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SPERM CRYOPRESERVATION THROUGHOUT THE SPAWNING SEASON IN *Prochilodus lineatus*

LAVRAS – MG

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Dissertation presented to Universidade Federal de Lavras, as required by the Programa de Pós-Graduação em Ciências Veterinárias, concentration area on Fisiologia e Metabolismo Animal, to obtain the degree of Master in Veterinay Sciences.

Prof. Dr. Luis David Solis Murgas
Advisor

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Egger, Renata Catão.
Sperm cryopreservation throughout the spawning season in *Prochilodus lineatus* / Renata Catão Egger. - 2019.
57 p. : il.

Orientador(a): Luis David Solis Murgas.

Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2019.
Bibliografia.

1. Reproduction. 2. Neotropical fish. 3. Sperm. I. Murgas, Luis David Solis. II. Título.

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SPERM CRYOPRESERVATION THROUGHOUT THE SPAWNING SEASON IN *Prochilodus lineatus*

CRIOPRESERVAÇÃO ESPERMÁTICA AO LONGO DO PERÍODO REPRODUTIVO EM *Prochilodus lineatus*

Dissertation presented to Universidade Federal de Lavras, as required by the Programa de Pós-Graduação em Ciências Veterinárias, concentration area on Fisiologia e Metabolismo Animal, to obtain the degree of Master in Veterinay Sciences.

Aproved on August 22th, 2019.

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LAVRAS – MG
2019

This work is dedicated to

My beloved parents, Maria Christina and Carlos Eduardo,

My beloved sister and best friend Alice.

AKNOWLEDMENTS

To God, my guide and protector.

To my family, my parents, Maria Christina and Carlos Eduardo, and my sister, Alice. For the unconditional support and love.

To Dr. Luis Murgas, for your guidance and friendship all these years, and for trusting on my work. You are a great inspiration.

To Dr. Cristina Delarete Drummond and Dr. Alexmiliano Vogel de Oliveira, for their availability and contributions to this work.

To the Federal University of Lavras, especially the Department of Veterinary Medicine, for the opportunity and support for this work. And to the professors of the Graduate Program in Veterinary Sciences, for the knowledge they have shared with me.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES), for the Masters scholarship. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

To my laboratory mates, for the good moments we lived together and for all I learned with them. Especially to my friends Naiara and Thales, who were fundamental in this work, thanks for your friendship and companionship. To Luciana and Isabela, for their friendship and support during the Master's degree.

To Dr. Jacqueline and Weslley, who opened the doors of the Atherosclerosis and Nutrition Biochemistry Laboratory at UFMG to assist in the analysis of this project.

To the technicians and employees of UFLA, EPAMIG and CEMIG who helped me and taught me so much. Especially William Cesar Cortês, Fidélis Antônio da Silva Júnior, Jardell Peixoto, Geraldo Francisco, José Lopes, José do Carmo and Jaílson Silva.

To the animals, the real meaning for my career.

Thank you!

“Inspiration exists,

but it has to find you working.”

Pablo Picasso

ABSTRACT

The aim of this study was to determine the quality of *Prochilodus lineatus* sperm cryopreserved throughout the reproductive season (November to March). Males (n=43) were monthly hand-stripped after carp pituitary treatment. Fresh sperm was subjectively analyzed for its motility rate, motility quality score, duration of motility and morphology. Sperm was cryopreserved on 0.5 mL straws (n=258) using methyl glycol as cryoprotectant and glucose solution as extender. Post-thaw sperm was analyzed for its motility rate, velocities (curvilinear – VCL; straight-line – VSL; average path – VAP), and beat cross frequency (BCF) using a Computer-Assisted Sperm Analyzer (CASA). Frozen sperm morphology, oxidative stress (lipid peroxidation – LPO; reactive oxygen species – ROS; superoxide dismutase – SOD; catalase – CAT), and fertilization capacity (fertilization and hatching rates) were also evaluated. Seminal plasma was analyzed for pH, osmolality and ion concentration (Na^+, K^+, Ca^{2+} and Mg^{2+}). *P. lineatus* fresh sperm presented good quality during the reproductive season, exhibiting motility rates above 80%, and post-thaw sperm quality was affected throughout the spawning season. Post-thaw sperm yielded higher ($p<0.05$) motility and VCL on December to March. Negative correlations were observed between sperm concentration and frozen sperm motility, sperm concentration and VCL, and Ca^{2+} concentration on seminal plasma and frozen sperm motility ($p<0.01$). Sperm concentration was positively correlated with Ca^{2+} concentration, CAT and ROS ($p<0.01$). CAT and fertility correlated positively ($p<0.05$). Increased CAT was efficient in reducing ROS and LPO in samples frozen in November, and maintained sperm fertilization capacity, although ROS activity affected sperm motility and VCL. Climate factors registered before the reproductive season also affected the quality of samples frozen at the beginning of the season. Although both fresh and post-thaw semen presented lower percentages of normal sperm cells on March, samples yielded good quality parameters such as motility rate, motility quality score, and VCL on this month. *P. lineatus* sperm cryopreserved from December to March exhibits better characteristics to undergo the stress induced by cryopreservation.

Keywords: Spermatozoa. Motility. Catalase. Seminal plasma. Concentration. Neotropical fish.

RESUMO

O objetivo deste trabalho foi determinar a qualidade do sêmen de curimba (*Prochilodus lineatus*) criopreservado ao longo do período reprodutivo (novembro a março). O sêmen foi coletado mensalmente dos animais (43 machos) após indução hormonal com extrato bruto de hipófise de carpa. O sêmen fresco foi avaliado subjetivamente quanto a sua motilidade, vigor, duração da motilidade e morfologia. O sêmen foi criopreservado em palhetas de 0,5 mL (n=258) utilizando-se metilglicol como crioprotetor e solução de glicose como diluidor. O sêmen descongelado foi avaliado quanto a sua motilidade, velocidades (curvilínea – VCL, linear progressiva – VSL, média – VAP) e frequência de batimento flagelar cruzado (BCF) por meio de um sistema de análise espermática computadorizada (CASA). O sêmen descongelado também foi analisado quanto a sua morfologia, estresse oxidativo (peroxidação lipídica – LPO, espécies reativas de oxigênio – ROS, superóxido desmutase – SOD e catalase – CAT) e capacidade de fertilização (taxas de fertilização e eclosão). O plasma seminal foi analisado quanto ao seu pH, osmolalidade e concentração iônica (Na^+, K^+, Ca^{2+} e Mg^{2+}). O sêmen fresco de curimba apresentou alta qualidade durante o período reprodutivo, com taxa de motilidade espermática superior a 80%, e a qualidade do sêmen congelado foi afetada ao longo da estação reprodutiva. O sêmen criopreservado apresentou maiores ($p<0.05$) motilidade e VCL de dezembro a março. Correlações negativas ($p<0.01$) foram observadas entre a concentração espermática e a motilidade espermática, concentração espermática e a VCL, e concentração de íons Ca^{2+} no plasma seminal e motilidade espermática. A concentração espermática foi positivamente correlacionada ($p<0.01$) com a concentração de íons Ca^{2+} no plasma seminal, CAT e ROS. CAT e fertilidade também se correlacionaram positivamente ($p<0.05$). O aumento da CAT foi eficiente em reduzir as ROS e a LPO nas amostras criopreservadas em novembro, e manteve a capacidade de fertilização espermática, apesar de a atividade das ROS ter afetado a motilidade e a VCL. Fatores climáticos registrados no período que antecedeu a estação reprodutiva também afetaram a qualidade das amostras criopreservadas no início do período. Apesar de o sêmen fresco e criopreservado terem apresentado baixo percentual de células normais em março, as amostras apresentaram boa qualidade neste mês, com alta motilidade, vigor e VCL. O sêmen de *P. lineatus* criopreservado de dezembro a março exibe características mais favoráveis para ser submetido ao estresse induzido pela criopreservação.

Palavras-chave: Espermatozoide. Motilidade. Plasma seminal. Concentração. Peixe neotropical.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BCF	Beat cross frequency
BW	Body weight
CASA	Computer Assisted Sperm Analysis
CAT	Catalase
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein diacetate
DEC	December
FEV	February
h	Hour
INMET	Instituto Nacional de Meteorologia – Brasil
JAN	January
Kg	Kilogram
L	Litre
LPO	Lipid peroxidation
MAR	March
MDA	Malondialdehyde
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
mmol	Millimole
mOsm	Milliosmole
nm	Nanometer
nmol	Nanomole
NOV	November
pH	Power of hydrogen
pmol	Picomole
ROS	Reactive Oxygen Species

s	Second
SOD	Superoxide dismutase
SNK	Student-Newman-Keuls test
S.D.	Standard deviation
TBARS	Thiobarbituric acid reactive substances
U	Unit
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
µm	Micrometre
µM	Micromolar

SUMMARY

FIRST PART

1 INTRODUCTION

Reproduction of neotropical migratory fish is characterized by the reproductive displacement of animals with a seasonal pattern, synchronized with the rainy season, higher temperatures and longer daylength (RESENDE et al., 1996; WINEMILLER, 1989). The interaction of biotic and abiotic factors exerts long and short term effects on these animals reproduction (VENTURIERI; BERNARDINO, 1999). Fish gonadal maturation, gametogenesis, oocyte maturation, sperm and spawning are regulated by the hypothalamus-pituitary-gonad axis, which is under environmental influence (BROMAGE; PORTER; RANDALL, 2001; MYLONAS; FOSTIER; ZANUY, 2010). Environmental stimuli reflect on fish gamete characteristics, so that the variation of environmental factors throughout the reproductive season affects the quality of these cells (PIRES et al., 2017; RAHMAN; RAHMAN; HASAN, 2011; SILVA et al., 2009).

