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A RELAÇÃO DA MORFOLOGIA ESPERMÁTICA COM A CINÉTICA DOS ESPERMATOZOIDES DE *Danio rerio* E *Brycon hilarii*

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Orientador: Danilo Pedro Streit Jr

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A RELAÇÃO DA MORFOLOGIA ESPERMÁTICA COM A CINÉTICA DOS ESPERMATOZOIDES DE *Danio rerio* E *Brycon hilarii*[1]

Autor: Bruna Bitencourt da Costa

Orientador: Danilo Pedro Streit Jr.

Resumo – A criopreservação espermática de peixes é uma técnica importante para a conservação de material genético de espécies com importância econômica e ecológica, bem como para otimização do manejo de machos geneticamente superiores e sincronização da disponibilidade de gametas. Porém, o sucesso da criopreservação depende da mitigação dos danos caudados às células espermáticas durante o processo através da otimização dos protocolos, e para isso, o conhecimento das características espermáticas das espécies se faz necessário. Assim, o estudo da biologia espermática apresenta-se como uma ferramenta importante. A qualidade espermática é de fundamental importância no processo de reprodução artificial. Os parâmetros mais utilizados para avaliação são motilidade e viabilidade. No entanto, a morfologia espermática deve ser considerada. O conhecimento das características morfológicas e funcionais dos espermatozoides é essencial para o estudo da biologia reprodutiva e para o desenvolvimento de técnicas relacionadas à conservação de espécies nativas. Assim, no primeiro estudo, utilizando o modelo biológico zebrafish (*Danio rerio*), o objetivo principal foi avaliar os danos morfológicos causados pela criopreservação aos espermatozoides. Os resultados obtidos mostraram que a criopreservação aumentou o percentual de anormalidades espermáticas, trazendo a discussão sobre a necessidade de uma nova classificação das anormalidades espermáticas de peixes. Com objetivo de categorizar os danos morfológicos causados pela criopreservação aos espermatozoides de peixes de água doce e auxiliar no desenvolvimento de protocolos mais eficientes para conservação de gametas, realizamos uma revisão sistemática (RS). Os resultados deste segundo estudo mostraram uma alta variabilidade entre os estudos incluídos, em virtude dos protocolos espécie-específicos e da diversidade de espécies de peixes de água doce estudadas. Todos os estudos incluídos apresentaram efeitos negativos da criopreservação na morfologia espermática, sugerindo também uma discussão quanto à uma nova classificação morfológica, sendo possível identificar limitações e tendências para novas pesquisas. Estes dois primeiros estudos mostraram a necessidade de se conhecer as características espermáticas das espécies, para que se possa desenvolver de protocolos mais eficientes para conservação espermática. Assim, no terceiro estudo, descrevemos os parâmetros espermáticos qualitativos (*in natura*) e suas correlações, com foco na morfologia e cinética do movimento espermático, de uma espécie nativa sul-americana (*Brycon hilarii*), com resultados interessantes para o conhecimento da biologia reprodutiva desta espécie.

Palavras-chave: espermatozoide; morfologia espermática; qualidade espermática; sêmen.

[1] Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (175 p.) Junho, 2022.

THE RELATIONSHIP OF SPERMATIC MORPHOLOGY WITH THE KINETICS OF SPERMATOZOA FROM *Danio rerio* AND *Brycon hilarii*[1]

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Advisor: Danilo Pedro Streit Jr.

Abstract - Sperm cryopreservation of fish is an important technique for the conservation of genetic material of species with economic and ecological importance, as well as for optimizing the management of genetically superior males and synchronization of gamete availability. However, the success of cryopreservation depends on mitigating the damage caused to sperm cells during the process through the optimization of protocols, and for that, knowledge of the sperm characteristics of the species is necessary. Thus, the study of sperm biology presents itself as an important tool. Sperm quality is of fundamental importance in the process of artificial reproduction. The most used parameters for evaluation are motility and viability. However, sperm morphology must be considered. The knowledge of the morphological and functional characteristics of spermatozoa is essential for the study of reproductive biology and for the development of techniques related to the conservation of native species. Thus, in the first study, using the zebrafish biological model (*Danio rerio*), the main objective was to evaluate the morphological damage caused by cryopreservation to sperm. The results obtained showed that cryopreservation increased the percentage of sperm abnormalities, bringing the discussion about the need for a new classification of sperm abnormalities in fish. In order to categorize the morphological damage caused by cryopreservation to freshwater fish spermatozoa and assist in the development of more efficient protocols for gamete conservation, we performed a systematic review (SR). The results of this second study showed a high variability among the included studies, due to the species-specific protocols and the diversity of freshwater fish species studied. All included studies showed negative effects of cryopreservation on sperm morphology, also suggesting a discussion about a new morphological classification, making it possible to identify limitations and trends for further research. These first two studies showed the need to know the sperm characteristics of the species, so that more efficient protocols for sperm conservation can be developed. Thus, in the third study, we describe the qualitative sperm parameters (in natura) and their correlations, focusing on the morphology and kinetics of sperm movement, of a native South American species (*Brycon hilarii*), with interesting results for the knowledge of the reproductive biology of this species.

Keywords: spermatozoa; sperm morphology; sperm quality; semen

[1] Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (175 p.) June, 2022.

