GUILHERME ANTONIO DE GOUVÊA LOPES

PROTEOMIC ANALYSIS OF CRYOPRESERVED *Prochilodus lineatus* SEMINAL PLASMA: EFFECTS OF MELATONIN ON SPERM QUALITY PRESERVATION

LAVRAS – MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Fisiologia e Metabolismo Animal, para a obtenção do título de Mestre.

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GUILHERME ANTONIO DE GOUVÊA LOPES

ANÁLISE PROTEÔMICA DE PLASMA SEMINAL CRIOPRESERVADO DE *Prochilodus lineatus:* EFEITOS DA MELATONINA NA CONSERVAÇÃO DA QUALIDADE SEMINAL

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LAVRAS – MG
2025

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Resumo

Este estudo avaliou os efeitos da suplementação com melatonina na criopreservação do sêmen e na proteômica do plasma seminal do peixe neotropical *Prochilodus lineatus*. A criopreservação de sêmen é uma técnica fundamental para a aquicultura e a conservação de espécies, mas frequentemente causa estresse oxidativo, danos estruturais e prejuízos metabólicos nas células espermáticas. Neste estudo, amostras de sêmen ($n = 9$ por grupo) foram criopreservadas com ou sem 2 mM de melatonina, seguidas de análises pós-descongelamento da motilidade, vigor, integridade de membrana, morfologia e perfil proteômico. A melatonina melhorou significativamente a qualidade espermática após o descongelamento, aumentando a motilidade de 43,15 ± 16,95% para 65,72 ± 16,32% ($p = 0{,}042$), bem como a velocidade em linha reta (VSL; 10,53 ± 4,19 para 16,57 ± 5,64 µm/s; $p = 0{,}033$) e a velocidade média do trajeto (VAP; 15,73 ± 5,48 para 22,90 ± 6,75 µm/s; $p = 0{,}046$), enquanto a velocidade curvilínea (VCL; $p = 0{,}075$) e a frequência de batimento cruzado (BCF; $p = 0{,}061$) apresentaram tendências positivas, porém não significativas. A avaliação morfológica revelou redução de caudas fortemente enroladas, enquanto a integridade de membrana (42–84%) e as taxas totais de anormalidades (49–51%) não foram afetadas. A análise proteômica identificou 1.238 proteínas, incluindo oito expressas exclusivamente sob tratamento com melatonina, principalmente associadas ao metabolismo energético, defesa antioxidante e proteostase. A melatonina regulou positivamente enzimas relacionadas à produção de ATP, ao equilíbrio redox e à degradação proteica, ao mesmo tempo em que regulou negativamente proteínas associadas a danos induzidos pela criopreservação. Esses achados demonstram que a melatonina exerce um efeito protetor multifacetado durante a criopreservação espermática, preservando o metabolismo energético, reforçando a capacidade antioxidante, mantendo a integridade estrutural e genômica e melhorando a motilidade pós-descongelamento. De modo geral, este estudo fornece o conjunto de dados proteômicos mais abrangente do plasma seminal de *P. lineatus* até o momento e destaca a melatonina como um aditivo promissor para o aprimoramento de protocolos de criopreservação de sêmen na aquicultura e na conservação.

Palavras-chave: Curimba, peixe, reprodução, macho

Abstract

This study evaluated the effects of melatonin supplementation on sperm cryopreservation and seminal plasma proteomics in the neotropical fish *Prochilodus lineatus*. Sperm Cryopreservation is a key technique for aquaculture and species conservation but often causes oxidative stress, structural damage, and metabolic impairment in sperm cells. In this study, sperm samples (n = 9 per group) were cryopreserved with or without 2 mM melatonin, followed by post-thaw analyses of motility, vigor, membrane integrity, morphology, and proteomic profile. Melatonin significantly improved sperm quality after thawing, increasing motility from 43.15 ± 16.95% to 65.72 ± 16.32% (p = 0.042), as well as straight-line velocity (VSL; 10.53 ± 4.19 to 16.57 ± 5.64 µm/s; p = 0.033) and average path velocity (VAP; 15.73 ± 5.48 to 22.90 ± 6.75 µm/s; p = 0.046), while curvilinear velocity (VCL; p = 0.075) and beat-cross frequency (BCF; p = 0.061) showed positive but non-significant trends. Morphological evaluation revealed a reduction in strongly coiled tails, whereas membrane integrity (42–84%) and total abnormality rates (49–51%) were unaffected. Proteomic analysis identified 1,238 proteins, including eight exclusively expressed under melatonin treatment, mainly associated with energy metabolism, antioxidant defense, and proteostasis. Melatonin upregulated enzymes related to ATP production, redox balance, and protein degradation, while downregulating proteins linked to cryo-induced damage. These findings demonstrate that melatonin exerts a multifaceted protective effect during sperm cryopreservation by preserving energy metabolism, reinforcing antioxidant capacity, maintaining structural and genomic integrity, and improving post-thaw motility. Overall, this study provides the most comprehensive proteomic dataset of *P. lineatus* seminal plasma to date and highlights melatonin as a promising additive for enhancing sperm cryopreservation protocols in aquaculture and conservation.

Key Words: Curimba, fish, reproduction, male

INDICADORES DE IMPACTO

Objetivou-se com este trabalho avaliar os efeitos da suplementação com melatonina na criopreservação do sêmen de *Prochilodus lineatus*, associando análises funcionais da qualidade espermática à caracterização proteômica do plasma seminal, com vistas ao aprimoramento de biotecnologias reprodutivas aplicadas à aquicultura e à conservação de peixes nativos. Os resultados demonstraram impactos tecnológicos e científicos diretos, evidenciados pelo aumento significativo da motilidade espermática pós-descongelamento, da velocidade em linha reta e da velocidade média do trajeto, bem como pela redução de defeitos morfológicos específicos, indicando maior eficiência reprodutiva. No âmbito molecular, a identificação de mais de 1.200 proteínas e a modulação positiva de vias relacionadas ao metabolismo energético, defesa antioxidante e proteostase configuram o conjunto de dados proteômicos mais abrangente já descrito para a espécie, contribuindo de forma inédita para a compreensão da fisiologia reprodutiva de peixes neotropicais. Esses avanços apresentam impacto potencial direto sobre a produtividade aquícola, ao permitir a otimização de protocolos de criopreservação, a formação de criobancos genéticos e a racionalização do uso de reprodutores, reduzindo custos operacionais e ampliando a eficiência de programas de melhoramento genético e reprodução artificial. Do ponto de vista ambiental, os resultados fortalecem estratégias de conservação da biodiversidade aquática, ao fornecer subsídios técnicos para a preservação do material genético de populações naturais e para programas de repovoamento, especialmente em bacias hidrográficas brasileiras sujeitas a pressões antrópicas. O trabalho apresenta caráter extensionista potencial ao gerar conhecimentos aplicáveis a estações de piscicultura, órgãos ambientais e produtores, com impacto indireto sobre comunidades dependentes da pesca e da aquicultura continental. A pesquisa foi desenvolvida no estado de Minas Gerais, envolvendo diretamente docentes, pesquisadores e discentes de graduação e pós-graduação, contribuindo para a formação de recursos humanos qualificados. Os impactos do estudo enquadram-se principalmente nas áreas temáticas de tecnologia e produção, meio ambiente, saúde e trabalho, e estão alinhados aos Objetivos de Desenvolvimento Sustentável da ONU, em especial os ODS 2 (fome zero e agricultura sustentável), 12 (consumo e produção responsáveis), 14 (vida na água) e 15 (vida terrestre), reforçando o papel da ciência aplicada no desenvolvimento sustentável e na gestão responsável dos recursos naturais.

SUMÁRIO

1.0 INTRODUCTION

Prochilodus lineatus (Curimba) is a widely distributed neotropical fish found in major South American river basins. As a detritivore, it plays a key role in nutrient cycling and food webs. Despite its migratory behavior, the species adapts well to captivity, serving as a valuable model for reproductive studies, including sperm cryopreservation. It also holds economic importance, being farmed for aquaculture and fisheries in South America (Galdioli et al., 2000; Viveiros et al., 2009).

Fish sperm cryopreservation is a key biotechnology for aquaculture and conservation, offering benefits such as overcoming broodstock asynchrony, optimizing sperm use, simplifying breeding management, enabling long-distance transport of genetic material, and supporting cryobank establishment for genetic improvement and endangered species preservation (Cabrita et al., 2010). It also ensures preservation of wild genetic diversity, more efficient restocking programs, continuous availability of gametes and protection against population losses from environmental or pathological events (Cabrita et al., 2014).

However, the process involves exposure to ultralow temperatures (−196 °C) and thawing, which can damage plasma membranes, flagella, mitochondria, DNA, and RNA of spermatozoa (Xin et al., 2020). Cryodamage mechanisms include ice crystal formation, osmotic stress, protein denaturation, mitochondrial dysfunction, and cytoskeletal disruption (Cabrita et al., 2010, 2014). These alterations lead to increased production of reactive oxygen species (ROS), causing lipid peroxidation and nucleic acid fragmentation (Sandoval-Vargas et al., 2021). Although sperm possess enzymatic (catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic (vitamin E, glutathione) antioxidant defenses, their effectiveness is reduced by dilution in cryoprotective media, creating oxidative imbalance (Sandoval-Vargas et al., 2021; Xin et al., 2020). This stress ultimately compromises sperm viability, motility, fertilization capacity, and embryonic development.

