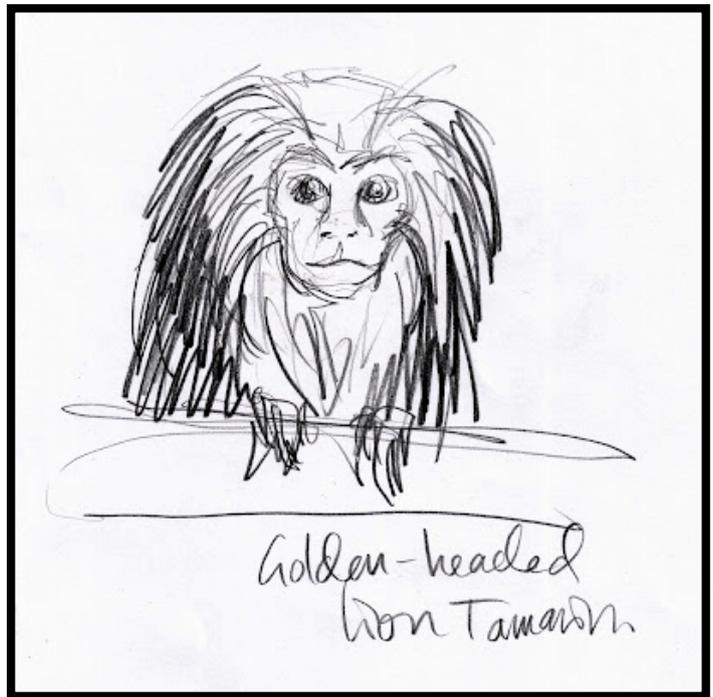
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# General Conclusion

## 6 GENERAL CONCLUSION

Our results showed that spontaneous in vitro dissolution of seminal coagulum from golden-headed lion tamarin (*Leontopithecus chrysomelas*) does not happens. However, even without the complete dissolution of the coagulum, it is possible to obtain samples with sufficient number of spermatozoa to carry out the proposed analyses, and also to cryopreserve it.

Sperm-egg-binding assay and non-fluorescent staining for the evaluation of plasma membrane integrity, acrosome integrity and mitochondrial activity were validated, resulting in accurate results for sperm evaluation in this species.

Semen collections from captive golden-headed lion tamarin can be achieved during both dry and rainy seasons, and despite the fact that in rainy season samples were of better quality, ejaculates from both seasons presented the necessary attributes to the application of cryopreservation.

It is possible to cryopreserve sperm from golden-headed lion tamarin using either BotuBOV® or Freezing Medium with Glycerol and Gentamicin Test Yolk Buffer®, and to obtain quality thawed samples, especially regarding total motility.

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