Cryopreservation consists in the process of conservation of biological material at low temperatures (AGARWAL, 2011), and provides numerous benefits to fish assisted reproduction (AGCA, 2012). Despite its benefits, the cryopreservation process causes damage to the spermatozoon, compromising its fertilizing capacity. These damages are related to sperm metabolism and structure, and may be caused by increased reactive oxygen species (ROS) generation, osmotic stress, impaired cell membrane integrity, inactivation of enzymes, alteration in mitochondrial activity, modifications in sperm adenosine triphosphate (ATP) molecules concentration, and variation in intracellular calcium ion homeostasis (FIGUEROA et al., 2019).

Prochilodus lineatus has been the focus of studies for sperm cryopreservation, since this species has demonstrated good results on assisted reproduction programs, and a large amount of scientific work has been published in this area (FRANCISCATTO; MURGAS; MILIORINI, 2002; MURGAS et al., 2007; PEREIRA et al., 2009). *P. lineatus* is a migratory fish of great ecological importance for the neotropical aquatic ecosystems (CASTRO; VARI, 2004; FLECKER, 1996), and it is among the main fish species of interest for fishing in Brazil (CASTRO; VARI, 2004; REIS; KULLANDER; FERRARIS JUNIOR, 2003). In addition, *P. lineatus* plays an important role as a biological model for studies applied to neotropical fish (BARBIERI; SALLES; CESTAROLLI, 2000; CAPELETI; PETRERE JR, 2006; VASCONCELOS et al., 2015). Studies conducted with this model species show that, according to the characteristics of the environment, the duration of their reproductive period varies from

October to February and, similarly, the quality of their gametes changes during the season (HARDT; PERET; PEREIRA-SILVA, 2006; RAMOS et al., 2010; SILVA et al., 2009).

Thus, the definition of the best time for collection and cryopreservation of *P. lineatus* sperm allows optimizing the use of breeders during the reproductive season, as well as the cryopreservation and assisted reproduction techniques of this species. Thus, the objective of this work was to evaluate the characteristics of *P. lineatus* cryopreserved sperm throughout the reproductive season, aiming to determine the best moment for sperm cryopreservation using the analysis of sperm quality.

2 LITERATURE REVIEW

2.1 Characterization of the species: *Prochilodus lineatus*

Taxonomic classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Ordee: Characiformes

Family: Prochilodontidae

Subfamily: Prochilodontinae

Genus: *Prochilodus*

Species: *Prochilodus lineatus* (VALENCIENNES, 1836)

Prochilodus lineatus is popularly known in Brazil by the names “curimba”, corimba”, “curimbatá”, “carimbatá”, “corimbatá”, “curimatã”, “grumatã” and “papa-terra”. In other countries in South America it is known as “sábalo”, and internationally as “streaked prochilod”. Some of these names are also used for other species of the genus *Prochilodus* in Brazil, such as *P. vimboides, P. argenteus, P. costatus, P. nigricans*. In this work, the name curimba is used to refer to *P. lineatus*.

Briefly, *P. lineatus* was characterized by Sverlij; Ros; Orti (1993) as a greenish gray fish, darker in the dorsal region and lighter in the ventral region, with narrow and tall body, concave head and circular shaped mouth located in the rostral region of the head, presenting two rows of very small teeth (Figure 1).

Figure 1 – *Prochilodus lineatus* (curimba)

Source: The Author (2018).

P. lineatus is widely distributed in the South American basins (CASTRO; VARI, 2004). Due to its detritivorous eating habits, this species is considered of great ecological importance, as it participates in the main route of energy flow and material cycling in neotropical aquatic ecosystems (FLECKER, 1996) and it can also be highlighted as a key species in the trophic chains because carnivorous species feed on this fish. In fish farming, *P. lineatus* larvae can be used as live fed for carnivorous fish species (MURGAS et al., 2003) and adults can be reared with other fish to assist in cleaning the bottom of the tanks, improving water quality, reducing costs and effluent discharge in nature (MEDEIROS; MORAES, 2013). *P. lineatus* is also considered an important species for commercial, artisanal and subsistence fishing, and figures among the main species fished in Brazil and other South American countries (CASTRO; VARI, 2004; REIS; KULLANDER; FERRARIS JUNIOR, 2003).

Due to its high tolerance to different physical, chemical and biological conditions (VAZZOLER; AGOSTINHO; HAHN, 1997) *P. lineatus* is considered as a good biological model for studies applied to neotropical fish. *P. lineatus* is used as a model species for research in neotropical fish physiology (JENSCH-JUNIOR et al., 2006), reproduction (MILIORINI et al., 2011; MURGAS et al., 2007; VASCONCELOS et al., 2015), toxicology (RODRIGUES; RANZANI-PAIVA; JULIANO, 2001) and ecology (BARBIERI; SALLES; CESTAROLLI, 2000; CAPELETI; PETRERE JR, 2006). Therefore, the close relationship between *P. lineatus* and *Prochilodus vimboides* (SANTOS, 2014) is important, as the latter is in danger of extinction (ICMBio, 2016) e and studies performed on the model species may help on the recovery of *P. vimboides* natural stocks. Although *P. lineatus* is not on the list of threatened species, studies have shown that populations of *P. lineatus* suffer from heavy fishing pressure in their natural habitat (PESOA; SCHULZ, 2010).

In natural environment, *P. lineatus* migrates upstream the rivers for reproduction (VAZZOLER; AGOSTINHO; HAHN, 1997), and it is characterized as a long-distance

migratory species (RESENDE et al., 1996; SIMABUKU, 2005). During their upstream migration, the displacement and gonadal development require energy and lipid reserves are used as energy source, therefore, when they are found at the spawning areas, these reserves are completely depleted (CAPELETI; PETRERE JR, 2006; RESENDE et al., 1996).

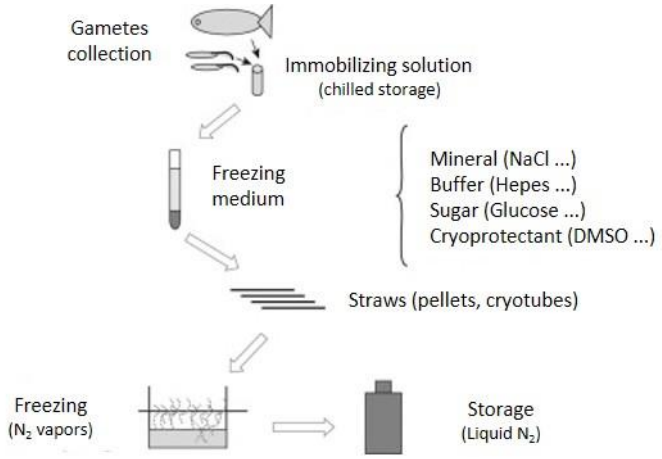
P. lineatus migration for reproduction begins in September and October (BARBIERI; SALLES; CESTAROLLI, 2000; CAPELETI; PETRERE JR, 2006) and its reproduction occurs during the months of abundant rain, high temperature and longer photoperiod. The length of the reproductive season is described in different ways by researchers, both in natural environment and in captivity. Resende et al. (1996) identified the reproductive season of *P. lineatus* from December to February (Mato Grosso), Vazzoler, Agostinho and Hahn (1997) in October to February (Paraná and Mato Grosso do Sul), Barbieri, Salles and Cestarolli (2000) in November and December (São Paulo), Simabuku (2005) in November to January (São Paulo), and Ramos et al. (2010) in October to January (São Paulo). Hardt; Peret; Pereira-Silva (2006) identified *P. lineatus* males with maximum gonad somatic index (IGS) values in March and April, indicating that these animals were able to reproduce at this time (Goiás), while Capeleti and Petrere Jr. (2006) identified specimens of *P. lineatus* migrating during March and April (São Paulo), but did not attribute reproductive purpose to the displacement of the animals at this time. The variation in the definition of the *P. lineatus* reproductive season is mainly related to differences in environmental factors, which change between the years and places where the animals live.

2.2 Fish sperm cryopreservation

Cryopreservation is the process of conserving biological material (cells and tissues) at very low temperatures (usually –196°C in liquid nitrogen) (AGARWAL, 2011; MARTÍNEZ-PÁRAMO et al., 2017). The cryopreservation technique involves interconnected steps that include biological material collection, dilution, cryoprotectant selection, freezing, storage, thawing and material viability assessment (LABBÉ; ROBLES; HERRAEZ, 2013; TIERSCH et al., 2007) (Figure 2). The development of cryopreservation protocols with effective, fast and accurate steps, as well as their standardization and careful control of its quality are indispensable for the success of the cryopreservation technique (TORRES; HU; TIERSCH, 2016; YANG et al., 2016). The effectiveness of each step of the cryopreservation process must be carefully monitored individually. In addition, the steps must be integrated to ensure that the thawed material is viable.

Agca (2012) described the main benefits of this biotechnology, which can be summarized as: easier transportation of genetic material (since the displacement of live animals causes stress, presents higher cost and requires quarantine – time factor), reduced breeding costs, continued supply of gametes and advantages for breeding programs. Viveiros and Godinho (2009) pointed that this technique provides a diverse range of biological material, ensuring greater genetic variability of animals generated by assisted reproduction. Specifically for migratory fish species, the use of cryopreserved sperm assists on the synchronization of males and females, as male gametes can be stored until female gametes are available (VIVEIROS; ORFÃO; LEAL, 2014).