The supplementation of exogenous antioxidants in sperm cryoprotective media has emerged as a promising strategy to mitigate oxidative stress caused by excessive ROS production during cryopreservation, with studies demonstrating improvements in post-thaw sperm motility, velocity, membrane integrity, reductions in abnormalities, DNA fragmentation, lipid peroxidation, and ROS levels (Felizardo et al., 2011; França et al., 2020; Lahnsteiner; Mansour, 2010; Motta et al., 2022; Palhares et al., 2020; Viveiros et al., 2016). A wide range of antioxidants have been tested, including ascorbic acid, α-tocopherol, glutathione, taurine, resveratrol, and quercetin, but melatonin (N-acetyl-5-methoxytryptamine) stands out due to its amphiphilic properties that allow action in both hydrophilic and lipophilic environments, particularly at the mitochondrial level (Reiter et al., 2010; Carpentieri et al., 2012; H.-M. Zhang & Zhang, 2014). Melatonin enhances antioxidant defenses by stimulating key enzymes like catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase, while inhibiting pro-oxidant enzymes such as nitric oxide synthase, thereby amplifying its overall protective capacity (Cruz et al., 2014; Özgür et al., 2020; Motta et al., 2022).

In fish sperm cryobiology, melatonin supplementation has been investigated and reported for cryopreservation protocols in several species including *Prochilodus lineatus* (curimba) (Lima Assis, 2019), *Brycon orbignyanus* (piracanjuba) (Palhares et al., 2021, 2020), and for short-term storage applications in *Capoeta trutta* (trout barb) (Özgür et al., 2020). Recent studies have also explored melatonin's protective effects in marine species such as *Sparus aurata* (gilthead seabream*), Dicentrarchus labrax* (European seabass), and various salmonid species, however, no studies have yet investigated the proteomic effects of melatonin supplementation in a Brazilian native fish species.

In *P. lineatus*, melatonin supplementation during sperm cryopreservation shows dose-dependent effects. Low doses improve motility parameters, intermediate doses enhance antioxidant protection, while high concentrations impair morphology and fertility. Earlier studies found no improvement in motility alone (Lima Assis, 2019), but recent findings highlight melatonin's broader influence on sperm quality (Motta et al., 2022). Building upon these findings, the present research will be the first to investigate, at the proteomic level, the effects of melatonin on sperm cryopreservation in this species. Given that the underlying molecular mechanisms of melatonin's action remain poorly understood, proteomic analysis represents a powerful approach to uncover the specific proteins and pathways involved.

Proteomics, a key post-genomic technology, enables comprehensive analysis of proteins and their interactions, offering critical insights into cellular physiology and reproduction (Kovac; Pastuszak; Lamb, 2013). In fish, proteomic studies have advanced the understanding of male reproductive biology, including the effects of cryopreservation, mechanisms of sperm motility initiation and impacts of domestication on semen quality (Ciereszko; Dietrich; Nynca, 2017a; Nissa et al., 2021). Such approaches provide biomarkers of reproductive health, support sperm cryopreservation optimization and contribute to conservation and aquaculture sustainability, particularly in species like *P. lineatus*. Label-free proteomic methods, especially when combined with advanced acquisition and separation techniques, enhance sensitivity, reproducibility, and protein coverage, establishing proteomics as a powerful tool for studying sperm function and male gamete biology in aquatic organisms (Nynca et al., 2014; Nynca, et al., 2014; Ciereszko et al., 2017a; Gombar et al., 2017; Dietrich, Arnold, Dietrich & Ciereszko, 2018; Jaiswal et al., 2023).

This study resulted in the first proteome data set of the seminal plasma from *P. lineatus* to investigate protein abundance differences between male alternative reproductive freezings. The quantitative proteomic data offers insights into potentially unique mechanistic aspects of *P. lineatus* alternative reproductive tactics employed for sperm competition and fertilization success.

In spite of growing evidence that seminal plasma plays an important role in influencing sperm quality and fertilization competition, there is limited information regarding its protein composition in *P. lineatus*. This study aims to enhance the understanding of the seminal plasma proteome of *P. lineatus* and to identify functional effects of Melatonin in cryopreservation of this species within wild spawning populations using label-free quantitative proteomics. In addition, it tests the hypothesis that melatonin acts as an antioxidant cryoprotectant, capable of mitigating oxidative stress and protein damage during sperm cryopreservation. Simultaneously, this research contributes to the advancement of reproductive biotechnologies applied to the conservation and sustainable management of neotropical fish genetic resources, potentially establishing optimized protocols for enhanced sperm cryopreservation in this economically and ecologically important species.

2.0 THEORETICAL FRAMEWORK

The streaked prochilod *P. lineatus*, commonly known as curimba or curimbatá, is a neotropical Characiform fish with extensive distribution across major South American hydrographic basins, including the Paraná, Paraguay, Uruguay, São Francisco, and Amazon River systems (Motta et al., 2022). *P. lineatus* (curimba) is one of the most extensively studied south american native fish species in relation to reproduction and larviculture. Under optimal farming conditions, it exhibits high growth rates and good meat quality. Due to its rusticity, precocity, and favorable response to hormonal induction, it is considered an excellent model species for aquaculture studies (Viveiros et al., 2009; Egger et al., 2021).

Its detritivorous feeding habit allows the use of *P. lineatus* in polyculture systems as a secondary species, since it can take advantage of the organic residues produced by the main species. This characteristic increases system biomass without significantly raising feed costs, thus contributing to sustainable production and higher economic value(Motta et al., 2022).

In natural environments, *P. lineatus* can reach up to 4 kg of body weight. Studies with juveniles have reported daily weight gains of 3.6 to 4.5 g, with an initial weight of 2.4 g, and a feed conversion ratio of 1.6 over a 30-day period. These results highlight the potential of adequate management practices, such as fertilization strategies, to further enhance growth performance. Regarding processing potential, specimens weighing 250 g can yield approximately 69% carcass, 59% skin-on fillet, and 50% skinless fillet. These values are superior to those reported for tilapia (*Oreochromis niloticus*), which typically show lower skinless fillet yields. Despite its potential, market acceptance of *P. lineatus* is still limited by the presence of intramuscular bones and the risk of off-flavor (earthy taste). Nevertheless, these issues can be mitigated through processing, since bones can be removed and off-flavor controlled during industrialization (Galdioli et al., 2000).

Despite being a highly migratory species that undertakes extensive reproductive journeys covering hundreds of kilometers during spawning seasons, *P. lineatus* demonstrates remarkable adaptability to captive conditions, making it an invaluable model species for scientific research, particularly in studies focusing on cryopreservation of neotropical fish sperm Additionally, this species holds significant economic importance in South American aquaculture and fisheries, being extensively cultured for both commercial production and stock enhancement programs

(Maria et al., 2006; Viveiros et al., 2009; França et al., 2020; Egger et al., 2021; Motta et al., 2022).

The cryopreservation of fish sperm represents a revolutionary biotechnology that provides numerous strategic advantages for modern aquaculture and conservation biology. These benefits include: elimination of gonadal asynchrony between broodstock, optimization of sperm economy through precise artificial insemination dosing, substantial simplification of breeding management protocols, enhanced facility for long-distance genetic material transport, and establishment of comprehensive cryobanks for genetic improvement programs and endangered species conservation (Cabrita et al., 2010). Furthermore, this technology enables the preservation of genetic diversity from wild populations, development of more efficient restocking programs, year-round availability of high-quality genetic material, and the possibility of creating genetic repositories that can safeguard against catastrophic population losses due to environmental disasters or disease outbreaks (Cabrita et al., 2014).

However, despite these substantial advantages, the cryopreservation process involves exposure to extreme freezing temperatures (typically -196°C in liquid nitrogen) and subsequent thawing procedures that can induce both lethal and sublethal cellular alterations in spermatozoa. The primary mechanisms of cryodamage affect critical cellular components including the spermatozoa plasma membrane, flagellar structures, midpiece mitochondria, nuclear DNA, and mitochondrial RNA (Xin et al., 2020). These damages encompass intracellular ice crystal formation, osmotic shock during freezing and thawing phases, protein denaturation, alterations in membrane fluidity and permeability, mitochondrial dysfunction, and disruption of cytoskeletal elements essential for sperm motility (Cabrita et al., 2010, 2014).

The cellular damage occurring during cryopreservation procedures significantly induces increased generation of reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, and hydroxyl radicals, resulting in extensive lipid peroxidation in both plasma and mitochondrial membranes, as well as DNA and RNA fragmentation (Cabrita et al., 2010). Although spermatozoa possess endogenous antioxidant defense systems, including enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase, along with non-enzymatic antioxidants like vitamin E and glutathione, the cryopreservation process simultaneously generates a substantial reduction in these protective agents due to dilution of sperm and seminal plasma in cryoprotective media, creating a critical imbalance between pro-oxidant and antioxidant forces (Maria et al., 2006; Xin et al., 2020). This oxidative stress cascade ultimately compromises sperm viability, motility parameters, fertilization capacity, and embryonic development potential.

Consequently, to mitigate the oxidative stress induced by excessive ROS production, the strategic addition of exogenous antioxidants to sperm cryoprotective media has emerged as a promising approach currently being investigated across numerous fish species (França et al., 2020; Motta et al., 2022). The implementation of antioxidant supplementation has been consistently associated with significant improvements in post-thaw sperm quality parameters, including enhanced motility rates, increased swimming velocities, improved membrane integrity, reduced morphological abnormalities, decreased DNA fragmentation, minimized lipid peroxidation, and reduced ROS formation (Felizardo et al., 2011; Lahnsteiner; Mansour, 2010; Viveiros et al., 2016). Various antioxidants have been successfully tested, including ascorbic acid, α-tocopherol, reduced glutathione, taurine, and natural compounds such as resveratrol and quercetin (Martínez-Páramo et al., 2012).