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RELAÇÃO DE ABREVIATURAS

ALH: Amplitude de deslocamento lateral da cabeça
ASMA: Automated Sperm Morphology Analysis
ATP: Trifosfato de adenosina
BCF: Frequência de batida cruzada
Ca^{+2}: Cálcio
CASA: Computer Assisted Sperm Analysis
Cl^{-}: Cloro
DNA: Ácido desoxirribonucleico
EROs: Espécies reativas de oxigênio
Hz: Hertz
K^{+}: Potássio
LIN: linearidade
LPO: Peroxidação lipídica
Na^{+}: Sódio
pH - potencial hidrogeniônico
PI: Iodeto de propídio
PROG: Espermatozoides móveis progressivos
RS: Revisão sistemática
s: segundo
STR: retilinearidade
VAP: Velocidade média da trajetória
VCL: Velocidade curvilinear
VSL: Velocidade em linha reta
WOB: Índice de oscilação
%: percentual
°C: Graus centigrados
µm: Micrometro

CAPÍTULO I

1. INTRODUÇÃO GERAL

A criopreservação do sêmen de peixes é um procedimento importante para melhorar a conservação do material genético de espécies com importância econômica e ecológica (Figueroa et al., 2016), na conservação da biodiversidade e preservação do sêmen de animais ameaçados de extinção ou na otimização do manejo de machos geneticamente superiores (Cabrita et al., 2010; Nynca et al., 2015; Dietrich et al., 2017). Na aquicultura, a conservação dos recursos genéticos é importante para o controle reprodutivo de diferentes espécies, principalmente, para minimizar as dificuldades quanto à sincronização da disponibilidade de gametas (Cabrita et al., 2010). Entretanto, esta técnica pode resultar em alterações letais e subletais no espermatozoide, que afetam a qualidade espermática, reduzindo a eficiência da fertilização, mesmo com o uso das melhores técnicas disponíveis (Costa et al., 2019; Xin et al., 2020). Assim, o sucesso da criopreservação depende da mitigação dos danos caudados às células espermáticas durante o processo, através da otimização dos protocolos. No entanto, o desenvolvimento de protocolos depende da padronização de métodos mais adequados de coleta e diluição, determinação de diluidores e crioprotetores mais eficazes, taxas de resfriamento, congelamento e descongelamento, que são variáveis e espécie-específicas (Suquet et al., 2000; Liu et al., 2006). Pesquisas vêm sendo realizadas a respeito da criopreservação de sêmen de peixes, porém os resultados obtidos apresentam alta variabilidade. Para permitir que a criopreservação de sêmen de peixes se torne uma prática eficiente e rotineira em larga escala dentro do setor produtivo, há necessidade de maior aporte de informações e avanços tecnológicos.

O estudo da biologia espermática é uma ferramenta importante, pois permite a reprodução em condições de cativeiro, sendo importante tanto para atender o manejo para produção comercial quanto para o manejo e conservação em programas de repovoamento (Carolsfeld et al., 2003; Silva et al. al., 2009; Viveiros & Godinho, 2009). O sucesso reprodutivo dos peixes depende do aprimoramento das técnicas de reprodução artificial e da preservação dos gametas. Além disso, a produção de estoques de peixes para aquicultura depende estritamente da qualidade dos gametas e, consequentemente, da qualidade das larvas (Kucharczyk et al., 2019). Toth et al.

(1997) e Lahnsteiner (2000), ressaltaram a importância de se conhecer as características morfológicas e funcionais dos espermatozoides, para o estudo básico da biologia reprodutiva e para a produção em cativeiro de qualquer espécie de peixe bem como, para o desenvolvimento de técnicas relacionadas à conservação de espécies nativas. Assim, o conhecimento quanto à qualidade espermática, os padrões de movimento e morfologia, bem como as relações entre estes parâmetros são de extrema importância na reprodução artificial e para o sucesso reprodutivo, além de fornecer informações valiosas sobre a biologia reprodutiva das espécies nativas e contribuir para o estabelecimento de protocolos de conservação de sêmen padronizados (Silva et al., 2014). Conhecer a qualidade espermática é a primeira consideração para padronização de protocolos para manipulação *in vitro* e para o desenvolvimento de protocolos de armazenamento de sêmen (Caldas & Godoy, 2019). Segundo Fauvel et al. (2010), a avaliação sistemática, quantitativa e precisa de diferentes parâmetros que influenciam a fertilidade permitiria melhorar o controle da reprodução de peixes em cativeiro e no desenvolvimento de meios eficientes para preservação e fertilização de gametas em programas de aquicultura e conservação de espécies

A qualidade espermática é de fundamental importância no processo de reprodução artificial, refletindo diretamente no sucesso da fertilização (Figueroa et al., 2016). Motilidade e viabilidade são os parâmetros mais comumente usados para avaliar a qualidade espermática (Cosson et al., 2008a; Cosson et al., 2008b). Como alternativa, a morfologia espermática pode ser utilizada devido à facilidade de uso da técnica e à associação com a velocidade de natação e a capacidade de fertilização dos espermatozoides na maioria das espécies estudadas (Chenoweth, 2005; Tuset et al., 2008). A motilidade é essencial para a fertilização (Kavamoto et al., 1999; Figueroa et al., 2016) e depende de vários aspectos, como o estado fisiológico da célula, produção de ATP (adenosina trifosfato), integridade do canal da membrana plasmática, estrutura do flagelo e da energia previamente armazenada pelas mitocôndrias (Dzyuba & Cosson, 2014). A morfologia dos espermatozoides de peixes pode ser afetada por vários fatores, tais como nutrição, idade e estágio de maturação gonadal do reprodutor, componentes genéticos e ambientais vivenciados por um indivíduo durante o desenvolvimento, método de coleta e preparação das amostras,

osmolaridade do meio de ativação e pelo processo de criopreservação (Butts et al., 2010; Miliorini et al., 2011). A avaliação morfológica dos espermatozoides também é importante na investigação da falta de sucesso reprodutivo em animais aparentemente com boa capacidade de fertilização com base na análise de motilidade (Gwo & Arnold, 1992; Butts et al., 2010).