Figure 2 – Main steps for sperm cryopreservation process



Source: Adapted from de Labbé; Robles e Herraez (2013)

Sperm dilution is performed for its cryopreservation due to high sperm concentration (VASCONCELOS et al., 2015), however, it reduces the concentration of seminal plasma constituents, making sperm more sensitive to oxidative lesions resulting from the cryopreservation process (MARTÍNEZ-PÁRAMO et al., 2012a). Salts and/or sugars are added to the diluting solution (extender), to maintain its osmolality and pH similar to the seminal plasma (MURGAS et al., 2015; TIERSCH et al., 2007). Thus, the extender ensures an optimum environment for the maintenance of sperm cells, as it keeps it immotile and makes its lifespan longer (TORRES; HU; TIERSCH, 2016).

During the freezing and thawing processes, the sperm cell is damaged due to differences in osmotic pressure and intra- and extra-cellular ice formation (AGARWAL, 2011). Extracellular fluid begins to freeze before intracellular fluid, and the formation of extracellular ice crystals causes increased concentration of salts in the extracellular medium. As a result,

spermatozoa suffer from dehydration due to osmosis, since the intracellular fluid is not yet frozen. When the intracellular medium freezes, the formation of ice crystals also occurs inside the spermatozoon causing damages. Freezing speed adjustment is essential to reduce this damage. Thus, the optimal freezing speed should be slow enough to reduce ice crystal formation and fast enough to minimize the exposure time of cells to an increasingly hyperosmotic environment (TIERSCH et al., 2007).

To reduce the damage caused by the formation of intracellular ice crystals, cryoprotectants are added to the freezing medium. According to their molecular weight, cryoprotectants may be permeable or not and act inside or on the cell surface, respectively, protecting the sperm membrane integrity (TORRES; HU; TIERSCH, 2016). Internal cryoprotectants act by reducing the intracellular freezing temeperature, thereby reducing ice crystal formation, while external cryoprotectants cover the cell surface, stabilize the sperm membrane and are able to restore damaged phospholipids during thermal shock (SALMITO-VANDERLEY et al., 2012). Among the main cryoprotectant substances used for fish sperm cryopreservation are dimethylsufoxide (DMSO), methylglycol, methanol, dimethylacetamide (DMA), chicken egg yolk and skim milk. The use of extenders and cryoprotectants reduces the negative effects of cryopreservation, but the quality of frozen sperm is still lower than fresh.

Sperm motility rate is considered to be the most appropriate parameter for sperm quality evaluation (FAUVEL; SUQUET; COSSON, 2010; KOWALSKI; CEJKO, 2019), since it is correlated to the fertilization capacity of these cells (GALLEGO; ASTURIANO, 2018). The identification of factors that influence sperm motility contributes to the understanding of the physiology of this cell and permits optimization of the cryopreservation technique, thus ensuring its benefits. Recent studies have allowed a better understanding of the mechanisms responsible for interfering with sperm quality during cryopreservation, providing information on the nature of the damage caused by low temperature (MARTÍNEZ-PÁRAMO et al., 2017; FIGUEROA et al., 20018).

Oxidative stress is recognized as an important source of damage to cryopreserved sperm (CABRITA et al., 2014; FIGUEROA et al., 2018; LAHNSTEINER; MANSOUR; KUNZ, 2011; MARTÍNEZ-PÁRAMO et al., 2012a; WANG et al., 2016). Sperm damage occurs because, during cryopreservation, there is an imbalance between the production of reactive oxygen species (ROS) and the action of the seminal antioxidant defense system. This is due to the exposure of sperm cells to low temperature and dilution of sperm in the freezing medium, which reduces the availability of antioxidant agents (CABRITA et al., 2011; MARTÍNEZ-PÁRAMO et al., 2012a). The presence of a large amount of polyunsaturated fatty acids in the

sperm membrane makes this structure more sensitive to the action of ROS, which are highly reactive molecules and cause lipid peroxidation, compromising cell structure and metabolism (FIGUEROA et al., 2018). During cryopreservation, ROS activity is also recognized as affecting mitochondrial activity and sperm DNA integrity, which enhances its influence on sperm quality (FIGUEROA et al., 2019; VALCARCE; ROBLES, 2016).

Seminal plasma is an important source of protection for spermatozoa and it is the main supplier of fish sperm antioxidant defense system (CABRITA et al., 2014; DIETRICH et al., 2019; LAHNSTEINER, 2007; LAHNSTEINER; MANSOUR, 2010). The analysis of sperm oxidative stress is considered an important tool for sperm quality assessment, as it contributes to a better understanding of the mechanisms that affect these cells quality (CABRITA et al., 2014). Therefore, the analysis of ROS production and lipid peroxidation, as well as the activity of the enzymes catalase, superoxide dismutase and glutathione involved in antioxidant defense, is used as a marker of sperm quality in studies with cryopreserved sperm (FIGUEROA et al., 2019; HAGEDORN et al., 2012; LAHNSTEINER; MANSOUR; KUNZ, 2011; MARTÍNEZ-PÁRAMO et al., 2012a; WANG et al., 2016).

2.3 Sperm quality and its variation throughout the reproductive season in fish

The quality of gametes is essential for the success of fish breeding (VIVEIROS; GODINHO, 2009). The quality of these cells is influenced by environmental factors (BROMAGE; PORTER; RANDALL, 2001; MYLONAS; FOSTIER; ZANUY, 2010) and the variation of these factors throughout the reproductive season influences their characteristics (PIRES et al., 2017; RAHMAN; RAHMAN; HASAN, 2011; SILVA et al., 2009).

The evaluation of sperm characteristics is essential for assisted reproduction of fish (MURGAS et al., 2011), since the identification of good quality sperm allows the optimization of its use in artificial fertilization. Seminal evaluation can be performed by subjective or objective methods, and the parameters considered most important for sperm evaluation after freezing and thawing are fertilization rate, sperm motility rate and morphology (MURGAS et al., 2011; SALMITO-VANDERLEY et al., 2014). The analysis of these parameters can also be associated with the evaluation of other sperm characteristics such as sperm motility duration, sperm membrane integrity, sperm trajectory and velocity, and larval hatching and survival rates (MELO-MACIEL et al., 2012; SALMITO-VANDERLEY et al., 2014; VIVEIROS; GODINHO, 2009).

Sperm motility rate is the main parameter used for sperm quality assessment in both aquaculture and research (MURGAS et al., 2015), it indicates the percentage of motile

spermatozoa after activation (VIVEIROS; GODINHO, 2009) and is correlated to sperm fertilization capacity (GALLEGO; ASTURIANO, 2018). In migratory fish from Brazil, the initial sperm motility of fresh sperm is usually greater than 60 % (VIVEIROS; GODINHO, 2009) and it is influenced by the period of the reproductive season in which sperm is released (MURGAS et al., 2015).

Differences in fresh sperm quality over the reproductive season in *Solea senegalensis*, *Salvelinus namaycush* and *Prochilodus lineatus* (BEIRÃO et al., 2011; JOHNSON et al., 2013; SILVA et al., 2009) have been associated with climatic variations, mainly to temperature and precipitation. Silva et al. (2009) identified higher sperm concentration in *P. lineatus* when the temperature was higher and, according to these authors, higher temperatures stimulated the hypothalamus-pituitary-gonad axis and induced the release of a larger number of spermatozoa.

Lenz et al. (2018) and Pires et al. (2017) reported that fresh sperm from *Colossoma macropomum* presented better quality at the beginning of the reproductive season and associated the decrease in sperm quality over the season with repeated use of the male. On the other hand, Kuradomi et al. (2016) showed that the species *Piaractus mesopotamicus*, despite having decreased sperm quality during the reproductive season, could be submitted to more than one sperm collection during the same season without significant losses in sperm quality.

The variation in seminal plasma composition over the reproductive season also influences sperm quality. Cejko et al. (2018) and Viveiros et al. (2019) associated variations in seminal plasma characteristics and sperm quality in *Cyprinus carpio* L. and *P. lineatus* throughout the reproductive season. In these species, changes in pH, osmolality and activity of seminal plasma enzymes during the reproductive season were correlated with sperm quality parameters such as sperm motility and velocity. Seminal plasma constituents also include enzymatic and non-enzymatic components of the sperm antioxidant defense system. Variations in these components throughout the reproductive season, as well as changes in the quantification of indicators of lipid peroxidation and generation of reactive oxygen species (ROS) in fish sperm were associated with changes in sperm quality of *Dicentrarchus labrax*, *Cyprinus carpio* and *Oncorhynchus mykiss* (MARTÍNEZ-PÁRAMO et al., 2012; SHALIUTINA-KOLEŠOVÁ et al., 2018). In these species, increased production of ROS or reduction of antioxidant components in sperm caused a decrease in sperm quality, mainly due to damage caused by lipid peroxidation in different sperm structures. However, while in *D. labrax* lipid peroxidation was higher in the early season, in *C. carpio* and *O. mykiss*, this effect was greater in the late season, demonstrating that variations in the status of sperm oxidative stress among different species occurs at different times of the reproductive season.

Most studies on sperm quality variation throughout the reproductive season in fish are conducted with fresh sperm. Cryopreserved sperm from two Brazilian native species (*Prochilodus lineatus* and *Brycon orbignyanus*) was evaluated for motility and sperm velocities and no differences were reported during the reproductive period (DI CHIACCHIO et al., 2017). Although few studies have evaluated cryopreserved sperm during the reproductive season, this type of analysis may allow the identification of the best time for sperm cryopreservation and, consequently, the optimization of animal management.