Among the diverse array of available antioxidants, the neurohormone melatonin (N-acetyl-5-methoxytryptamine) stands out due to its unique amphiphilic properties, enabling it to act effectively at multiple cellular levels, including both hydrophilic cytoplasmic environments and lipophilic membrane structures, as well as providing specific protection at the mitochondrial level (Anisimov et al., 2006; Reiter; Tan; Lorena, 2010). Melatonin, originally discovered as a pineal gland hormone regulating circadian rhythms, possesses exceptional ability to cross cellular barriers due to its dual lipophilic and hydrophilic nature. This remarkable molecule functions as a highly efficient scavenger of various toxic radicals, including ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and reactive nitrogen species (RNS) including nitric oxide and peroxynitrite (Motta et al., 2022; Özgür et al., 2020). Moreover, several metabolites generated from melatonin's interaction with free radicals, including cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), and N1-acetyl-5-methoxykynuramine (AMK), also demonstrate significant radical-scavenging capabilities, creating a beneficial antioxidant cascade effect. Additionally, melatonin stimulates the activity and expression of several crucial antioxidative enzymes, including catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase, while simultaneously inhibiting pro-oxidant enzymes such as nitric oxide synthase, thereby substantially amplifying its overall antioxidant potential (Cruz et al., 2014; Motta et al., 2022; Özgür et al., 2020).

Melatonin supplementation has consistently demonstrated promising results in fish sperm cryopreservation across multiple species. This innovative approach has successfully provided significant improvements in post-thaw sperm quality parameters, including increased sperm viability and survival rates, enhanced motility percentages, extended motility duration, improved sperm swimming velocities and kinematic parameters, increased fertilization rates and embryonic development success, as well as substantial reductions in sperm morphological abnormalities, lipid peroxidation levels, and reactive oxygen species formation (Félix; Oliveira; Cabrita, 2021; Lima Assis, 2019; Makris et al., 2023; Motta et al., 2022; Özgür et al., 2020).

In a recent comprehensive study examining post-thaw sperm quality in *P. lineatus*, melatonin supplementation did not demonstrate significant improvements in kinetic motility parameters; however, the investigation did not evaluate the effects on oxidative stress-related parameters, membrane integrity, or long-term sperm functionality (Lima Assis, 2019).

Recent studies have shown that supplementation of the cryoprotective medium with melatonin during sperm cryopreservation in *Prochilodus lineatus* affects sperm kinetic, structural, and oxidative parameters. A 2.00 mM dose-maintained motility similar to the control but increased. Sperm curvilinear velocity (VCL) and average path velocity (VAP), preserving membrane integrity and morphology. Medium concentrations mM reduced lipid peroxidation without altering SOD activity and decreased catalase activity, while higher doses impaired morphological quality and fertility. Thus, low doses optimize kinetic performance, intermediate doses enhance antioxidant effects, and high concentrations are detrimental (Motta et al., 2022).

Effective fertilization requires a spermatozoon capable of undergoing a series of sequential and essential processes that ultimately lead to the formation of a viable embryo: sperm capacitation, hyperactivation, penetration through the cumulus mass, adhesion to and penetration through the zona pellucida (ZP), sperm-oocyte membrane fusion, and successful formation of interacting pronuclei (Dacheux,2012). These processes are closely related to changes in the expression and/or configuration of proteins that surround the sperm membrane and interact with membrane structural proteins during epididymal maturation and ejaculation (Khan et al., 2024). Since these changes are associated with fertility-related outcomes, proteomic analysis of seminal plasma (SP) and spermatozoa has emerged as a crucial tool for identifying potential fertility biomarkers (Ashwitha et al., 2023; Khan et al., 2024; Kovac; Pastuszak; Lamb, 2013).

Proteomics represents a significant advancement in the study of proteins, focusing on the comprehensive analysis of protein products expressed by the genome. Emerging as a leading technology in the post-genomic era, proteomics has gained prominence due to the central role of proteins and their interactions in cellular physiology and biological processes (Kovac; Pastuszak; Lamb, 2013). In the context of fish reproduction, the initial applications of proteomic research have begun to reveal critical insights into various aspects of male reproductive biology (Ciereszko; Dietrich; Nynca, 2017b; Nissa et al., 2021). These applications include investigations into the effects of cryopreservation on the semen proteome, the molecular mechanisms that govern the initiation of sperm motility, and the impact of domestication on semen quality in cultured species. By integrating proteomic techniques into the study of male reproduction in fish, researchers are presented with a unique opportunity to unravel the physiological mechanisms that are vital for sperm function, including motility and fertilizing ability (Ciereszko; Dietrich; Nynca, 2017b).

Such biomarkers could provide valuable insights into reproductive health and success, ultimately contributing to the development of more effective reproductive biotechnologies(Kovac; Pastuszak; Lamb, 2013). This is especially relevant for species like *P. lineatus*, where understanding the seminal plasma proteome can inform conservation efforts and optimize sperm cryopreservation protocols. By leveraging proteomic approaches, researchers can better understand the functional differences between alternative reproductive tactics within wild spawning populations, thereby advancing the sustainable management of neotropical fish genetic resources and improving the overall success of aquaculture practices (Ciereszko; Dietrich; Nynca, 2017b; Nissa et al., 2021).

Label-free techniques generally involve comparing normalized precursor ion intensities across various biological and technical replicates to identify differential protein levels. Additionally, when label-free methods are combined with data-independent acquisition modes and orthogonal separation techniques, such as ion mobility separation, they significantly improve reproducibility, sensitivity, and sequence coverage compared to data-dependent spectral counting methods. Consequently, the potential of proteomics has established it as a powerful tool for analyzing protein dynamics in the male gametes of aquatic organisms (Ciereszko; Dietrich; Nynca, 2017b; Dietrich et al., 2014a; Gombar et al., 2017; Jaiswal et al., 2023; Nynca et al., 2014).

3.0 MATERIALS AND METHODS

3.1. Fish handling and sperm collection

This study was approved by the Ethical Committee for Animal Use at the Federal University of Lavras (UFLA) under protocol number 023/18, conducted in Lavras, Minas Gerais, Brazil. Prochilodus lineatus males (n = 9), approximately 4 years old, were selected from fish cultured in earthen ponds at the Minas Gerais Power Company Fish Culture Station (CEMIG) in Itutinga (21°19′16"S; 44°36′54"W), Minas Gerais State, Brazil, during the 2022/2023 spawning season. The fish were fed a commercial extruded feed (28% crude protein) ad libitum, twice daily. Males with body weight (BW) of 606.0 ± 146.7g, releasing a few drops of sperm under gentle abdominal pressure, received a single intraperitoneal dose of carp pituitary extract (CPE; Argent Chemical Laboratories, Redmond, USA) at 4 mg/kg BW, 8 hours before sperm collection. These protocols align with the reproduction practices routinely employed at the Fish Culture Station to induce spermiation.

Sperm and seminal plasma were collected at room temperature (~27 °C) of 9 animals. Immediately after collection, sperm samples were stored in tubes placed in a cooler with chemical ice (~6–9 °C) for a maximum of 30 minutes. Strict measures were taken to prevent contamination with water, urine, or feces during collection. Each sperm sample was subjectively evaluated for motility, expressed as the percentage of motile sperm (Gonçalves, 2013). Motility analysis was performed after activation in a 150 mOsm/kg glucose solution (Viveiros, 2016), using a light microscope (Olympus® CX22LED, Tokyo, Japan) at 100× magnification. Only samples achieving at least 90% motility were included in subsequent cryopreservation procedures. Additionally, 500 µL of seminal plasma was collected from each sample for subsequent proteomic analysis. The samples were centrifuged at 1600 × g for 30 min using a CAPP (model CAPPRondo CR-68, Denmark) benchtop centrifuge, and the supernatant was carefully collected and stored for further analysis.

3.2 Sperm characterization

The assessment of membrane integrity was performed using the nigrosin-eosin staining technique. Sperm was stained with nigrosin-eosin (2 µL sperm: 4 µL nigrosin-eosin), and 200 cells were counted in distinct histological slide fields under light microscope (Olympus®

CX22LED, Tokyo, Japan) at × 400 magnification. Two wet preparations per sperm were analyzed. White spermatozoa (unstained) were considered to have an intact membrane and those with pink or red heads were considered to have damaged membranes. Sperm membrane integrity was calculated as the percentage of unstained cells, the result was given by the formula: membrane integrity (%) = (number of unstained cells/200 cells counted) × 100 (Maria et al., 2012).

For morphological sperm analysis, the sperm was diluted at a ratio of 1: 1000 in citrate formaldehyde solution (2.9% sodium citrate, 4% commercial solution of formaldehyde 35% and distilled water; Vetec Química Fina Ltd., Duque de Caxias, Brazil). The diluted sample was stained with 4% Rose Bengal (3: 20; stain: sperm) and two wet preparations per sperm sample were analyzed (Motta et al., 2022). Each sample was observed under a light microscope (Olympus® CX22LED, Tokyo, Japan) at × 1000 magnification and the morphology of 200 sperm cells were 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 (Table 1) (Motta et al., 2022; Murgas et al., 2007). Data were recorded as the percentage of abnormal sperm cells.

Table 1. Morphological damage evaluated in post-thaw sperm in *Prochilodus lineatus*.