Considerando estas questões, inicialmente objetivamos avaliar o padrão de resposta para as anormalidades espermáticas após a criopreservação em uma espécie modelo experimental (zebrafish), e com o uso de uma revisão sistemática, categorizar os danos morfológicos causados pela criopreservação ao sêmen de peixes de água doce para auxiliar no desenvolvimento de protocolos mais eficientes para conservação de gametas. As observações obtidas através destes estudos, mostraram a necessidade de se conhecer os padrões e qualidade espermática das espécies de peixes de água doce, para que se possa, futuramente, desenvolver protocolos mais eficientes para conservação de sêmen. Assim, em um segundo momento, objetivamos o desenvolvimanto de novos estudos voltados para a descrição dos parâmetros espermáticos qualitativos (*in natura*) e suas correlações, com foco na morfologia e cinética do movimento espermático, de uma espécie nativa sul-americana, *Brycon hilarii*.

2. REVISÃO BIBLIOGRÁFICA

2.1 Espécies modelos dos estudos

2.1.1. *Danio rerio*

O zebrafish (*Danio rerio*) é um teleósteo tropical de água doce, de origem asiática, pertencente à família *Cyprinidae* (Engeszer et al., 2007). A espécie apresenta tamanho pequeno, podendo atingir em torno de 4-5cm de comprimento (Spence et al., 2008). Em seu habitat natural apresenta estação reprodutiva entre abril e agosto (monções), variando em função de altitude e condições climáticas, e a desova ocorre em pequenas áreas alagadas adjacentes a córregos (Engeszer et al., 2007). A reprodução é fortemente regulada pelo fotoperíodo (Lawrence, 2007). Geralmente a desova ocorre ao amanhecer, nas primeiras horas do dia, tanto em laboratório quanto na natureza (Lawrence, 2007; Spence et al., 2008).

É utilizado como modelo experimental em pesquisas com vários compostos, testes de segurança com fármacos, avaliação de riscos ambientais e toxicidade durante o desenvolvimento, pois apresenta características favoráveis, como a alta fecundidade, pequeno tamanho, rápido tempo entre gerações, transparência óptica durante a embriogênese e homologia genética com humanos (Lawrence, 2007). Além disso, o custo de criação e manutenção, e o estabelecimento e manutenção de instalações de cultivo (Lawrence, 2007), é inferior à de outros animais utilizados na pesquisa, como os roedores. Um fator importante à ascensão do zebrafish como modelo experimental é sua tolerância a uma ampla gama de condições ambientais em cativeiro (Lawrence, 2007). Com a extensa homologia de seu genoma com o de outras espécies de vertebrados, como os humanos, que apresentam cerca de 70% dos genes em comum com o zebrafish (Howe et al., 2013), a espécie tornou-se um organismo popular no estudo de genética, biologia do desenvolvimento e biomedicina (Kalueff et al., 2014).

2.1.2. *Brycon hilarii*

O gênero *Brycon* está distribuído da América Central à América do Sul, pelas principais bacias hidrográficas brasileiras tais como Amazônica, Paraná, Paraguai e Araguaia-Tocantins (Antunes et al., 2010). São espécies de grande importância ecológica e comercial, mas são extremamente sensíveis a alterações no ambiente natural (Sanches & Galetti, 2007). As espécies são reofílicas, habitando rios de águas claras e ricas em oxigênio (Sanches & Galetti, 2007).

A piraputanga, *Brycon hilarii*, é uma espécie migratória de médio porte, originária da bacia do Prata e encontrada no médio Paraná e por toda a Bacia do rio Paraguai, que se reproduz entre a primavera e o verão (Sanches & Galetti, 2007). É apreciada pela qualidade da carne e pela pesca esportiva, e pode ser encontrada em pisciculturas em várias regiões do país (Sanches et al., 2007; Reys et al. 2009; Antunes et al., 2010). Assim como outras espécies do gênero *Brycon*, as populações de *B. hilarii* diminuíram, principalmente devido à pesca excessiva e atividades antrópicas, como a poluição, desmatamento e a construção de hidrelétricas que interrompem o fluxo migratório necessário à reprodução (Sanches & Galetti, 2007; Bignardi et al., 2016), e ameaçam os ciclos biológicos locais, afetando negativamente a reprodução (Röpke et al., 2017). Programas de repovoamento podem ajudar na reconstrução das populações afetadas (Bignardi et al., 2016). Assim, a conservação *ex situ* pode ser a única alternativa para evitar o risco de ameaça às espécies.

Como uma espécie de peixe migrador, a piraputanga necessita de estímulos ambientais para maturação gonadal e reprodução, assim a eficiência da reprodução em cativeiro se torna difícil (Mylonas et al., 2010). Espécies migradoras tem como característica a não capacidade de desova em ambientes lênticos devido à falta de estímulos adequados para tal, como o exercício da migração em si, profundidade ou corredeiras (Mylonas et al., 2010).