3 FINAL CONSIDERATIONS

From this work, it was confirmed that specimens of *Prochilodus lineatus* bred in the Zona da Mata region of Minas Gerais produce high quality sperm throughout the reproductive season. In addition, it was shown that male individuals are able to reproduce in March, thus extending the reproductive season of this species.

The present study demonstrated that, differently from fresh sperm, cryopreserved sperm quality is affected throughout the reproductive season. Cryopreserved sperm characteristics were influenced by sperm concentration, oxidative stress-related damage, seminal plasma composition and climatic conditions.

In this study, it was possible to identify and understand mechanisms that affect sperm quality, indicating which factors should be managed to improve cryopreserved sperm quality. Therefore, due to variations in sperm characteristics during the reproductive season, the optimal period for cryopreservation of *P. lineatus* sperm is between December and March, when sperm presents better characteristics to be subjected to cryopreservation-induced stress

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SECOND PART – MANUSCRIPT

MANUSCRIPT 1

Sperm cryopreservation of *Prochilodus lineatus* throughout the same reproductive season

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According to the journal Animal Reproduction Science

Sperm cryopreservation of *Prochilodus lineatus* throughout the same reproductive season

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Abstract

The aim of this study was to determine the effect of seasonality on post-thaw sperm quality in *Prochilodus lineatus*. Therefore, sperm was collected from 43 males throughout the spawning period (November – March). Fresh sperm was subjectively analyzed for its motility rate, motility quality score, duration of motility and sperm morphology. Post-thaw sperm motility rate, velocities (curvilinear – VCL; straight-line – VSL; average path – VAP), and beat cross frequency (BCF) were analyzed using a Computer-Assisted Sperm Analyzer. Post-thaw sperm morphology, oxidative stress (lipid peroxidation – LPO; reactive oxygen species generation – ROS; superoxide dismutase activity – SOD; catalase activity – CAT), and fertilization capacity were also evaluated. Seminal plasma was analyzed for pH, osmolality and ion concentration (Na^+, K^+, Ca^{2+} and Mg^{2+}). *P. lineatus* presented high-quality fresh sperm during the reproductive period (motility > 80%), and post-thaw sperm quality changed throughout the season, with higher ($p<0.05$) motility (63.2–72.3 %) and VCL (55.9–59.2 µm/s) on December to March. Negative correlations were observed between sperm concentration and sperm motility and VCL, Ca^{2+} concentration and frozen sperm motility ($p<0.01$). Sperm concentration was positively correlated with Ca^{2+} concentration, CAT and ROS ($p<0.01$). CAT and fertility correlated positively ($p<0.05$). Increased CAT was efficient in reducing ROS and LPO in sperm samples frozen in November, and maintained sperm fertilization capacity, although LPO affected sperm motility and VCL. In order to face seasonal influence, the optimal period to cryopreserve *P. lineatus* spermatozoa is from December to March, when sperm exhibits characteristics which make spermatozoa more prone to resist to cryodamage.

Keywords: Spermatozoa; CASA; Oxidative stress; Seminal plasma; Concentration; Neotropical fish.

1 Introduction

The streaked prochilod *Prochilodus lineatus* (Prochilodontidae, Characiformes) is a migratory neotropical fish with a large distribution in the main hydrographic basins of South America (Castro and Vari, 2004). The Prochilodontidae family comprises detritivorous fish important to community dynamics in tropical streams and *Prochilodus* larvae can be used as natural feed in the larviculture of endangered carnivorous fish such as piracanjuba (*Brycon orbignyanus*), dourado (*Salmius brasiliensis*) and jaú (*Zungaru jahu*) (Flecker, 1996; Murgas et al., 2003). The annual migration and spawn of the *P. lineatus* occurs during the rainy months, when water temperatures are higher and the daylength is longer (Vazzoler et al., 1997). During the reproductive period, *P. lineatus* male and female characteristics change, and fresh gamete features exhibit seasonal variation (Hardt et al., 2006; Silva et al., 2009).

Reproduction in fish integrates the hypothalamic-pituitary-gonadal axis and environment information such as temperature, precipitation and photoperiod, the interaction among these internal and external factors influence spermatogenesis and final sperm maturation (Bromage et al., 2001; Mylonas et al., 2010). Therefore, environmental changes throughout the year and during the reproductive season affect sperm quality. Sperm characteristics variation throughout the reproductive period has been demonstrated in teleost fish with discontinuous reproductive pattern (Cejko et al., 2018; Johnson et al., 2013; Kuradomi et al., 2016; Pires et al., 2017). The importance of seminal plasma constituents to spermatozoa protection and metabolism is proven, and variations on its components throughout the reproductive period has been associated with external factors and sperm characteristics (Cejko et al., 2018; Dietrich et al., 2019; Lahnsteiner and Mansour, 2010; Viveiros et al., 2019a). Martínez-páramo et al. (2012a) and Shaliutina-Kolešová et al. (2018) reported differences in sperm quality and oxidative stress status during the spawning season in European sea bass (*Dicentrarchus labrax*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*).

Reactive oxygen species (ROS) are produced during normal metabolism of living cells, but a status of oxidative stress can be established during sperm cryopreservation due to an imbalance between the generation of ROS and sperm antioxidant capacity, affecting sperm quality (Cabrita et al., 2014, 2011; Figueroa et al., 2018; Lahnsteiner et al., 2011; Martínez-Páramo et al., 2012b; Wang et al., 2016). In spite of being a valuable tool used in the fish farming industry, in laboratories maintaining important strains of

model fish species, and in the conservation of threatened species to preserve genetic material, the cryopreservation technique induces damage and changes in the spermatozoon, at both structural and physiological levels (Figueroa et al., 2019, 2016).

Therefore, the assessment of sperm quality associating external and internal factors that influence fresh and post-thaw sperm characteristics is indispensable to guarantee the success of sperm cryopreservation. In the present work, sperm from *P. lineatus* was cryopreserved throughout the reproductive period and characterized with the aim to determine the effect of seasonality on parameters influencing post-thaw sperm quality.

2 Materials and methods

2.1 Fish handling and sperm collection

The handling of animals and the experiments conducted were carried out in strict accordance with international guidelines for animal experimentation and all procedures were conducted with approval by Animal Experimentation Ethics Committee of the Federal University of Lavras (UFLA), Lavras, MG, Brazil (Protocol N. 23/2018).

Prochilodus lineatus (males n = 43, 0.375 ± 0.12 kg of body weight, and females n = 2, 0.518 ± 0.02 kg of body weight, BW) with approximately three years of age were selected from fish cultured in earthen ponds at the Fish Culture Station of the Agricultural Research Company of Minas Gerais (EPAMIG) in the city of Leopoldina (21°28'34"S; 42°43'17"W), Minas Gerais State, Brazil, during the spawning season 2017–2018. The fish were fed commercial extruded feed (28% crude protein) twice a day ad libitum. Males releasing a few drops of thick milt under soft abdominal pressure received a single intramuscular dose of carp pituitary extract at 4 mg/kg BW and females presenting well-rounded and soft abdomen, and a protruding and reddish urogenital papilla received two intramuscular doses of carp pituitary extract at 0.5 and 5.0 mg/kg BW in a 12 h interval, which is the routine method currently used to induce spermiation and oocytes extrusion in the Fish Culture Station.

Sperm was collected monthly throughout the spawning season of the species (Hardt et al., 2006; Silva et al., 2009), on November (n = 7), December (n = 8) (2017), January (n = 9), February (n = 11), and March (n = 8) (2018) on the first week of each month. For cryopreserved sperm fertilization tests, oocytes were collected on December (2018), when females presented better characteristics for spawning. During the season, climate conditions (temperature and precipitation) of the region were analyzed according

to the Brazilian National Institute of Meteorology (INMET website, http://www.inmet.gov.br, Muriaé Automatic Station, 21°07'50"S; 42°21'59"W). The climate conditions of October were also considered as this month precedes the spawning season and thus influences physiological changes on fish reproductive endocrine system.

For sperm and oocytes collection, the urogenital papilla was dried and contamination with water, urine, feces or blood was carefully avoided. The sperm from each male was collected in graduated test tubes. The volume was recorded and soon after collection, the sample was maintained in a polystyrene box containing crushed ice (5 ± 2°C). The oocytes were collected in dry plastic containers, total spawning was weighed and 0.1 g oocytes per straw was used for sperm fertilization tests.

2.2 Fresh sperm analysis and characterization of seminal plasma

Immediately after collection, each sperm sample was subjectively evaluated for motility rate (expressed as percentage of motile sperm), motility quality score (assigned using an arbitrary grading system ranging from 0 to 5 – no movement to rapidly swimming sperm) and duration of sperm motility (seconds) (Gonçalves et al., 2013; Viveiros and Godinho, 2009). Samples were analyzed after activation in 150 mOsm/kg glucose solution, using a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×200 magnification. All samples possessed motility above 80%.

An aliquot of 1 μL of sperm was diluted (1:1000) in citrate formaldehyde solution (2.9% sodium citrate, 4% commercial solution of formaldehyde 35% and distilled water; Vetec Química Fina Ltda, Duque de Caxias, Brazil) for posterior evaluation of sperm concentration, determined using a Neubauer-type hemacytometer chamber (Boeco, Hamburg, Germany), and sperm morphology.