Spermatozoa Segment	**Primary Damages**	**Secondary Damages**
Head	Macrocephaly	Free normal
	Microcephaly	
	Degenerated	
Midpiece	Degenerated	Proximal droplet
Tail	Fractured	Simple bent
	Stump	Distal droplet
Tail	Strongly coiled	

3.3 Sperm Cryopreservation and post-thaw sperm analysis

Individual male samples were used for sperm cryopreservation, which was conducted within 45 minutes of collection, following the methodology described by (Motta et al., 2022) with melatonin supplementation. The cryoprotective medium consisted of 5% glucose (325 mOsm/kg; pH 7.6) diluted in reverse osmosis water as an extender, with methyl glycol [CH3O(CH2)2OH] serving as the cryoprotectant. Chemicals were obtained from Vetec Química Fina Ltd. (Duque de Caxias, Rio de Janeiro, Brazil).

Each sample was diluted in the cryoprotective medium at a ratio of 1:8:1 (50 µL sperm [10% v/v] + 400 µL extender [80% v/v] + 50 µL methyl glycol [10% v/v]). Melatonin (N-acetyl-5-methoxytryptamine, M5250; molecular weight 232.28, Sigma-Aldrich, Missouri, USA) was dissolved in the cryoprotective medium, comprising the extender and methyl glycol, at a final concentration of 2.00 mM. The control group included sperm samples combined with the cryoprotective medium without melatonin (PSC). All samples were used in the cryopreservation protocols, both for each melatonin (PSCM) concentration and for the control group (PSC).

Samples were loaded into 0.5 mL straws (total of 180 straws; 9 samples × 10 replicate straws × 2 treatments). Loaded straws were kept for 10 min at room temperature (~27 °C) during equilibration time (Viveiros et al., 2016) and placed on racks. The racks were frozen 5 cm from the surface of the liquid nitrogen for 12 min and then transferred to a cryogenic tank (BioCane 34 Thermo Fisher Scientific, Dubuque, USA) at -196 C for storage (Motta et al., 2022)

Straws were individually thawed in a water bath (Water-bath MA 127, Marconi, São Paulo, Brazil) at ~ 60 C for 8 s (Viveiros et al., 2016).

3.3.1. Computer-Assisted Sperm Analysis (CASA)

Straws (n = 3 straws per sperm pool) 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 measured using the Computer-Assisted Sperm Analysis (CASA) system, following the methodology described by Motta et al., 2022. 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 acA 1300-200uc, 106754-26, 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™, Microptics, S.L. Version 6.6, Barcelona, Spain). Each straw was analyzed in duplicate. 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 spermatozoon was individually followed throughout the recorded video images from which sperm trajectories were evaluated.

3.4 Sample collection and protein extraction

For the preparation of samples for proteomic analysis it was used a pool of the 9 animals per treatment, and 500 µL of fresh semen was centrifuged at 1600 × *g* for 30 minutes using a centrifuge from CAPP (model CAPPRondo CR-68, Denmark) was used. The supernatant (seminal plasma) was then carefully collected and stored for subsequent analysis. For proteomic concentration, each male pool (n=9) sample (2.22 mL) was mixed with a precipitation solution [50% acetone, 49.5% ethanol, 0.5% acetic acid] at a 1:4 ratio and incubated overnight (24 hours) at -20°C.

The samples were then centrifuged at 2,000 × *g* for 20 minutes at 4°C (Megafuge 16R, Thermo Fisher Scientific, USA). The supernatant was discarded, and the resulting pellets were washed with cold acetone to remove residual impurities. After this, the pellets were air-dried at room temperature for 10-15 minutes to remove residual solvent completely. Finally, the prepared samples were aliquoted and sent to a specialized laboratory for proteomic analysis.

3.5 Sample Preparation

Initially, the samples were combined and designated as follows: Sample 1: PSF (Fresh); Sample 2: PSC (Control Cryopreserved); and Sample 3: PSCM (Cryopreserved Melatonin). For protein precipitation and separation, 200 µL of each sample was utilized, to which 1 mL of a solution composed of acetone, ethanol, and formic acid (in a ratio of 50:49.5:0.5) was added. The samples were subsequently stored in a freezer overnight. Following this, centrifugation was performed at 12,000 × *g* for 10 minutes at 4°C, resulting in a pellet containing the proteins. This pellet was dried using a SpeedVac for 5 minutes and then resuspended in 50 µL of 150 mM Tris-HCl buffer (pH 8.8) containing 8 M urea and 0.5% octyl glucoside (OG). Protein quantification was then conducted.

3.6 Protein Quantification

To ascertain the protein mass present in the samples, quantification was performed using the Bradford method with the Protein Assay Dye Reagent Concentrate (Bio-Rad, catalog #500-0006). A standard curve was established using various dilutions of bovine serum albumin (BSA), sourced from a commercially acquired stock (Protein Standard 200 mg/mL, Sigma, Catalog #P5369-10 mL). The samples were diluted 10x or 20x and distributed in duplicates (20 µL each) onto a microplate. Absorbance was measured at 595 nm using a spectrophotometer (Molecular Devices, SpectraMax Plus 384). From the obtained data, the total protein mass in the samples was estimated.

3.7 Sample Preparation for Proteomic Analysis

The preparation of samples for advanced mass spectrometry involved three primary steps: i) reduction and alkylation of proteins, ii) enzymatic digestion of proteins with trypsin, and iii) clean-up/desalting of the samples. A total of 35 µg of proteins was utilized. In summary, the samples underwent reduction of disulfide bridges in the proteins by adding 50 µg of dithiothreitol (DTT) and incubating for 1 hour at 37°C, followed by alkylation with the addition of 250 µg of iodoacetamide (I.A.) and incubation for 1 hour at room temperature in the dark. The sample volume was then diluted eightfold with 0.05 M Tris-HCl buffer (pH 8.8). The samples were subsequently incubated with 0.5 µg of trypsin (Promega, V511A) at 37°C overnight. Prior to mass spectrometry analysis, clean-up/desalting was performed using the OASIS HLB Cartridge 1cc column (catalog number: 186000383, Waters), following the manufacturer's instructions. Briefly, the column was equilibrated with a 2% acetonitrile solution containing 0.1% formic acid, and the material of interest was eluted with 50% acetonitrile. The samples were then dried in a SpeedVac in low protein-binding tubes (Eppendorf) and prepared for mass spectrometry analysis.

3.8 Advanced Mass Spectrometry Analysis

The prepared sample, as described above, was analyzed using a state-of-the-art mass spectrometer: Orbitrap Eclipse (ThermoFisher), coupled with a nano-flow chromatography system (Dionex Ultimate 3000 RLSCnano System, Thermofisher). Approximately 1 µg of the sample was injected for analysis. Peptides were separated on a Nanoease MZ peptide BEH C18 column (130A, 1.7 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nL/min using a gradient of 4-50% acetonitrile over 90 minutes. Data were acquired in MS1 mode within the M/Z range of 375–1500 (120,000 resolution, AGC target 1E6 with a maximum injection time of 100 ms). The most abundant ions were subjected to MS/MS analysis with a collision energy of 30%, a precursor isolation window of 1.2 m/z, AGC target of 1E5, and a resolution of 15,000. The raw data files (.raw) were converted to mzXML format and processed using the Comet algorithm (v. 2018). The UniProt database for *Prochilodus magdalenae* (genome ASM24036415.1) was utilized because no proteomic or genomic data were available for *Prochilodus lineatus*. This approach was adopted since *P. magdalenae* is a closely related species within the same genus, allowing for reliable protein identification and annotation. To our knowledge, this is the first study to investigate the proteomic profile of *P. lineatus*, providing a foundational reference for future research on sperm cryopreservation in this species. The peptides identified that met the maximum error criterion of 3% as determined by Peptide Prophet were used for intensity extraction for quantification via the Xpress algorithm. The peptides and their corresponding intensities were grouped to generate the protein intensity using a script in the R programming language.

3.9 Bioinformatic Analysis

Among the identified proteins, those exhibiting at least a twofold increase in relative abundance (>2×) were classified as upregulated, whereas those showing a reduction to half or less of their abundance (≤0.5×) were classified as downregulated when comparing the control and melatonin-treated groups. Proteins meeting these fold-change thresholds and showing statistical significance after False Discovery Rate (FDR) correction (FDR < 0.05) were defined as *differentially expressed proteins* (DEPs). This dual criterion combines quantitative and statistical evidence, minimizing false-positive identifications and ensuring the reliability of proteomic interpretation.

Functional enrichment analyses were then performed separately for the up- and downregulated protein sets. For each comparison (PSC/PSF and PSCM/PSF, PSCM/PSC), the five Gene Ontology (GO) terms with the lowest FDR-adjusted p-values were selected for discussion, representing the most significantly enriched biological processes and molecular functions. The enrichment results revealed highly significant FDR values (ranging from 10^{-6} to 10^{-35}), highlighting pathways primarily associated with protein folding, catabolic and metabolic processes, ATP hydrolysis activity, and proteasome function.

These enriched categories correspond to the most robust molecular signatures modulated by melatonin supplementation, providing insight into the cellular mechanisms potentially involved in sperm protection and functional maintenance during cryopreservation.

3.10 Statistical analyses

Data were expressed as mean ± standard deviation (SD). Prior to statistical comparisons, data were assessed for normality using the Shapiro–Wilk test and for homoscedasticity using the Levene test (homogeneity of variances). When these assumptions were met, paired *t*-tests were performed to evaluate significant differences between groups. Statistical analyses were conducted using GraphPad Prism software version 10.3, and the level of significance for all tests was set at 5% ($P < 0.05$).