2.2 Aspectos básicos da fisiologia e mobilidade espermática

O sêmen é uma mistura de espermatozoides e plasma seminal, que atua como diluidor e carreador dos espermatozoides, e é responsável por muitos processos biológicos relacionados com a maturação, armazenamento e/ou envelhecimento dos espermatozoides (Kowalski & Cejko, 2019), como controlar o pH e a osmolaridade

(Petrunkina et al., 2001), estimular a motilidade espermática (Baas et al., 1983), conter agentes antioxidantes que servem como mecanismo de defesa contra o estresse oxidativo (Pesch et al., 2006; Kowalski & Cejko, 2019), agir como bactericida e ter efeito imunomodulatório (Strzemienski, 1989), além de, em condições naturias, manter a capacidade de movimentação dos espermatozoides (Kowalski & Cejko, 2019). O plasma seminal desempenha papéis críticos na criação de um ambiente ideal para os espermatozoides (Xin et al., 2020), e sua composição é bastante singular, contendo basicamente de ácido cítrico, ácido lático, ergotioneína, frutose, sorbitol, glicerilfosforilcolina, ácido ascórbico, aminoácidos, peptídeos, lipídeos, ácidos graxos, enzimas e proteínas (Garner & Hafez, 2004; Cosson, 2010). A composição iônica do plasma seminal é variável entre as espécies de peixes. No entanto, Na^+, K^+ e Cl^- são íons predominantes em peixes de água doce e marinhos (Cosson, 2010; Alavi et al., 2019). A alta concentração de K^+ no plasma seminal é um importante inibidor da motilidade espermática em algumas espécies (Alavi & Cosson, 2006). O Ca^{2+} extracelular é um pré-requisito para o início da motilidade dos espermatozoides em ciprinídeos (Alavi & Cosson, 2006; Cosson, 2010). No entanto, a composição iônica pode mudar durante a estação reprodutiva (Alavi & Cosson, 2006; Cosson, 2010).

Dados sobre a composição iônica do plasma seminal e do sêmen dos ciprinídeos revelam ampla variabilidade intra e interespécies (Billard et al., 1995, Alavi & Cosson, 2006), o que pode implicar em diferenças no mecanismo preciso de iniciação da motilidade (Alavi & Cosson, 2006). Os espermatozoides de peixes, geralmente, são imóveis no trato reprodutivo (Stoss, 1983), e sua mobilidade inicia-se quando expostos ao ambiente aquático (Coward et al., 2002). A osmolaridade e a composição do plasma seminal geralmente previnem a motilidade nos dutos espermáticos dos peixes e protege os espermatozoides (Billard, 1986).

Como a principal função de um espermatozoide é transmitir genoma masculino, os espermatozoides devem acessar, ligar e penetrar em um oócito, para uma fertilização bem-sucedida (Cosson, 2019). Portanto, a maior parte da atividade fisiológica dos espermatozoides de peixes é orientada para a motilidade (Cosson, 2019). A motilidade espermática é sustentada pela hidrólise do ATP, catalisada pela dineína ATPase que conduz o deslizamento de duplas adjacentes de microtúbulos no flagelo, levando à geração de batimento flagelar (Gibbons, 1968; Fauvel et al., 2010).

Os mecanismos envolvidos na ativação da motilidade espermática são considerados de vital importância na regulação de processos como fertilização artificial e criopreservação (Figueroa et al., 2015). Em ciprinídeos, o principal fator de controle da motilidade é a osmolaridade (Alavi & Cosson, 2006). A osmolaridade tem sido identificada como o principal fator para o gatilho da motilidade espermática, independente da temperatura, pH ou a composição iônica do meio externo (Cabrita et al., 2010). Essas alterações ocorrem quando os espermatozoides entram em contato com o meio externo, após sua liberação durante o processo de reprodução. A osmolaridade do plasma seminal dos peixes de água doce é de cerca de 300mOsm/L, sendo que a ativação do movimento ocorre em condições de hipo-osmolaridade (Browne et al., 2015). O pH do sêmen de peixes varia de 6,4 a 8,4, sendo que nestes valores podem contribuir para o movimento flagelar (Alavi et al., 2019). O decréscimo no pH intracelular dos espermatozoides inibe a atividade da ATPase (responsável pela hidrólise do ATP), interferindo na motilidade espermática (Browne et al., 2015).

De modo geral, a motilidade é acionada pelo decréscimo da concentração de K^+ no meio extracelular após o choque osmótico, causando um efluxo desse íon através de canais específicos da membrana, levando a sua hiperpolarização, e iniciação da motilidade (Alavi et al., 2019). Assim como o influxo de Ca^{+2}, através de canais de cálcio é responsável pela sinalização da atividade motora da dineína ATPase, que hidrolisa o ATP, fornecendo energia para o início da motilidade do flagelo com alta velocidade (Figueroa et al., 2015). O tempo de motilidade dos espermatozoides de peixes de água doce dura de poucos segundos a menos de 2 minutos, sendo finalizado quando os estoques energéticos intracelulares se tornam limitantes ao movimento espermático (Browne et al., 2015).

O processo de flexão do axonema é provocado pelo deslizamento entre duas duplas adjacentes de microtúbulos externos que deslizam relativamente um ao outro devido à força motriz, gerada pela atividade motora da dineína, gerando ondas (Cosson, 2019). A propagação destas ondas, da cabeça à ponta do flagelo, provoca a translação de todo o espermatozoide para frente, iniciando assim, o movimento espermático (Cosson, 2019). Com o início da motilidade, o transporte de energia (ATP - produzido pela respiração mitocondrial) da mitocôndria para o flagelo, ocorre

principalmente, através da dineína-ATPases (Alavi et al., 2019). A enzima atua ao longo do axonema gerando e propagando ondas ao longo do flagelo (Cosson, 2019).