Sperm morphologic analysis was performed according to Miliorini et al. (2011) methodology with slight modifications. Briefly, the fixed sample was stained with Rose Bengal (3:20; stain: sperm) and two wet preparations per sample were analyzed (Melo-Maciel et al., 2015). For each sample, two slides (a duplicate) were viewed using a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×1000 magnification and the morphology of two hundred sperm cells was evaluated. Primary (head degeneration, midpiece degeneration, tail stump, fractured tail, strongly coiled tail, macrocephaly, and microcephaly) and secondary (free normal head, simple bent tail, proximal and distal droplet) damages were considered (Miliorini et al., 2011). Data were recorded as percentage of abnormal sperm cells.

Approximately 0.5 mL of each sperm sample was centrifuged (K14-0602 Kasvi, São José dos Pinhas, Brazil) at 2000 g for 30 min, and the seminal plasma (supernatant) was collected and frozen (-20°C) to be subsequently evaluated. Seminal plasma was analyzed for pH using a pH meter (DM22 Digimed, São Paulo, Brazil), osmolality by a vapor pressure osmometer (Wescor Vapro 5520, Logan, USA) and ionic composition (Na^+, K^+, Ca^{2+} and Mg^{2+}) by an inductively coupled plasma optical emission spectrometer (Spectro Blue ICP-OES, Kleve, Germany).

2.3 Sperm cryopreservation

Sperm from each male was individually cryopreserved within 30 minutes after collection following the methodology described for *P. lineatus* by Viveiros et al. (2009). Briefly, the freezing medium was composed of 325 mOsm/kg glucose solution (pH adjusted to 7.6) as extender and methyl glycol [$CH_3O(CH_2)_2OH$] as cryoprotectant agent. Chemicals were purchased from Vetec Química Fina Ltda (Duque de Caxias, RJ, Brazil). Sperm was diluted in the freezing medium to a ratio of 1 sperm: 8 extender: 1 cryoprotectant and loaded into unsealed 0.5 mL straws (total of 258 straws; 43 males × 6 replicate straws), frozen in a nitrogen vapor vessel (Dry Vapor Vessel YDH-8, Cryofarm, Itu, SP, Brazil) at –170°C for 24h (approximately –36°C/min; Maria et al., 2006), and then transferred to a cryogenic tank (BioCane 34 Thermo Fisher Scientific, Dubuque, Iowa, USA) at –196°C for storage.

2.4 Post-thaw sperm analysis

Post-thaw sperm analyses were carried out at the Division of Physiology and Pharmacology of the Department of Veterinary Medicine, Federal University of Lavras (UFLA) and at the Laboratory of Atherosclerosis and Biochemistry Nutritional of the Biological Sciences Institute, Federal University of Minas Gerais (UFMG).

2.4.1 Computer-Assisted Sperm Analysis (CASA)

Straws (n=3 straws per male) were individually thawed in a water bath (Water-bath MA 127, Marconi, São Paulo, Brazil) at 60°C for 8 s. Post-thaw sperm features were estimated using Computer-Assisted Sperm Analysis (CASA) system, following the methodology described by Viveiros et al. (2012). Briefly, sperm motility was triggered in a 150 mOsm/kg glucose solution at a ratio of 1:10 (1 µl post-thaw sperm: 10 µl activating solution) at approximately 27°C directly in a Makler™ counting chamber (Sefi-Medical Instruments ltd, Haifa, Israel) placed under a phase-contrast microscope (Nikon™ Eclipse E200, Tokyo, Japan) at ×100 magnification, green filter and phase one

position. The microscope was connected to a video camera (Basler Vision Technologies™ A780-54FC, Ahrensburg, Germany) generating 50 images/s; video recording started approximately 10 s post-activation. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™ 2013, Microptics, S.L. Version 5.4, Barcelona, Spain). Motility rate, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), and beat-cross frequency (BCF) were considered for analysis. To determine these parameters, each individual spermatozoon (*n* = 1363 ± 278 sperm per straw) was followed throughout the recorded video images from which sperm trajectories were evaluated. From each thawed straw, an aliquot of sperm was diluted at a ratio of 1:1000 in citrate formaldehyde solution for post-thaw sperm morphologic analysis, performed as described for fresh sperm.

2.4.2 Indices of oxidative stress

Post-thaw sperm samples were also assayed for oxidative stress indices and antioxidant activity. Straws (n=2 straws per male) were thawed in a polystyrene box containing crushed ice (5 ± 2°C) and analyses were carried out on total sperm (seminal plasma was not separated). The results were normalized to the total protein content determined by the Lowry method (Lowry et al., 1951).

The levels of thiobarbituric acid reactive substances (TBARS) was measured as an index of oxidative stress resulting from lipid peroxidation (LPO), following the protocol described by Buege and Aust (1978). The absorbance of the samples was read in duplicates using a microplate reader at 535 nm (Synergy 2; Bio-Tek, Winooski, USA). The concentration of malondialdehyde (MDA) was read from a standard calibration curve plotted using 1, 1, 3, 3-tetramethoxypropane (Sigma-Aldrich, St. Louis, USA), and the results were expressed as nanomoles of MDA per milligram of protein.

Determination of reactive oxygen species (ROS) content in the sperm was determined by fluorescence probe 2,7-dichlorofluorescine diacetate (DCFH-DA; Sigma Aldrich, St. Louis, USA) based on the method of Driver et al. (2000) with slight modifications. The final concentration of the DCFH-DA (10 μM) solution was made by diluting the stock solutions in 50 mM phosphate buffer (pH 7.2). Conversion of DCFH-DA to dichlorofluorescein (DCF) was measured using a microplate reader (Synergy 2, Bio-Tek, Winooski, USA) at 485/ 530 nm (excitation/ emission), samples were assayed in duplicates. The free radical content was quantified using a DCF standard curve and the results were expressed as DCF fluorescence per milligram of protein per minute.

Evaluation of superoxide dismutase (SOD) activity in sperm was determined based on the autoxidation of pyrogallol by the method of Dieterich et al. (2000) with slight adaptations for fish sperm. The absorbance of the samples was read in duplicates using a microplate reader at 570 nm (Synergy 2; Bio-Tek, Winooski, USA). The total SOD activity was expressed in units per milligram of protein, where one unit of SOD activity is defined as the amount of the enzyme necessary to produce 50% dismutation of the superoxide radical per min.

The catalase (CAT) activity was measured according to a spectrophotometric method adapted from Aebi (1984), following the decrease in absorbance at 240 nm by H_2O_2 consumption. The absorbance (240 nm) was measured every 15 s for 1 min (at 25°C, pH 7.2 and path length 10 mm), using a quartz cuvette in a spectrophotometer (Shimadzu UV-160, Japan), samples were assayed in duplicates. The specific activity is reported as units per milligram protein (one unit is defined as 1 pmol of H_2O_2 consumed per minute).

2.4.3 Fertilization tests

For fertilization tests, 0.1 g oocytes of two females were weighed into 50 mL disposable cups and sperm was thawed (60°C for 8 s) and added to the cups (one straw per cup). Fresh sperm from two males was collected and used as control. To activate fertilization, 5 mL of 150 mOsm/kg glucose solution was added, circular motions were performed for 90 seconds, and the oocytes were randomly transferred to experimental incubators. The incubators were arranged in a tank with constant water renewal and oxygenation, where oocytes remained in movement.

The fertilization rate was determined 8 h after fertilization, when the blastopore closure can be observed (Ninhaus-Silveira et al., 2006) by analyzing all oocytes from each incubator using a trinocular stereomicroscope (Q7740SZ-T, Quimis, Diadema, Bazil) at ×10 magnification and the result given by the formula: fertilization rate (%) = (number of fertilized oocytes/ total number of oocytes) × 100. The hatching rate and the malformation rate of larvae were estimated 22 h (Ninhaus-Silveira et al., 2006) after fertilization and the results were given by the formulas: hatching rate (%) = (number of hatched larvae/ total number of oocytes) × 100, and malformation rate of larvae (%) = (number of larvae presenting malformation/ total number of larvae) × 100.

2.5 Statistical analysis

Data were expressed as mean ± standard deviation (SD). Data were tested for normal distribution using test Shapiro–Wilk and for significant differences using

ANOVA, followed by Student–Newman–Keuls test, when applicable. The level of significance for all statistical tests was set to 5% (P<0.05). Statistical analyses were conducted with the R software version 3.3.2 (R Core Team, 2016). Possible relationships among seminal plasma characteristics and post-thaw sperm features were analyzed by Pearson's correlation test (P<0.05).

3 Results

3.1 Climate data

Among the months of October and March, the average daily minimum and maximum temperatures were 21.15°C and 30.03°C, respectively, with a mean of 25.6 ± 5.2°C, and precipitation values presented a total mean of 197.67 ± 108.13 mm a month (Figure 1).

3.2 Fresh sperm analyses and characterization of seminal plasma

Mean sperm volume was similar (P>0.05) throughout the reproductive season, whereas concentration values were highest (P<0.05) in November than the other months. *P. lineatus* fresh sperm yielded similar (P>0.05) motility rates throughout the reproductive season. Motility quality score was significantly higher (P<0.05) in November, January and February, and duration of motility was higher in January. Regarding the fresh sperm morphological analysis, the percentage of normal cells observed in February was higher (P<0.05) than in March. Values of primary damages observed in February were lower (P<0.05) than in March, while the lowest (P<0.05) values of secondary damages were observed in November, January and February. Fresh sperm characteristics are presented in Table 1.