4.0 RESULTS

4.1 Computer-Assisted Sperm Analysis

Each fresh sperm was individually evaluated, and yielded mean values for motility rate of 96.52 ± 3.84%, VCL of 133.70 ± 32.08, VSL of 46.51 ± 10.32, VAP of 82.49 ± 17.88 and BCF of 11.64 ± 00.93. The average sperm concentration of the pools was 25.2 ± 5.37 × 10^9 spermatozoa/mL.

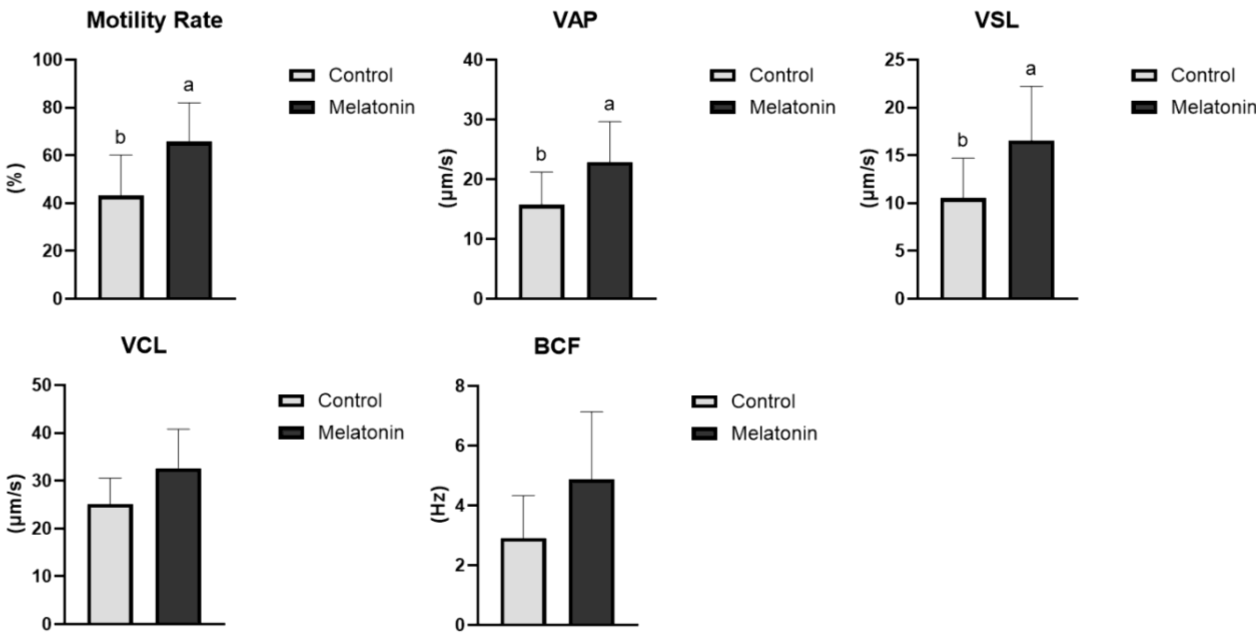


Fig. 1. Post-thaw motility rate, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), and beat-cross frequency (BCF) of *Prochilodus lineatus* sperm fresh and cryopreserved in solutions without (control) and with melatonin (2.00mM). Bars indicate mean ± standard deviation; n = 9 males. ^{a,b} Means followed by different superscript differ (P < 0.05 (student t test)).

Table 2: Post-thaw Motility (%), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), and beat-cross frequency (BCF) of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with Melatonin 2mM.

	Control (PSC)	Melatonin (PSCM)	p value
Motility (%)	43,15 ± 16,95^{b}	65,72 ± 16,32^{a}	**0,042**
VCL (µm/s)	25,17 ± 05,39	32,63 ± 08,19	0,075
VSL (µm/s)	10,53 ± 04,19^{b}	16,57 ± 05,64^{a}	**0,033**

VAP (µm/s)	15,73 ± 05,48^{b}	22,90 ± 06,75^{a}	**0,046**
BCF (Hz)	02,91 ± 01,42	04,88 ± 02,25	0,061

Data were expressed as mean ± standard deviation (SD); n = 9 males, ^{a,b} Means followed by different superscript in the same column differ (P < 0.05, Student t test).

Similarly, the parameters of straight-line velocity (VSL) and average path velocity (VAP) were significantly higher in sperm treated with melatonin (p = 0.042 and p = 0.049, respectively), suggesting that this antioxidant contributed to maintaining the integrity and efficiency of sperm movement after the freezing and thawing process.

4.2 Sperm Membrane Integrity and Morphology

Table 3: Post-thaw membrane integrity and morphology of *Prochilodus lineatus* sperm cryopreserved in solutions with Melatonin 2mM (PSCM) and without melatonin (PSC).

	Membrane Integrity (%)	Primary Damages (%)	Secundary Damages (%)	Normal Cells (%)
PSC	73,64 ± 12,20%	33,52 ± 13,61	18,11 ± 1,94	48,37 ± 11,99
PSCM	71,92 ± 13,94%	28,89 ± 10,25	20,59 ± 3,54	50,52 ± 13,61

Note: Primary damage includes head degeneration, midpiece degeneration, truncated tail, fractured tail, strongly coiled tail, macrocephaly, and microcephaly; Secondary damage includes normal free head, simple folded tail, proximal droplet, and distal droplet. The data are expressed as mean ± standard deviation (SD); n = 9 males.

No significant difference was observed in membrane integrity between the standard freezing group and the melatonin-treated group (P > 0.05) (Table 3). Regarding morphology, no morphological differences were observed for primary and secondary damage (P > 0.05) between treatments. Primary damage was mainly represented by degenerated head (11.61%) and degenerated midpiece (8.66%), while secondary damage was primarily represented by simple folded tail (15.08%) and normal free head (2.34%). However, among the primary defects, a significant difference was observed in the defect of strongly coiled tail, being lower for the melatonin-treated frozen group ($P < 0.05$). The percentage of normal cells did not differ between the control and the melatonin-supplemented samples.

4.3 Proteomics Analysis

The protein quantity was determined using the Bradford method.

Protein concentration (mg/mL) in the fresh, frozen, and frozen with melatonin groups. Results are shown as the average obtained in mg/mL in the test; SD – standard deviation among repetitions; CV – coefficient of variation in percentage; Dilution – sample dilution in the test; Protein concentration – mg/mL (Average Result x Dilution) (Table 4).

Table 4: Protein concentration in semen of *Prochilodus lineatus*: fresh semen (PSF), standard cryopreservation (PSC), and cryopreservation with 2 mM melatonin (PSCM)

Sample	Wells	Value	Mean Results	SD	CV	Dilution	Protein Concentration (mg/mL)
PSF	A3	0.139	0.039	0.007	17.9	10	0.4
	A4	0.115					
PSC	B3	0.508	0.0274	0.046	16.8	10	2.7
	B4	0.609					
PSCM	C3	0.9	0.029	0.029	5.4	20	10.7
	C4	0.954					

The area of the identified peaks (ions) was used as an approximate indicator of protein abundance. A total of 1,238 proteins were identified. In the 1,238 proteins, with 128 being exclusive to the fresh group, 38 in control group and 8 exclusives to the frozen melatonin group (Figure 3).

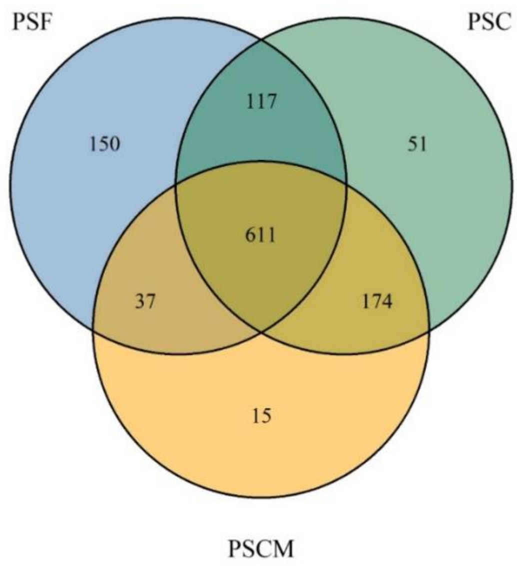


Figure 3: Venn diagram illustrating the quantification and overlap of proteins identified in the seminal plasma of the fresh (PSF), standard cryopreserved (PSC), and melatonin-supplemented cryopreserved (PSCM) groups, highlighting proteins that are unique to each group and those shared among treatments.

Gene Ontology (GO) bioinformatic analysis provides an overview of the dominant functional traits of isolated proteomes. For the biological process category, Cellular process and metabolic process are most represented in the proteome (Fig 1). With regards to molecular function, the predominant gene function activities represented in the *Prochilodus lineatus* seminal fluid are binding activity and catalytic activity (Fig 1). A similar GO profile was observed in the closely related Chinahook salmon (Gombar et al., 2017), rainbow trout seminal plasma (Nynca et al., 2014) and carp seminal plasma proteome studies (Dietrich et al., 2014a), providing a level of validation for the identified proteins.