Após o início da motilidade em peixes de água doce, os parâmetros de cinética do movimento diminuem, enquanto o número de ondas e seu grau de curvatura aumentam, e a diminuição de motilidade e velocidades dos espermatozoides ocorrem, principalmente, devido à depleção dos estoques de ATP intracelulares necessários para o batimento axonemal, que é diretamente proporcional à atividade dos motores dineína-ATPase (Cosson, 2010; Alavi et al., 2019). No período inicial de natação, a velocidade de natação é alta, assim como a frequência de batimento flagelar e o movimento é caracterizado por ondas totalmente desenvolvidas que prosseguem por todo o comprimento flagelar com uma amplitude quase constante (Cosson, 2010). Na parte intermediária do período de motilidade os flagelos apresentam padrão de onda completo, e neste momento, a amplitude da onda é menor e o comprimento da onda é maior em relação ao período anterior (Alavi et al., 2019). Depois de algum tempo de motilidade, o padrão de onda evolui, e ocorre o chamado “amortecimento” (Alavi et al., 2019). Esse “amortecimento das ondas”, leva a uma diminuição da eficiência de natação (Cosson, 2010). A partir deste momento, o ATP começa a esgotar na parte distal do flagelo, pois está mais longe do local de produção (mitocôndria) e de origem das ondas (Alavi et al., 2019), e a diminuição da frequência de batimento do flagelo e, consequentemente, da velocidade são acompanhadas por uma mudança na forma da onda restringindo as ondas à região proximal à cabeça (Cosson, 2010; Alavi et al., 2019). Esse processo é acelerado à medida que o tempo avança dentro do período de motilidade, pois as ondas viajam em uma parte cada vez mais restrita do flagelo proximal, enquanto uma parte distal cada vez mais longa se torna inativa e reta, até que o movimento cesse, identificando o fim do período de motilidade (Cosson, 2010; Alavi et al., 2019). A duração da motilidade também é limitada por danos que aparecem durante o período de motilidade resultantes de defeitos de membrana gerados por estresse osmótico (Cosson, 2010)

2.3 Estrutura e morfologia do espermatozoide de peixes de água doce

O espermatozoide de peixes teleósteos divide-se, basicamente, em cabeça, peça intermediária e cauda, e geralmente não possuem acrossoma. Na cabeça, quase com forma quase esférica para a maioria das espécies, se encontra o um núcleo esférico com cromatina homogênea altamente condensada, com material de DNA paterno (Cosson, 2019). A peça intermediária é pequena, com ou sem um canal citoplasmático, e composta por centríolos e mitocôndrias (geralmente de 2 a 9 por cada espermatozoide), cuja função é gerar energia para o batimento flagelar. O complexo centriolar da peça intermediária consiste nos centríolos proximal e distal, que formam o corpo basal do flagelo, utilizado para ancorar o flagelo à cabeça do espermatozoide (Cosson, 2019).

A cauda, ou flagelo, é longa e responsável pela movimentação do espermatozoide (Cosson, 2019). O comprimento flagelar do esperma dos peixes é variável. A flexão flagelar é gerada por um sistema cilíndrico de microtúbulos, denominado axonema, com um padrão 9 + 2 consistindo de nove pares de microtúbulos periféricos e dois microtúbulos centrais, que emana do corpo basal (Inaba, 2003; Cosson, 2019). As conexões estruturais entre as nove duplas externas periféricas e a bainha que envolve o par central ocorrem através dos raios radiais (Cosson, 2019). Os pares periféricos são ligados a duas fileiras de dineína ao longo de todo o comprimento do axonema, que atuam gerando e propagando ondas ao longo do flagelo e representam o motor básico que aciona todo o axonema se estendendo de um dubleto externo em direção a um dubleto adjacente (Cosson, 2019). A membrana celular envolve os elementos de toda a célula espermática, fornecendo proteção do meio circundante e medeia a recepção do sinal (iônico ou osmótico) do meio e sua transdução para o axonema (Bondarenko & Cosson, 2019).

2.3.1. Anormalidades espermáticas em peixes

A morfologia espermática de peixes pode ser afetada por vários fatores, tais como nutrição, idade e estágio de maturação gonadal do reprodutor, componentes genéticos e ambientais vivenciados por um indivíduo durante o desenvolvimento, método de coleta e preparação das amostras, osmolaridade do meio de ativação e pelo processo de criopreservação (Butts et al., 2010; Miliorini et al., 2011; Costa et al.,

2019). Estudos sobre as anormalidades morfológicas dos espermatozoides não são tão comuns para as espécies de peixes como para mamíferos (Chenoweth, 2005) e são observadas uma grande variação quanto a este resultado nos estudos, mas a morfologia espermática é um importante indicador da qualidade espermática (Costa et al., 2019). De acordo com Boryshpolets et al. (2018), a motilidade e a morfologia dos espermatozoides são os parâmetros mais importantes da biologia reprodutiva dos peixes. No entanto, para avaliar as alterações morfológicas, a morfologia e a morfometria normais dos espermatozoides devem ser conhecidas (Maria et al., 2010).

Diversos são os fatores que causam defeitos morfológicos nos espermatozoides de peixes, algumas anormalidades podem ter sua origem na espermatogênese e outras através de questões relacionas ao ambiente e manejo dos machos e do sêmen. As principais anormalidades avaliadas nos espermatozoides de peixes de água doce são: cauda quebrada, cauda fortemente enrolada, cauda enrolada distalmente, cauda curta, cauda dobrada, gotas citoplasmáticas proximal e distal, peça intermediária degenerada cabeça solta, cabeça degenerada, microcefalia e macrocefalia (Tabela 1, Figura 1). No entanto, ainda não existe uma classificação específica para as anormalidades morfológica dos espermatozoides de peixes. A classificação utilizada por muitos autores, tem como base a classificação estabelecida por Blom (1973) para bovinos, em que classifica as anormalidades ocasionadas pela espermatogênese como primárias e as demais como secundárias. No entanto, alguns estudos com sêmen de peixes já provaram que anormalidades consideradas como primárias perante esta classificação podem ter origem em outros processos, como a criopreservação (Streit Jr. et al., 2006; Streit Jr. et al., 2009; Costa et al., 2019; Galo et al., 2019; Costa et al., 2020), fazendo-se necessário, assim, uma nova classificação específica considerando a estrutura e fisiologia dos espermatozoides de peixes.

TABELA 1 – Anormalidades espermáticas de peixes, adaptado de Milliorini et al. (2011).