Seminal plasma osmolality presented no significant difference (P>0.05) among the months, and plasma pH was higher in February and lower in January, while intermediate values were observed in the other months. Seminal plasma ionic composition fluctuated during the season (P<0.05). Seminal plasma characteristics are presented in Table 2.

3.3 Post-thaw sperm analysis

P. lineatus post-thaw sperm yielded higher (P<0.05) motility rates (63.20 – 72.30 %) from December to March (Figure 2a). Post-thaw sperm VCL (46.50 – 59.20 µm/s) was higher (P<0.05) in January and February than in November (Figure 2b). Post-thaw sperm VAP, VSL, and BCF were similar (P>0.05) among the months (32.70 – 38.40 µm/s of VAP, 22.50 – 24.60 µm/s of VSL and 3.00 – 3.20 Hz of BCF).

In respect to post-thaw sperm morphology, significantly less (P<0.05) normal sperm cells were observed in March (72.06 ± 2.49 %), while the lowest primary damages values were observed in December to February (11.21–13.78 %), and secondary damages were higher in March (8.94±2.04 %) than in February (5.07±1.56 %) (Figure 2c). The mid piece degeneration was the most observed damage in post-thaw spermatozoa.

Indices of oxidative stress are presented in Table 3. The highest (P<0.05) CAT activity values accompanied the lowest motility in November, whereas LPO, ROS, and the SOD activity values presented no significant difference (P>0.05) throughout the season.

Fertilization and hatching rates are presented on Figure 5. Post-thaw sperm fertilization (7.30 – 22.30 %) and hatching (5.40 – 14.50 %) rates presented no significant difference (P>0.05) throughout the reproductive season. Considerable variation in fertilization and hatching rates were observed among individuals, as evidenced by the large standard deviations in these samples.

The correlation matrices of post-thaw sperm and seminal plasma characteristics during the spawning season are presented on Tables 4, 5 and 6. A significant correlation was negative (P<0.01) between sperm concentration and motility, sperm concentration and VCL, and Ca^{2+} concentration on seminal plasma and motility. A significant correlation was positive between sperm concentration and ROS levels (P<0.01), sperm concentration and CAT activity (P<0.01), sperm concentration and Ca^{2+} concentration on seminal plasma (P<0.01), Ca^{2+} concentration on seminal plasma and ROS levels (P<0.05), and CAT activity and fertilization rate (P<0.05).

4 Discussion

The quality of initial fresh samples is known to influence cryopreserved sperm quality (Cabrita et al., 2009). In the present study, *Prochilodus lineatus* fresh sperm presented good quality throughout the spawning season (Table 1) and even when fresh samples presented a little decrease on motility parameters (on December and March), it did not influence frozen sperm quality negatively. High-quality sperm should yield motility above 80% (Kowalski and Cejko, 2019), and although high-quality fresh sperm was used in the cryopreservation process, post-thaw sperm quality changed during the reproductive period.

Post-thaw sperm quality was influenced by cell concentration (negatively correlated with motility and VCL), seminal plasma composition (especially the Ca^{2+} ion,

289 which was directly correlated motility), and catalase enzyme activity (positively
290 correlated with fertility rates).

291 Since higher sperm concentrations are associated with reduced sperm quality due
292 to lower concentrations of the cryoprotectant within the spermatozoa (Tiersch et al., 2007;
293 Torres et al., 2016), it is possible that the samples cryopreserved in November did not
294 have optimal cryoprotectant availability. During sperm cells cryopreservation, not only
295 the presence of the cryoprotectant is important, but also the seminal plasma components
296 (Wang et al., 2016). The high sperm concentration may also have caused a decrease in
297 the availability of seminal plasma constituents for spermatozoa.

298 Fish seminal plasma is an important source of protection to sperm cells and
299 represents the major source of the defense system against oxidative stress (Cabrita et al.,
300 2014; Dietrich et al., 2019; Lahnsteiner, 2007; Lahnsteiner and Mansour, 2010). The
301 analysis of sperm oxidative stress has been recognized as an useful tool for sperm quality
302 assessment, as it contributes to a better understanding of the mechanisms by which sperm
303 is affected (Cabrita et al., 2014). In this study, we analyzed sperm oxidative stress status
304 (lipid peroxidation – LPO, and reactive oxygen species – ROS) and oxidant defensive
305 enzymes (catalase – CAT, and superoxide dismutase – SOD) in *P. lineatus* sperm (Table
306 3), and a positive correlation was observed between CAT activity and sperm
307 concentration (Table 4).

308 The higher sperm concentration in the beginning of the reproductive period might
309 have reduced seminal plasma antioxidant system availability, producing an imbalance
310 between cell generation of ROS and both enzymatic and non-enzymatic antioxidants.
311 This agrees with data obtained on the correlation analysis, which identified a positive
312 correlation between sperm concentration and ROS production (Table 4). In addition, the
313 dilution of sperm in the freezing medium reduces the availability of seminal plasma
314 antioxidant components, and during cryopreservation the exposure of the spermatozoa to
315 thermal shock contributes to increase this imbalance (Cabrita et al., 2011; Martínez-
316 Páramo et al., 2012b), leading to lipid peroxidation of the sperm plasma membrane by
317 ROS (Figueroa et al., 2019, 2018; Wang et al., 2016).

318 Consequently, sperm catalase activity increased due to a need for a higher
319 defensive response against oxidative stress. Catalase acts as a mediator of hydrogen
320 peroxide radicals and its activity is important to mitigate the negative effects of oxidative
321 stress on spermatozoa (Hagedorn et al., 2012). Higher levels of catalase in seminal plasma

have been associated with increased lipid peroxidation, but also with a decrease in lipid peroxidation in fish sperm (Chen et al., 2010; Figueroa et al., 2019, 2018; Kutluyer et al., 2017). Antioxidant enzymes activity increase to oppose the peroxidation of lipids in sperm (Martínez-Páramo et al., 2012b; Shaliutina-Kolešová et al., 2018), and while in some cases it shows to be effective in reducing the oxidative stress status, in others it does not seem to be enough. In November, the ROS levels seemed to be increased when compared to the other months, but no significant difference was detected (Table 3). This agrees with the hypothesis that the increase in catalase activity was capable to oppose to LPO by reducing ROS values to lower levels. Similarly, LPO did not show significant differences among the months. Nevertheless, catalase could not block the damages caused by LPO in the spermatozoa, resulting in decreased sperm motility and VCL in November (Figures 2a, b). This adverse effect of lipid peroxidation in cryopreserved spermatozoa motility and velocity has been well reported in fish (Figueroa et al., 2019, 2018; Martínez-Páramo et al., 2012b), as well as in fresh sperm during the reproductive season (Martínez-Páramo et al., 2012a).

Increased activity of the antioxidant enzymes in fish cryopreserved sperm has been associated with a reduction in lipid peroxidation and greater sperm motility and velocities (Chen et al., 2010; Figueroa et al., 2019, 2018; Martínez-Páramo et al., 2012b). However, in our study, samples showing higher CAT activity exhibited lower motility and velocities, when compared to those with lower activity. This could be related to variations between species, as well as differences on the cryopreservation protocol since different extender solutions, cryoprotectant and freezing rates were used.

Similar to ROS production and LPO, fertilization rates also did not present significant differences throughout the season (Figure 3). A similar effect of catalase activity might have influenced sperm fertility, which was positively correlated to CAT activity (Table 5). It is possible that the antioxidant activity of this enzyme restrained some of the adverse effects of ROS to the spermatozoa, ensuring these cells fertilization ability. Thus, catalase showed to be efficient in reducing ROS and maintaining sperm fertility, despite lipid peroxidation-related damage. It is interesting to notice that the oxidative stress-related damaged might have occurred not only during the samples freezing process, but also throughout the storage in liquid nitrogen, since some studies have recognized that molecules remain mobile at low temperature (–196°C) and sperm biological activity does not cease during storage in liquid nitrogen (Chen et al., 2010;

355 Figueroa et al., 2019).

356 Fertilization tests were also performed with fresh sperm for control and yielded
357 intermediate results (40.8 ± 10.2 % of fertilization rate and 26.3 ± 6.1 % of hatching rate).
358 Although the eggs exhibited external characteristics of good quality (slightly graywish
359 eggs, translucent, granular-looking, not watery), these results indicate that oocytes
360 seemed to have produced a detrimental effect on fertility. Despite post-thaw sperm
361 exhibited good motility and velocities, which are features correlated with sperm fertility
362 (Figueroa et al., 2016; Gallego and Asturiano, 2018), fertilization with cryopreserved
363 sperm presented low results.

364 Sperm concentration was also positively correlated with the presence of Ca^{2+} in
365 seminal plasma, and this ion was negatively correlated with motility rates (Tables 4 and
366 6). Morita et al. (2006) and Viveiros et al. (2019b) described the formation of
367 agglutinations on fish sperm exposed to solutions containing high concentrations of Ca^{2+},
368 which produced reduction on sperm motility parameters. The mechanism by which these
369 agglutinations are formed is not clear, as well as the prejudicial levels of Ca^{2+} are not
370 established. The formation of agglutinations on sperm was not observed in this work
371 because Ca^{2+} concentration was reduced when the activating solution was added to sperm
372 on CASA and fertility tests, however, the lower *P. lineatus* post-thaw sperm quality is
373 related to an adverse effect of the concentration of Ca^{2+} in seminal plasma.