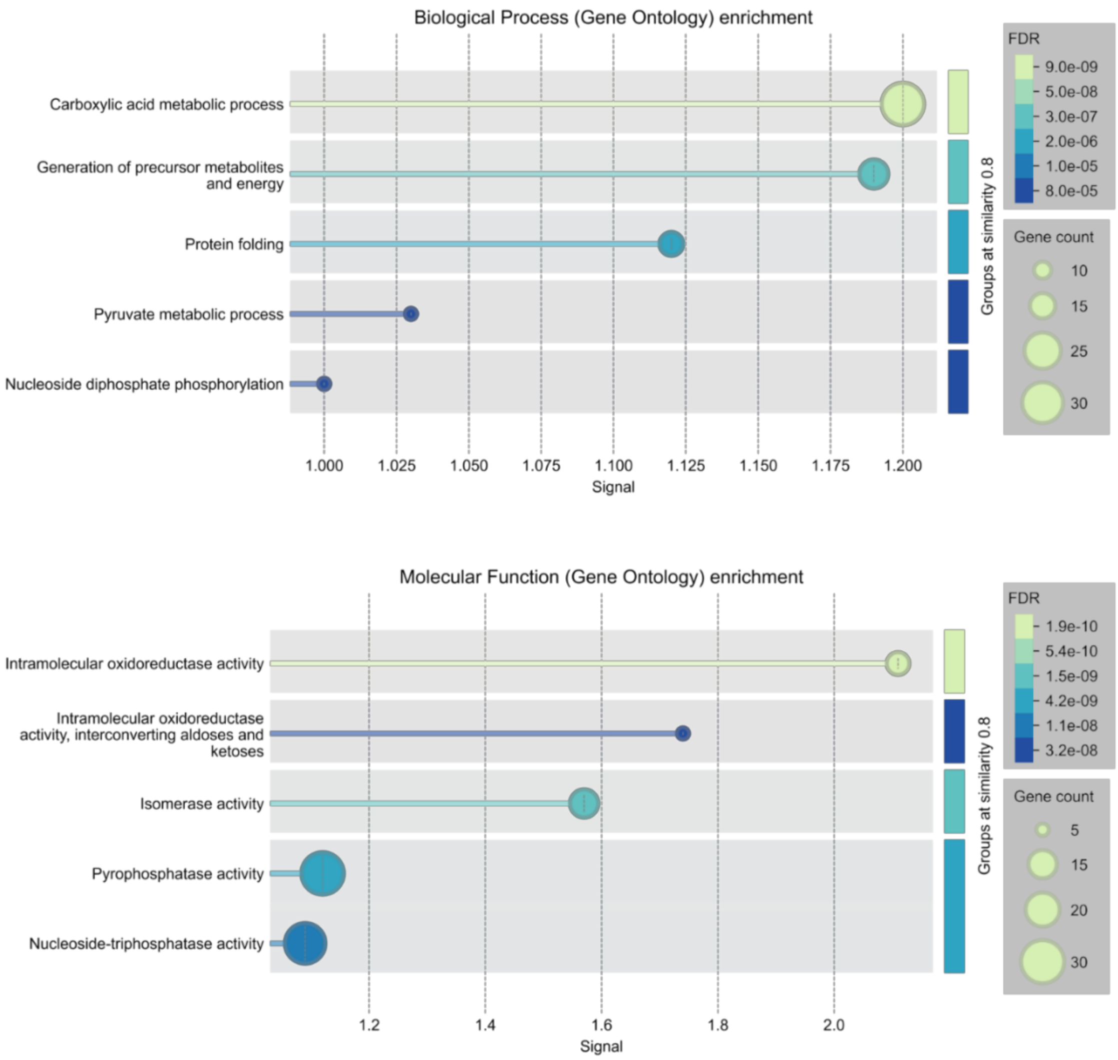
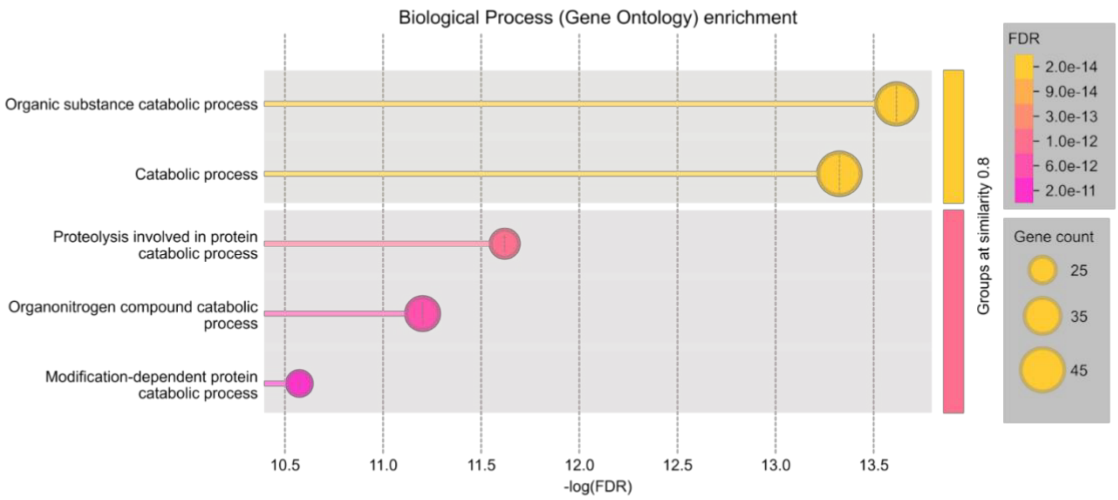


Figure 4: Proteins downregulated to 0.5 times lower in the standard freezing sample (PSC) compared to the fresh sample (PSF) in *Prochilodus lineatus*, with enrichment in biological processes and molecular functions.

For the enrichment of biological processes, the top five are: metabolic process of carboxylic acids, generation of precursor metabolites and energy, protein folding, metabolic process of pyruvate, and phosphorylation of nucleoside diphosphate. For molecular function, the top five are: intramolecular oxidoreductase activity, intramolecular oxidoreductase activity with interconversion of aldoses and ketoses, isomerase activity, pyrophosphatase activity, and nucleoside triphosphatase activity.



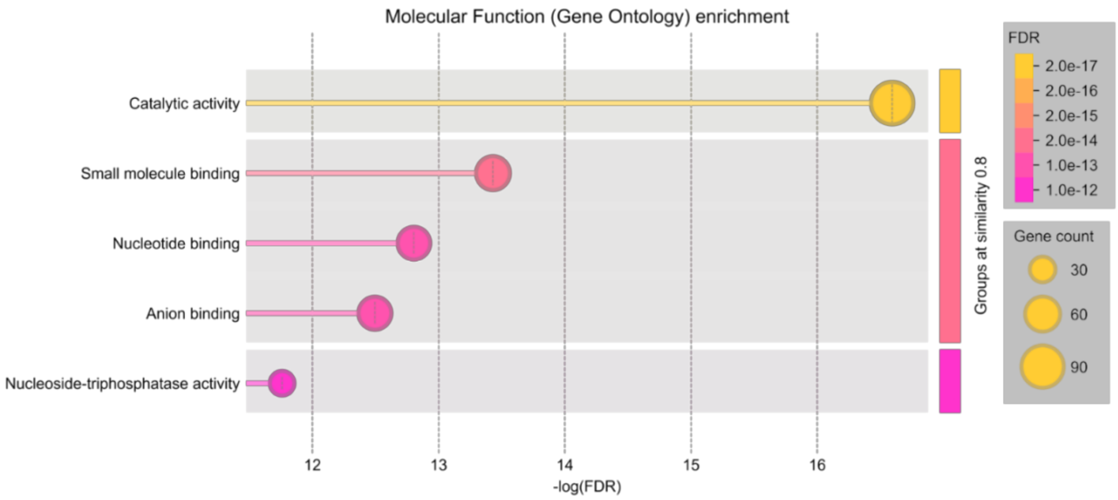


Figure 5: Proteins upregulated two times higher in the melatonin 2mM freezing sample (PSCM) compared to the standard freezing sample (PSC) in *Prochilodus lineatus*, with enrichment in biological processes.

Among these, the top five are: catabolic process of organic substances, catabolic process, catabolic process of proteins involving proteolysis, catabolic process of organonitrogen components, and catabolic process of protein modification. For the enrichment of molecular functions, the top five proteins are: catalytic activity, binding to small molecules, nucleotide binding, binding to anions, and nucleoside triphosphatase activity.

5.0 DISCUSSION

The results of the present study demonstrate that the addition of melatonin to the cryopreservation medium positively influenced the post-thaw sperm quality of *Prochilodus lineatus*. The significant increase in motility rate, as well as in VSL and VAP (Fig 1; Table 2) parameters, suggests that melatonin plays a protective role during the freezing and thawing processess. This effect is likely related to the antioxidant properties of melatonin, which help minimize the oxidative stress commonly generated during cryopreservation (Félix; Oliveira; Cabrita, 2021; Makris et al., 2023).

Cryopreservation induces the formation of reactive oxygen species (ROS) that can damage the sperm membrane, mitochondria, and DNA, ultimately impairing motility and fertilizing potential. Melatonin, being a potent free radical scavenger, may prevent these damages by reducing lipid peroxidation and preserving membrane integrity and mitochondrial function, ensuring better energy supply and motile activity after thawing (Makris et al., 2023; Shi et al., 2024).

Although not all kinematic parameters showed statistically significant differences, the general trend toward improved values in the melatonin group reinforces its beneficial role as a cryoprotective additive. Parameters like VCL and BCF, which are related to the oscillatory motion and beat pattern of the flagellum, may be less sensitive to oxidative stress or may require a higher concentration or longer exposure to melatonin to show significant differences These findings indicate that melatonin supplementation could be a promising strategy to enhance sperm cryoresistance and maintain reproductive performance in fish species subjected to cryopreservation (Anisimov et al., 2006; Motta et al., 2022).

This study investigated the protein abundance of proteins associated with protein degradation, energy metabolism, and the response to oxidative stress, emphasizing their relationship with spermatogenesis and sperm integrity, with higher expression observed in the melatonin group (PSCM). This indicates upregulation of these enzymes and may suggest that these proteins play a positive role in sperm function or semen quality (Aitken; Baker, 2008; Nynca et al., 2014).