Classificação	**Característica observada**
Macrocefalia	Espermatozoides que apresentam cabeça gigante, com contorno e formas anormais, sem aparente degeneração cromatínica ou vacuolar.

Microcefalia	Espermatozoides com tamanho de cabeça reduzido, com contorno e formas anormais, sem degeneração cromatínica ou vacuolar aparente.
Cabeça degenerada	Espermatozoides com cabeça de tamanho normal, mas que apresentam contorno irregular ou grumoso, e/ou degeneração cromatínica ou vacuolar aparente.
Cabeça solta	Cabeça observada sem cauda, porém sem qualquer alteração.
Peça intermediária degenerada (PID)	Alterações em espessura (terço médio da peça intermediária), densidade, difração, e comprimento da peça, envolvendo seu contorno e sua inserção na cabeça.
Cauda quebrada	Células espermáticas com fratura ou retenção de cauda.
Cauda fortemente enrolada	Dobradura e enovelamento da cauda sobre si mesma ou sobre a cabeça.
Cauda enrolada distalmente	Dobradura e enovelamento na extremidade final da cauda.
Cauda curta	Espermatozoides que apresentam descontinuidade da causa a partir da peça intermediária.
Cauda dobrada	Dobradura da cauda em diversos graus, sem envolver a si mesmo ou a cabeça.
Gota citoplasmática proximal	Persistência de uma gota citoplasmática na peça intermediária.
Gota citoplasmática distal	Persistência de gota citoplasmática na cauda, exceto na peça intermediária.

Anormalidades de cauda podem comprometer a progressão da célula, alterando a cinética do movimento, visto que a movimentação do espermatozoide para frente depende da capacidade dos flagelos de gerar ondas, que se propagam da junção cabeça-cauda em direção à ponta do flagelo, resultando no movimento para frente, a medida que maior parte do comprimento flagelar for coberta por estas ondas (Dzyuba et al., 2017). De acordo com Tuset et al. (2008), a velocidade de natação e a capacidade de fertilização dos espermatozoides estão relacionadas à sua morfologia. Neste caso, por exemplo, espermatozoides com flagelo dobrado têm

menos sucesso na fertilização devido à sua oscilação (Kavamoto et al., 1999) e ao movimento circular não progressivo.

Já as anormalidades de cabeça podem levar ao comprometimento da estrutura e função celular, por alterações na integridade do DNA, dano mitocondrial, deficiência metabólica, desintegração da membrana plasmática e vazamento de proteína, além de tornarem a célula menos hidrodinâmica, podendo assim, afetar negativamente a motilidade e a cinética do movimento. Além disso, a razão entre tamanho de cabeça e flagelo pode ser utilizada como um indicativo da velocidade de natação do espermatozoide (Humphries et al., 2008), o que pode ser afetado negativamente pelos danos morfológicos, tanto de cabeça como de cauda.

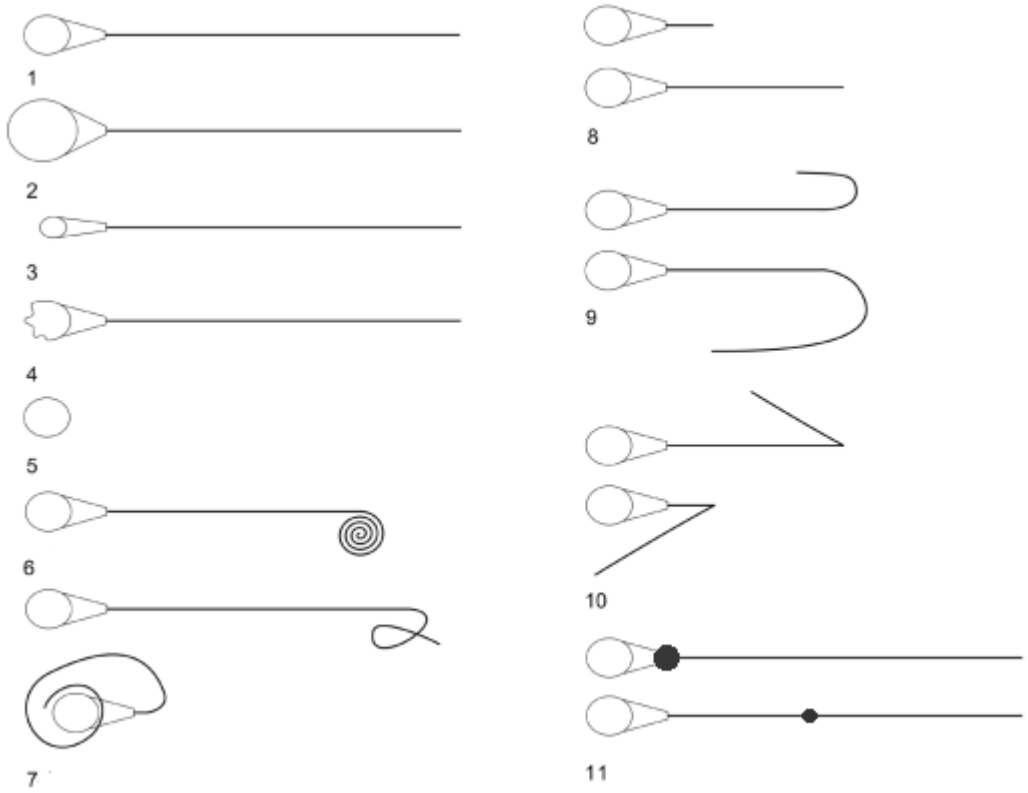

FIGURA 1. Principais alterações morfológicas nos espermatozoides de peixes. 1. Espermatozoide normal; 2. Macrocefalia. 3. Microcefalia. 4. Cabeça degenerada. 5. Cabeça solta. 6. Cauda enrolada distalmente; 7. Cauda enrolada e fortemente enrolada; 8. Cauda curta; 9. Cauda dobrada. 10. Cauda quebrada. 11. Gotas citoplasmáticas proximal e distal. Adaptado de Maria et al. (2017).