374 Some studies have demonstrated that the initiation of sperm motility in
375 characiforms is similar to cyprinids (Viveiros et al., 2019b, 2016), in which osmolality of
376 the medium is the trigger for sperm activation, as the hypo-osmotic shock promotes Ca^{2+}
377 influx and initiates sperm motility (Alavi and Cosson, 2006). The presence of
378 extracellular Ca^{2+} is critical for sperm motility activation, but it is possible that higher
379 concentrations of this ion produce an inhibitory effect on sperm motility. A similar effect
380 was demonstrated by Khara et al. (2014) in Common carp (*Cyprinus carpio*) sperm,
381 which also yielded lower motility in media with higher concentration of Ca^{2+}, compared
382 to solutions with lower concentrations of this ion. In addition, a positive correlation was
383 observed between Ca^{2+} concentration on seminal plasma and ROS levels (Table 4),
384 probably because this ion might have disturbed *P. lineatus* spermatozoa metabolism,
385 making these cells more vulnerable to ROS generation.

386 Seminal plasma osmolality is known to influence sperm quality (Cosson, 2010),
387 and it has been previously correlated to sperm motility in *P. lineatus* (Viveiros et al.,

2019a). However, in this work osmolality was not associated to any of the sperm quality markers evaluated. The maintenance of sperm osmolality between 296.3 and 350.4 mOsm/kg during the season (Table 2) characterizes optimum values for the species (Nascimento et al., 2012; Viveiros et al., 2016), thus providing a good environment for spermatozoa performance regardless of the month.

Sperm morphology is also an important factor that can affect the fertilizing capacity, and the analysis of this parameter contributes to the evaluation of sperm quality since even spermatozoa with visible motility may be unable to penetrate an oocyte due to morphological alterations (Miliorini et al., 2011). In our study, the midpiece degeneration was de most observed damage in post-thaw spermatozoa. Figueroa et al. (2019) also observed a greater proportion of structural alterations in the middle piece and associated it to loss of mitochondrial functionality in Atlantic salmon (*Salmo salar*) spermatozoa. The midpiece degeneration is considered as a primary damage because of its adverse effects on fertilization. Primary damages values were lower in samples cryopreserved in December, January and February, which also presented greater proportions of normal cells (Figure 2c). However, no differences were observed in fertility and hatching rates among months (Figure 3). This might be related to the percentage of total damages observed in post-thaw samples (16.3 – 27.9 %), since the critical proportion of fish sperm abnormalities for artificial fecundation is considered to be around 50% (Miliorini et al., 2011). Other studies have reported variations on sperm morphology during the spawning season. The percentage of normal cells were higher in the beginning and middle of the season in pacu (*Piaractus mesopotamicus*) and mandi (*Pimelodus britskii*) fresh sperm, while in tambaqui (*Colossoma macropomum*) fresh sperm morphology was similar throughout the season (Damasceno et al., 2015; Kuradomi et al., 2016; Pires et al., 2017).

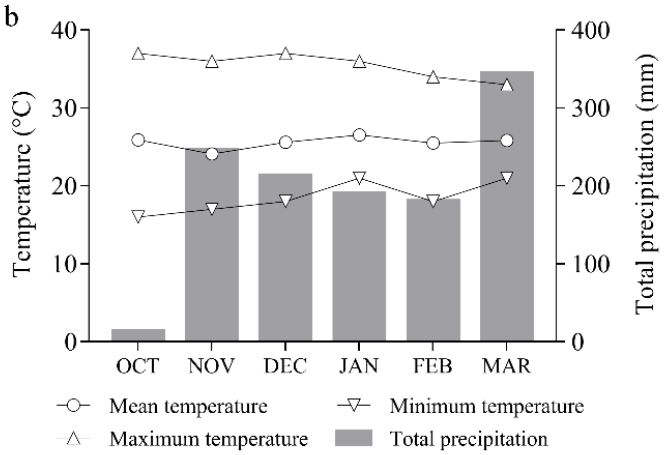
Environmental factors have a critical influence on spermatogenesis and consequently on sperm quality (Bromage et al., 2001; Mylonas et al., 2010). During this study, the lowest temperatures were registered in October and November (Figure 1). Although the minimum mean temperatures were similar in both months (mean of 19.9°C), on October the minimum temperatures remained constant along the weeks, while on November lower temperatures were registered on the first week and raised towards the end of the month. Moreover, mean and total precipitation registered in October were almost 10 times smaller than in the other months (Figure 1). Thus, it is possible that the lower temperatures and precipitation registered during the month of October influenced

the sperm frozen at the beginning of November, whereas the sperm cryopreserved in December did not seem to have been negatively affected. Di Chiacchio et al. (2017) did not observe seasonal influence in *P. lineatus* post-thaw sperm motility and velocities. Whereas Silva et al. (2009) and Beirão et al. (2011) reported that significant changes in *P. lineatus* and Senegalese sole (*Solea senegalensis*) fresh sperm parameters during the reproductive period were associated to variations on temperature among the months.

In our study, although fresh sperm presented good quality throughout the reproductive season, the quality of *P. lineatus* cryopreserved sperm showed seasonal variations. The quality of the post-thaw sperm was influenced by sperm concentration, oxidative stress-related damage, seminal plasma ionic composition, and climatic conditions.

Using techniques such as the analysis of oxidative stress is important in order to improve the knowledge on the mechanisms by which the cryopreservation process affects the sperm, thus permitting the management of the factors influencing sperm quality. In this study we identified that, in order to face seasonal influence, the optimal period to cryopreserve *P. lineatus* spermatozoa is from December to March, when sperm exhibits better characteristics to undergo the stress induced by cryopreservation.

Figure 1. Temperature and precipitation at Zona da Mata region from October, 2017 to March, 2018. Lines indicate mean values on each month and columns indicate total precipitation on each month.



Data source: Brazilian National Institute of Meteorology, Muriaé Automatic Station (http://www.inmet.gov.br).

443 Table 1. Body weight and fresh sperm features in *Prochilodus lineatus* throughout the reproductive season (from November to March).

Month	n	Body weight (kg)	Volume (mL)	Concentration (sperm × 10^9/mL)	Motility rate (% motile sperm)	Motility quality score[1] (0–5)	Duration of motility (s)	Sperm morphology (%)		
								Normal	Primary damages	Secondary damages
NOV	7	0.423±0.12	0.96±0.48	44.34±8.59^{a}	90.00±0.00	5.00±0.00^{a}	165.00±67.69^{b}	87.82±5.67^{ab}	8.71±4.47^{ab}	3.46±1.69^{b}
DEC	8	0.380±0.14	0.60±0.37	20.35±4.28^{b}	86.25±5.18	4.25±0.46^{b}	169.50±52.44^{b}	85.75±3.04^{bc}	9.41±2.95^{ab}	4.84±2.11^{a}
JAN	9	0.376±0.14	0.79±0.35	17.20±2.95^{b}	88.89±3.33	4.89±0.33^{a}	348.11±117.74^{a}	89.31±2.59^{ab}	7.19±2.39^{ab}	3.50±1.54^{b}
FEB	11	0.361±0.13	0.99±0.55	16.93±8.34^{b}	88.18±4.04	4.73±0.47^{a}	101.00±23.98^{b}	90.43±3.99^{a}	6.84±3.53^{b}	2.73±1.65^{b}
MAR	8	0.346±0.08	0.60±0.21	15.87±5.36^{b}	90.00±0.00	4.25±0.46^{b}	182.50±91.51^{b}	83.56±3.39^{c}	10.88±2.35^{a}	5.56±1.37^{a}