Functional enrichment analyses were then performed separately for the up- and downregulated protein sets. For each comparison (PSC/PSF, PSCM/PSF, and PSCM/PSC), the five Gene Ontology (GO) terms with the lowest FDR-adjusted p-values were selected for discussion, representing the most significantly enriched biological processes and molecular functions. The enrichment results revealed highly significant FDR values (ranging from 10^{-6} to 10^{-35}), highlighting pathways primarily associated with protein folding, catabolic and metabolic processes, ATP hydrolysis activity, and proteasome function. These enriched categories correspond to the most robust molecular signatures modulated by melatonin supplementation, providing insight into the cellular mechanisms potentially involved in sperm protection and functional maintenance during cryopreservation.

In the comparison between PSC and PSF, upregulated proteins were mainly associated with protein folding (GO:0006457) and catabolic processes (GO:0009056, GO:1901575, GO:1901565), all showing highly significant FDR values (10^{-8}–10^{-10}). These enrichments indicate an increased activity of protein quality control mechanisms, particularly involving molecular chaperones and proteolytic systems, which may help maintain protein integrity during cryopreservation. The enrichment of ATP hydrolysis and hydrolase activities suggests elevated energy consumption linked to protein refolding and degradation, consistent with a protective adaptive response promoted by melatonin to counteract stress-induced protein denaturation (Aitken; Baker, 2008).

Conversely, among the downregulated proteins in the PSC/PSF comparison, the most significant biological processes were also related to protein folding, catabolic, and proteolytic pathways, with FDR values between 10^{-6} and 10^{-10}. This downregulation may indicate that melatonin reduces the excessive activation of proteolysis and stress-related protein turnover, thereby preventing unnecessary protein degradation. The decreased abundance of proteins involved in unfolded protein binding and ATP-dependent chaperone activity suggests that melatonin contributes to maintaining redox and protein homeostasis, reducing the cellular demand for damage control mechanisms during freezing (Aitken; Baker, 2008; Zhang et al., 2025).

In the PSCM/PSF and PSCM/PSC comparisons, the upregulated proteins were enriched in carboxylic acid metabolic process, amino acid metabolism, and oxidoreductase and catalytic activities (FDR 10^{-6}–10^{-12}), indicating that melatonin supplementation enhances metabolic adaptability and antioxidant defense during cryopreservation. These pathways are consistent with the known role of melatonin in modulating cellular redox balance and energy metabolism. Conversely, downregulated proteins were enriched in proteasome-related and protein catabolic processes (FDR 10^{-11}–10^{-21}), suggesting that melatonin suppresses excessive proteolytic degradation, possibly conserving energy and reducing oxidative damage to sperm proteins. Altogether, these results indicate a shift from catabolic to protective and metabolic processes, supporting the role of melatonin in improving post-thaw sperm quality (Ciereszko; Dietrich; Nynca, 2017a; Li et al., 2023a; Zhang et al., 2025).

The data revealed a consistent and coordinated downregulation of enzymes involved in energy metabolism, antioxidant defense, protein folding, amino sugar and glycoprotein metabolism, and other processes critical to fertilization downregulation in PSC compared do PSF. Such alterations highlight that cryopreservation-induced damage is not limited to isolated molecular targets but affects entire metabolic and structural networks, leading to a multifaceted decline in sperm quality. These findings are consistent with previous reports identifying cryopreservation as a driver of profound, multifactorial metabolic dysfunctions (Aitken; Baker, 2008; Li et al., 2023a; Shi et al., 2024; Xin et al., 2020; Zhang et al., 2025).

A central observation concerns the disruption of energy metabolism. Downregulation of Krebs cycle enzymes in the PSC group, such as isocitrate dehydrogenase (IDH2), aconitase (ACO1), fumarate hydratase, citrate synthase, and succinyl-CoA ligase limit the generation of critical intermediates, including α-ketoglutarate, citrate, and NADPH (Amaral et al., 2013). These deficits directly compromise oxidative phosphorylation capacity and reduce ATP availability, essential for sperm motility, capacitation, and fertilization competence (Piomboni et al., 2012). Glycolytic enzymes—including aldolase (ALDOC), sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS), phosphoglycerate mutase, and triosephosphate isomerase—were also downregulated, restricting glycolytic flux, decreasing pyruvate production, and ultimately impairing mitochondrial ATP synthesis. Together, these alterations establish an energy-deficient state that severely affects the bioenergetic profile of frozen-thawed spermatozoa (Aitken; Curry, 2010; Amaral et al., 2013; Zhang et al., 2023).

Oxidative metabolism is further impaired by suppression of key enzymes in the pentose phosphate pathway (PPP), including 6-phosphogluconate dehydrogenase (PGD) and ribose-5-phosphate isomerase (RPIA), which are essential for generating NADPH. Reduced NADPH availability limits the cell’s ability to neutralize reactive oxygen species (ROS), contributing to oxidative stress. This antioxidant deficit, compounded by mitochondrial dysfunction and disrupted redox balance, promotes the accumulation of oxidative damage to DNA, lipids, and proteins—damage long recognized as one of the primary constraints of sperm cryosurvival (Du Plessis et al., 2015; Fang et al., 2014).

Parallel to these metabolic changes, there is a clear compromise in proteostasis. Downregulation of protein disulfide isomerases (PDIs), peptidyl-prolyl isomerases, thioredoxin domain-containing proteins (e.g., TXNDC5), and endoplasmic reticulum chaperones such as ERP44 indicates impaired protein folding, disulfide bond rearrangement, and structural maturation of acrosomal and membrane proteins (Fietz et al., 2024). These defects are likely to cause the accumulation of misfolded proteins, protein aggregation, and endoplasmic reticulum stress, which may activate apoptotic pathways and disrupt key sperm functions such as capacitation (Leahy et al., 2019; Xin et al., 2020; Zhang et al., 2025).

Alterations were also evident in amino sugar and glycoprotein metabolism. Downregulation of glucosamine-6-phosphate isomerase (GNPDA2) and mannose-6-phosphate isomerase (MPI) suggests impaired glycosylation of sperm surface proteins, a process essential for oocyte recognition, membrane fusion, and zona pellucida binding. This biochemical disruption is further aggravated by decreased activity of nucleoside triphosphatases, including ATPases, which play a crucial role in cytoskeletal dynamics, ion transport, and signal transduction during fertilization (Dzyuba et al., 2017; Li et al., 2024; Nynca et al., 2015).

The accumulation of inorganic pyrophosphate (PPi) inhibits Pi-dependent enzymes, including those of the glycolytic pathway, thereby further restricting ATP generation (Dzyuba et al., 2017). This inhibition, combined with the overall depletion of ATP, directly limits the energy available for critical processes such as motility, capacitation, and hyperactivation. In parallel, disturbances in calcium (Ca^{2+}) homeostasis impair both hyperactivation and the acrosome reaction, compromising fertilization potential (Naaby-Hansen et al., 2010). Furthermore, PPi accumulation promotes protein aggregation by inhibiting chaperone activity, which increases the burden of misfolded proteins and disrupts proteostasis. Together, these alterations establish a self-reinforcing loop in which energy deficiency and proteostatic failure intensify oxidative stress and functional deterioration in cryopreserved spermatozoa (Aitken; Baker, 2008; Ciereszko; Dietrich; Nynca, 2017b; Sandoval-Vargas et al., 2021; Zhang et al., 2025).

Collectively, these results demonstrate that cryopreservation induces a systems-level disruption of sperm metabolism and structure, in which impaired ATP generation, oxidative stress, altered ion homeostasis, and defective protein processing act synergistically to reduce sperm quality. This integrated perspective explains why post-thaw interventions limited to energy supplementation often fail to fully restore sperm function (Dietrich et al., 2015b; Nynca et al., 2015; Pini et al., 2018; Sandoval-Vargas et al., 2021).

Our study demonstrates that melatonin supplementation during sperm cryopreservation induces a broad upregulation of proteins involved in energy metabolism, protein degradation, oxidative stress defense, cytoskeletal organization, and structural maintenance. Such proteomic modulation appears to mitigate the deleterious effects typically associated with freeze–thaw cycles, thereby improving post-thaw sperm viability, motility, and potentially fertilization rates (Li et al., 2023a; Lima Assis, 2019).

A consistent finding was the enhanced expression of enzymes associated with ATP production. Glycolytic proteins such as glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDHS), phosphoglycerate kinase 1 (PGK1) (Rojas-Pirela et al., 2020), and lactate dehydrogenase A (LDHA), alongside mitochondrial components including ATP synthase subunit alpha (ATP5A1), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), citrate synthase (CS), and malate dehydrogenase (MDH1), were significantly upregulated. These results indicate that melatonin preserves both glycolytic and oxidative phosphorylation pathways, ensuring an adequate and continuous ATP supply necessary for motility and capacitation (Piomboni et al., 2012; Rojas-Pirela et al., 2020; Storey, 2008).

In parallel, proteins involved in antioxidant defense also showed increased abundance. Superoxide dismutase 2 (SOD2), catalase (CAT), peroxiredoxin 1 (PRDX1), aldehyde dehydrogenase 2 (ALDH2), and crystallin lambda 1 (CRYL1) were among the most upregulated (Fang et al., 2014; Motta et al., 2022). Given that SOD2 is a mitochondrial enzyme responsible for detoxifying superoxide radicals, its upregulation, together with the increased abundance of proteins from the electron transport chain, suggests a coordinated response in which melatonin may enhance mitochondrial efficiency while simultaneously reducing reactive oxygen species (ROS) accumulation. This dual action likely contributes to maintaining redox balance, preventing lipid peroxidation, membrane destabilization (O'Flaherty, 2014, 2018; Vasiliou; Pappa; Petersen, 2001).