Não existe um nível padrão de referência quanto ao percentual máximo de anormalidades morfológicas que comprometem a qualidade espermática de peixes.

Assim, a determinação de um percentual máximo aceitável de alterações morfológicas é um parâmetro importante de qualidade espermática de peixes a ser estabelecido. Este padrão é especialmente importante no que diz respeito às técnicas de preservação de gametas, pois algumas anormalidades morfológicas podem ser causadas durante a criopreservação, por exemplo (Figueroa et al., 2016; Costa et al., 2020). A fertilização bem-sucedida resulta da soma de vários fenômenos que levam à fusão dos gametas (Kholodnyy et al., 2020). No entanto, a curta vida de espermatozoides e óvulos, bem como as condições ambientais, tornam o sucesso reprodutivo bastante limitado, e este cenário é agravado pela existência de apenas um local na superfície do óvulo onde os espermatozoides podem entrar, a micrópila (Kholodnyy et al., 2020). Assim, se grande parte dos espermatozoides estiver comprometida pela presença de anormalidades morfológicas, o resultado da fertilização pode ser drasticamente afetado.

2.4 Caracterização espermática de peixes de água doce

O número de espécies aquáticas sob esforços de domesticação está aumentando rapidamente, devido ao desenvolvimento da aquicultura comercial e um dos pré-requisitos para a domesticação e o estabelecimento de uma indústria aquícola sustentável é a capacidade de controlar os processos reprodutivos de peixes em cativeiro e adquirir gametas de alta qualidade (ou seja, óvulos e espermatozoides) (Mylonas et al., 2010). A caracterização espermática é um passo fundamental no estudo da biologia reprodutiva, trazendo informações de extrema importância no controle e na eficiência da reprodução. No entanto, a eficiência reprodutiva ainda é um dos fatores limitantes da produção de peixes, visto que a obtenção de gametas e larvas de qualidade é uma tarefa difícil (Kucharczyk et al., 2019). Assim como, a qualidade dos gametas, que apesar da importância para a reprodução em cativeiro, é um dos fatores limitantes para o sucesso reprodutivo, pois é afetado por diversas variáveis ambientais (Bobe & Labbé, 2010; Morales-Gamba et al., 2019).

Diversos estudos têm sido desenvolvidos com ênfase no desenvolvimento de protocolos reprodutivos para coleta de gametas de alta qualidade e armazenamento de gametas a curto e longo prazo (Pardo-Carrasco et al., 2006; Streit et al., 2006;

Órfão et al., 2011; Viveiros et al., 2011; Carneiro et al., 2012; Streit et al., 2012; Varela Junior et al., 2012; Atencio-Garcia et al., 2013; Sanches et al., 2013; Araújo et al., 2014; Garcia et al., 2015a; Garcia et al., 2015b; Varela Junior et al., 2015; Vasconcelos et al., 2015; Viveiros et al. 2015; Varela Junior et al., 2015; Atencio-Garcia et al., 2017; Restrepo-Betancur et al., 2017; Pereira et al., 2019). O armazenamento do sêmen evita a coleta repetida durante a reprodução artificial e possibilita a formação de bancos de material genético para preservação de espécies ameaçadas de extinção, desempenhando importante papel ecológico (Rodrigues et al., 2020). Além disso, é especialmente importante em casos onde são utilizadas espécies não domesticadas, já que normalmente há um número limitado de reprodutores (Kucharczyk et al., 2019). No entanto, os dados gerados por estes estudos são geralmente reduzidos aos valores médios dos diferentes parâmetros que foram suficientes para revelar diferenças significativas nos protocolos experimentais, sem que se saiba o padrão para estes parâmetros espermáticos das espécies estudadas. Assim, estudos básicos de caracterização espermática, que ainda são pouco realizados, são fundamentais no desenvolvimento destes processos e protocolos, de extrema importância para avaliação dos danos que os processos de manejo e criopreservação causam aos espermatozoides de peixes e controle da qualidade espermática. Além de contribuir para estudos futuros tanto para fins comerciais como de conservação e na identificação de parâmetros preditivos ou marcadores da qualidade do esperma, essenciais para a reprodução artificial.

O perfil dos espermatozoides, incluindo volume, concentração, motilidade e vigor, bem como as características morfológicas, podem auxiliar na avaliação da qualidade espermática e otimizar seu uso para fertilização artificial (Routray et al., 2007). É extremamente difícil estimar com precisão a qualidade dos espermatozoides e correlacionar esses parâmetros de qualidade com a capacidade reprodutiva dessas células (Cabrita et al., 2014). No entanto, a caracterização e a avaliação da qualidade espermática podem ser relevantes para compreender melhor os mecanismos pelos quais os espermatozoides são afetados e para controlar alguns dos fatores que influenciam a qualidade geral dos gametas (Cabrita et al., 2014), inclusive para auxiliar no desenvolvimento de uma classificação quanto à morfologia e anormalidades espermáticas de peixes.

Embora existam estudos de caracterização dos espermatozoides de peixes, ainda se tem pouco conhecimento sobre estas características de muitas espécies nativas sul-americanas. Segundo Amman & Waberski (2014), é crucial lembrar que os espermatozoides de cada espécie diferem em tamanho, formato da cabeça, frequência de fixação abaxial da cauda, etc., e, portanto, possuem padrões de movimento diferentes. Além disso, qualidade espermática das espécies neotropicais migratórias apresenta um alto grau de variabilidade dentro e entre as espécies e é afetada por diversos fatores como idade, tamanho, época e estação do ano, frequência de coleta, indução hormonal e estado nutricional (Sanches et al., 2011; Araújo et al., 2014).