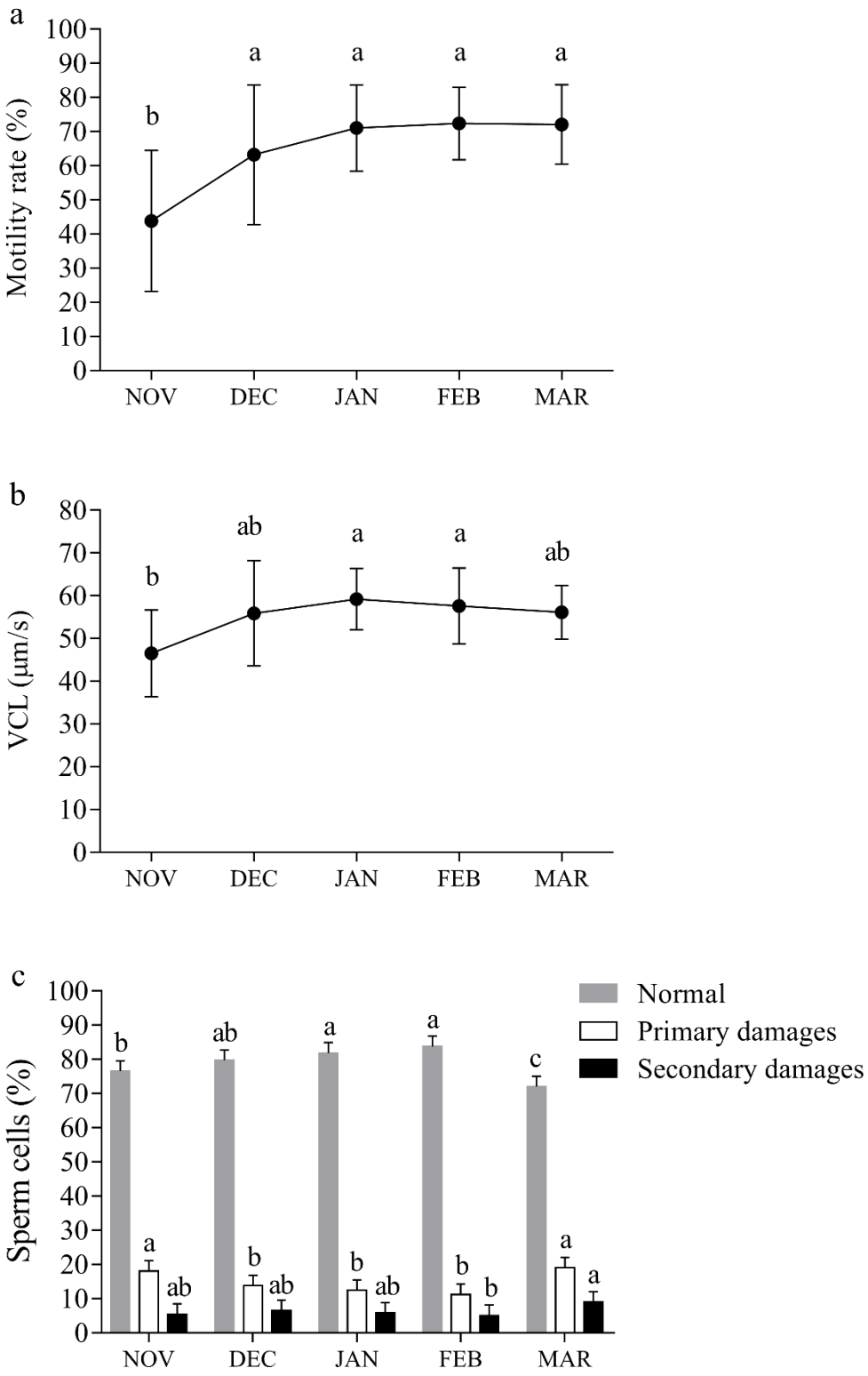
444 [1]The motility quality score (0–5) was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (to rapidly swimming sperm).
445 Different superscripts in the same row show differences between months for each parameter tested (ANOVA-SNK, $P<0.05$, mean values ± S.D.).

446 Table 2. Characteristics of the seminal plasma of *Prochilodus lineatus* throughout the reproductive season (from November to March). The ionic
447 concentrations of Na^+, K^+, Ca^{2+} e Mg^{2+} ions (mmol/L = mM) were determined by an inductively coupled plasma optical emission spectrometer.

Month	n	pH	Osmolality (mOsm/kg)	Na^+ (mmol/L)	K^+ (mmol/L)	Ca^{2+} (mmol/L)	Mg^{2+} (mmol/L)
NOV	7	8.56 ± 0.10^{ab}	350.40 ± 5.81	51.55 ± 1.26^{a}	32.31 ± 0.53^{c}	0.91 ± 0.13^{b}	4.68 ± 0.67^{a}
DEC	8	8.63 ± 0.08^{ab}	296.33 ± 78.50	40.49 ± 0.36^{c}	35.18 ± 0.34^{b}	2.03 ± 0.00^{a}	1.31 ± 0.01^{b}
JAN	9	8.45 ± 0.16^{b}	350.25 ± 12.76	41.60 ± 1.01^{c}	32.92 ± 0.91^{c}	0.92 ± 0.00^{b}	1.10 ± 0.01^{b}
FEB	11	8.95 ± 0.28^{a}	333.86 ± 7.38	44.37 ± 0.87^{b}	41.19 ± 0.26^{a}	0.44 ± 0.03^{d}	1.35 ± 0.00^{b}
MAR	8	8.55 ± 0.25^{ab}	323.00 ± 4.32	29.03 ± 0.42^{d}	24.43 ± 0.43^{d}	0.55 ± 0.00^{c}	1.08 ± 0.01^{b}

448 Different superscripts in the same row show differences between months for each parameter tested (ANOVA-SNK, $P<0.05$, mean values ± S.D.).

449 Figure 2. Motility rate (a) curvilinear velocity (b), and sperm morphology (c) of
450 *Prochilodus lineatus* sperm cryopreserved throughout the reproductive season (from
451 November to March). Data corresponds to mean values ± S.D. Different letters show
452 differences between months for each parameter (ANOVA-SNK, $P<0.05$).

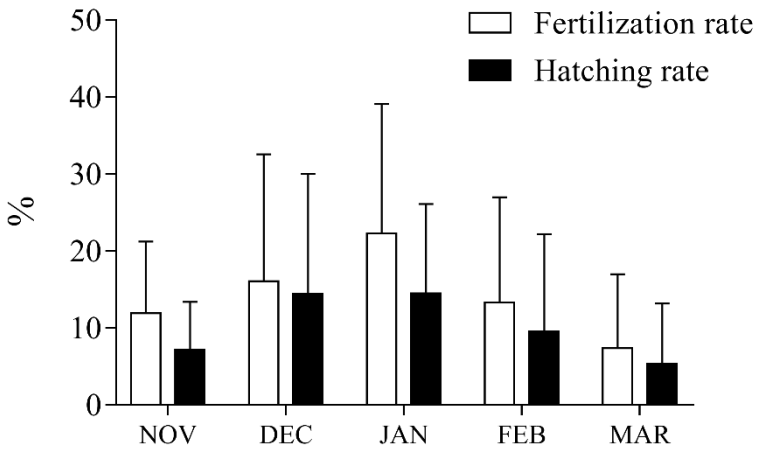


453 Table 3. Indices of oxidative stress in *Prochilodus lineatus* sperm cryopreserved
454 throughout the reproductive season (from November to March).

Month	n	LPO (nmol-MDA/ mg-protein)	ROS (DCF-fluorescence/ mg-protein)	SOD (U-SOD/mg-protein)	CAT (U-CAT/mg-protein)
NOV	7	0.4±0.2	7.1±3.9	1.7±0.6	3.9±2.0[a]
DEC	8	0.3±0.1	5.7±2.9	1.5±0.2	0.6±0.6[b]
JAN	9	0.4±0.1	5.6±2.3	1.8±0.5	1.3±1.3[b]
FEB	11	0.3±0.1	4.1±1.8	1.4±0.2	1.8±2.0[b]
MAR	8	0.3±0.1	5.2±2.7	1.5±0.4	2.2±1.3[b]

455 Different superscripts in the same row show differences between months for each
456 parameter tested (ANOVA-SNK, $p<0.05$, mean values ± S.D.). LPO: lipid peroxidation;
457 MDA: malondialdehyde; ROS: reactive oxygen species; DCF: dichlorofluorescein; SOD:
458 superoxide dismutase enzyme; CAT: catalase enzyme.
459

460 Figure 3. Fertilization and hatching rates of *Prochilodus lineatus* sperm cryopreserved
461 throughout the reproductive season (from November to March). Data corresponds to
462 mean values ± S.D. (ANOVA-SNK, $P<0.05$).



463 Table 4. Correlation matrix (Pearson's coefficient) of *Prochilodus lineatus* seminal
464 plasma and post-thaw sperm characteristics throughout the reproductive season.

	Motility	VCL	CAT	SOD	LPO	ROS
Ca^{2+}	-0.68**	-0.40	0.44	0.43	0.38	0.59*
K^{+}	-0.33	-0.27	-0.04	-0.16	-0.35	0.25
Mg^{2+}	-0.04	-0.22	0.59*	-0.19	-0.14	-0.25
Na^{+}	-0.23	-0.33	0.48	-0.34	-0.40	-0.04
pH	0.14	0.21	-0.05	-0.09	0.06	-0.16
Osmolality	-0.41	-0.32	0.02	0.24	0.11	0.21
Concentration	-0.54**	-0.41**	0.40**	0.28	0.24	0.45**

465 *p<0.05; **p<0.01. VCL: curvilinear velocity; CAT: catalase enzyme; SOD: superoxide
466 dismutase enzyme; LPO: lipid peroxidation; ROS: reactive oxygen species.
467

468 Table 5. Correlation matrix (Pearson's coefficient) of *Prochilodus lineatus* post-thaw
469 sperm features throughout the reproductive season.

	Motility	VCL	Fertilization rate	Hatching rate
CAT	-0.21	-0.18	0.32*	0.10
SOD	-0.18	0.07	0.09	0.08
LPO	-0.06	0.12	-0.06	-0.13
ROS	-0.27	-0.13	0.04	0.08

470 *p<0.05. CAT: catalase enzyme; SOD: superoxide dismutase enzyme; LPO: lipid
471 peroxidation; ROS: reactive oxygen species; VCL: curvilinear velocity.
472

473 Table 6. Correlation matrix (Pearson's coefficient) of *Prochilodus lineatus* seminal
474 plasma characteristics throughout the reproductive season.

	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}
pH	-0.21	-0.89**	-0.08	-0.63*
Osmolality	0.08	0.19	0.18	0.10
Concentration	0.81**	0.09	0.31	0.36

475 *p<0.05; **p<0.01.

Acknowledgements

The authors thank William César Cortês (UFLA) for assistance during laboratory analysis, and Jardell Peixoto, Geraldo Francisco, José Lopes and José do Carmo (EPAMIG) for assistance during fish manipulation.

Funding: This work was supported by the Brazilian fostering agencies Coordination of Improvement of Higher Level Personnel (Capes) [grant number 00002], Brazilian National Council for Scientific and Technological Development (CNPq) [grant number 304940/2014-3], FAPEMIG – Rede Mineira de Bioterismo [grant number 00001].

Conflicts of interest

The authors declare no actual or potential conflict of interest regarding the submitting manuscript.

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