Melatonin may stimulate the antioxidant response through activation of the Nrf2 (nuclear factor erythroid 2–related factor 2) signaling pathway. Under oxidative stress, Nrf2 dissociates from its cytoplasmic repressor Keap1 and translocates to the nucleus, where it binds to antioxidant response elements (ARE) in the promoter regions of target genes. This transcriptional activation increases the expression of antioxidant enzymes such as superoxide dismutase (SOD2), catalase (CAT), and peroxiredoxins, thereby enhancing the cell's capacity to detoxify reactive oxygen species and maintain redox homeostasis. Previous studies have shown that melatonin upregulates Nrf2 activity, protecting mitochondria and promoting cellular resistance to oxidative injury during cryogenic or stress conditions (Li et al., 2023a; Ruan et al., 2024). Thus, the observed increase in SOD2 and CAT abundance in melatonin-treated sperm may result from the activation of the Nrf2-mediated antioxidant defense pathway.

Moreover, melatonin promoted the upregulation of multiple proteasome subunits (PSMD3, PSMC5, PSMA5, PSMB1, PSMA3, PSMC2, PSMC4, PSMC6) and regulators of the ubiquitination cycle such as HUWE1, ubiquitin-like modifier activating enzyme 1 (UBA1), and ubiquitin-specific peptidase 7 (USP7) (Inaba; Morisawa; Morisawa, 1998; Li et al., 2023a; Zimmerman; Sutovsky, 2009). The coordinated activation of the ubiquitin–proteasome system likely facilitates the targeted degradation of damaged or misfolded proteins, preventing their aggregation and maintaining proteostasis (Druart; De Graaf, 2018; Mohanty; Swain; Samanta, 2015). In addition, the concurrent upregulation of molecular chaperones including heat shock protein 90 beta (HSP90B1), heat shock protein family A member 5 (HSPA5), and heat shock 60 protein 1 (HSPD1) further supports protein quality control, assisting in correct folding and preventing denaturation under cryogenic stress (Dietrich et al., 2014b, 2015a, 2016).

Structural and motility-associated proteins were also upregulated. These included tubulin alpha and beta chains (TUBB4B), septins (SEPTIN3, SEPTIN7A, SEPTIN10), and actin, which are essential for maintaining flagellar structure, morphology, and motility (Palander; El-Zeiry; Trimble, 2017; Torraca et al., 2023). Additionally, calcium-transporting ATPase (ATP2B4), a key regulator of intracellular calcium homeostasis, was upregulated, suggesting improved control of calcium-dependent signaling pathways involved in capacitation and the acrosome reaction (Dietrich et al., 2016; Ju; Huang, 2004; Ulloa-Rodríguez et al., 2017). Proteins implicated in DNA repair and genomic stability such as thyroid hormone receptor interactor 13 (TRIP13) and valosin-containing protein (VCP) were also upregulated, potentially contributing to the preservation of genetic integrity during cryostorage (Singh et al., 2025; Vanderhaeghe et al., 2024), but there are no studies confirming that hypothesis.

Identifying proteins exclusive to melatonin-treated samples can elucidate molecular mechanisms of protection against freeze-induced damage. In this context, we analyzed eight proteins detected only in the Cryopreserved Semen Preservation with Melatonin (PSCM) group, discussing their functions and potential impact on sperm quality.

Analysis of the identified proteins revealed a wide range of functions that collectively contribute to sperm protection and viability. One notable protein is leucine-rich repeatcontaining protein 23, involved in protein–protein interactions and cell signaling. It may play a role in protecting the integrity of the acrosome, an essential structure for fertilization (Li et al., 2023b; Zhang et al., 2021a). Endophilin-B1-like isoform X1 regulates mitochondrial dynamics and apoptosis, and melatonin may modulate its expression to reduce post-thaw cell death (Karbowski; Jeong; Youle, 2004).

Hypoxia up-regulated protein 1 responds to hypoxia and oxidative stress, and melatonin may induce its expression to protect against ROS-induced damage (Fang et al., 2014; Naaby-Hansen et al., 2010) Nucleobindin-2a, which regulates intracellular calcium and hormone secretion, may improve Ca2+ homeostasis, a crucial factor for sperm motility (Bystranowska et al., 2021). Finally, fructose-bisphosphate aldolase C-A, a glycolytic enzyme, generates the energy (ATP) required for sperm motility, and melatonin may optimize energy metabolism during freezing (Ashrafzadeh; Karsani; Nathan, 2013; Miki et al., 2004).

These proteins, exclusively identified under standard cryopreservation conditions, include UGGT1, which plays a critical role in repairing misfolded glycoproteins within the endoplasmic reticulum, as well as Cullin-5 and UBE3A, which are key components of the ubiquitination pathway responsible for the removal of damaged proteins. ITPA contributes to genomic stability by preventing the incorporation of incorrect nucleotides into DNA. The maintenance of sperm motility depends on ionic homeostasis, regulated by PMCA3b and SERCA2, and on Gelsolin A, which modulates actin polymerization in the flagellum. Alterations in these proteins can impair motility and consequently reduce fertility. Energy metabolism is also affected: NADP supports antioxidant defense mechanisms through NADPH production, while Phosphoglucomutase-2 provides the energy necessary for motility. Regarding genomic integrity, FACT complex subunits participate in chromatin remodeling and DNA repair, whereas nuclear pore complex proteins facilitate nucleocytoplasmic transport and chromatin reorganization (Dietrich et al., 2015a; Dietrich; Ciereszko, 2018; Nynca et al., 2015; Pini et al., 2018).

Together, these findings suggest that standard cryopreservation induces adaptive molecular responses related to cellular repair and DNA preservation; however, alterations in calcium-regulating and cytoskeletal proteins likely underlie the reduced motility observed following the process.

The exclusive presence of these proteins in the PSCM group suggests that melatonin can influence multiple protective pathways. Melatonin acts by counteracting oxidative stress, mediated by proteins such as hypoxia up-regulated protein 1 and nucleobindin-2a. Its action is also evident in maintaining cellular structural integrity via endophilin-B1. Finally, energy efficiency is improved by fructose-bisphosphate aldolase, which is essential for sperm motility. These findings corroborate previous studies demonstrating melatonin as a neuroprotective and antioxidant agent in cryopreservation (Zhang; Zhang, 2014; Zhang et al., 2021b).

Collectively, these results suggest that melatonin exerts a multifaceted cryoprotective action, combining preservation of energy metabolism, reinforcement of antioxidant defenses, enhancement of proteostasis, maintenance of membrane integrity, and safeguarding of genomic stability. This synergistic effect counteracts the major pathways of cryoinjury, which include ATP depletion, ROS overproduction, protein denaturation, lipid peroxidation, and DNA fragmentation. Based on these findings, melatonin emerges as a promising additive for semen cryopreservation protocols. Further studies should focus on optimizing its concentration, exploring possible synergistic effects with conventional cryoprotectants, and assessing species-specific responses in both aquatic and terrestrial reproductive biotechnology.

6.0 FINAL CONSIDERATIONS

The results obtained in this study demonstrate that melatonin supplementation during sperm cryopreservation of *Prochilodus lineatus* exerts a positive influence on post-thaw sperm quality, particularly on motility parameters and on the proteomic profile of seminal plasma. The integrated evaluation of sperm kinetics, morphology, and proteomic modulation allowed a broader understanding of the cellular and molecular mechanisms associated with cryopreservation-induced damage and its attenuation by antioxidant supplementation.

From the author's perspective, the findings indicate that melatonin acts through a multifaceted mechanism, contributing to the preservation of energy metabolism, redox balance, and proteostasis—processes that are essential for maintaining sperm functionality after thawing. The identification of differentially expressed proteins, including proteins exclusively detected in the melatonin-treated group, reinforces the potential of melatonin as a promising additive for sperm cryopreservation protocols.

Furthermore, this study contributes novel information to the field of fish reproductive biotechnology by providing the first comprehensive proteomic dataset of seminal plasma in *P. lineatus*. These results expand current knowledge on the molecular responses involved in sperm cryoresistance and support the use of proteomics as a powerful approach for elucidating mechanisms underlying sperm quality preservation in neotropical fish species.

7.0 CONCLUSION

This study demonstrated that melatonin supplementation in the cryopreservation medium improves post-thaw sperm quality of *Prochilodus lineatus* and induces significant modulation of the seminal plasma proteome. Standard cryopreservation promoted marked alterations in sperm motility and in proteins associated with energy metabolism, oxidative stress response, and protein homeostasis, reflecting the multifactorial nature of cryoinjury.

In contrast, melatonin supplementation attenuated several of these alterations, favoring the maintenance of metabolic pathways related to ATP production, antioxidant defense, controlled protein degradation, and structural integrity. These findings establish a clear relationship between experimentally observed data and theoretical frameworks describing oxidative stress, mitochondrial dysfunction, and proteostatic imbalance during sperm cryopreservation.

The main limitation of this study is related to the proteomic experimental design. The use of pooled seminal plasma samples, while appropriate for exploratory proteomic profiling, limits statistical inference, as biological variability among individual males could not be assessed. Consequently, the proteomic findings should be interpreted as hypothesis-generating rather than confirmatory, requiring validation in future studies using individual-level analyses, targeted proteomic approaches, and functional assays to confirm the biological significance of the identified proteins and pathways.

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