Estudos que avaliam as características espermáticas de espécies de peixes sul-americanos, como *Piaractus mesopotamicus* (Silveira et al., 1990), *Prochilodus lineatus* (Kavamoto et al., 1997; Kavamoto et al., 1999), *Leporinus obtusidens* (Murgas et al., 1999), *Steindachneridion scripta* (Luz et al., 2001), *Brycon insignis* (Andrade-Talmelli *et al.*, 2002), *Brycon opalinus* (Narahara et al., 2002), *Brycon siebenthalae* (Cruz-Casallas et al., 2005), *Brycon cephalus* (Ninhaus-Silveira et al., 2006), *Brycon amazonicus* (Pardo-Carrasco et al., 2006), *Salminus brasiliensis* (Streit et al., 2008) e *Prochilodus lineatus* (Silva et al., 2009), foram conduzidos, afim de se conhecer o real potencial de cada espécie para a piscicultura. Alguns novos trabalhos têm sido publicados nos últimos anos abordando este assunto em *Colossoma macropomum* (Maria et al., 2010)*, Brycon amazonicus* (Cruz-Casallas et al., 2007)*, Pseudoplatystoma reticulatum* (Streit et al., 2012)*, Brycon vermelha* (Faustino et al*.,* 2015)*, Brycon orbignyanus* (Oliveira et al., 2015)*, Brycon insignis* (Garcia et al., 2015a)*, Leiarius marmoratus* (Navarro & Pivato, 2016)*, Hypancistrus zebra* (Caldas & Godoy, 2019)*, Potamotrygon wallacei* (Morales-Gamba et al., 2019)*, Moenkhausia oligolepis* (Rodrigues et al., 2020). Porém os resultados obtidos ainda apresentam alta variabilidade. Para permitir que a reprodução artificial destas espécies nativas se torne uma prática eficiente e rotineira em larga escala dentro do setor produtivo, há necessidade de maior aporte de informações e avanços tecnológicos.

2.5 Qualidade espermática de peixes e principais parâmetros qualitativos de avaliação

A qualidade do espermatozoide é fundamental para o processo de reprodução refletindo diretamente no sucesso da fertilização (Cosson et al., 2008a; Cosson et al., 2008b). O controle da qualidade espermática é uma questão importante para a aquicultura, tanto para a produção de espécies comerciais bem estabelecidas quanto para a introdução de novas espécies com alto interesse comercial (Cabrita et al., 2014). Numerosos fatores determinam a qualidade espermática e o sucesso reprodutivo em peixes, como a dieta e idade dos reprodutores, estágio de maturação gonadal, fatores ambientais (mudanças sazonais de temperatura, fotoperíodo, salinidade da água e poluição), fatores decorrentes do manuseio da amostra (coleta e preparação), bem como, técnicas reprodutivas, como a criopreservação (Kowalski & Cejko, 2019).

O sucesso reprodutivo e a capacidade de fertilização de peixes são determinadas pela quantidade (volume e concentração) e qualidade espermática (motilidade, viabilidade, morfologia, composição e estabilidade da membrana e integridade do DNA) (Kowalski & Cejko, 2019). A qualidade espermática pode ser definida como a capacidade do espermatozoide de explorar sua capacidade de natação para alcançar e fertilizar o oócito (Fauvel et al., 2010). Portanto, qualquer característica quantificável diretamente correlacionada com o sucesso da fertilização poderia ser potencialmente usada como um biomarcador de qualidade espermática, como osmolaridade, pH e composição química do plasma seminal, atividade enzimática, concentração de ATP, concentração espermática, motilidade espermática, morfologia e ultraestrutura (Gallego & Austuriano, 2019). Embora alguns desses parâmetros sejam relativamente fáceis de avaliar e comumente usados (concentração, viabilidade e motilidade subjetiva), outros precisam de análises laboratoriais sofisticadas (análises bioquímicas), equipamentos caros (motilidade objetiva e quantitativa) (Gallego & Austuriano, 2019).

A avaliação da qualidade pode ser relevante para entender melhor os mecanismos pelos quais os espermatozoides são afetados e controlar alguns dos fatores que influenciam a qualidade geral dos gametas (Cabrita et al., 2014). Além disso, a estimativa da qualidade espermática é uma ferramenta fundamental em estudos quanto à biologia reprodutiva em espécies nativas (Carolsfeld et al., 2003;

Silva et al. al., 2009; Viveiros & Godinho, 2009) e espécies ameaçadas de extinção, onde espermatozoides de alta qualidade são necessários para realizar uma criopreservação bem-sucedida para bancos genéticos (Kowalski & Cejko, 2019), já que a qualidade do sêmen criopreservado é influenciada pela qualidade das amostras frescas iniciais (Cabrita et al., 2014). No entanto, apesar de a maioria das características espermáticas contribuir para a qualidade geral, nenhuma delas é suficientemente integrativa para descrever completamente a capacidade do espermatozoide de fertilizar óvulos (Fauvel et al., 2010), assim, avaliação dos diversos parâmetros e suas correlações, são importantes para uma avaliação mais completa e preditiva da qualidade espermática. De acordo com Cabrita et al. (2014), a gama de indicadores ideais de qualidade espermática, deve ser definida de acordo com as diferentes espécies, destino do sêmen ou estratégia reprodutiva, como fertilização artificial, criopreservação, banco de genes ou produção em massa.

O volume e a concentração espermática, associados à outras avaliações podem ser bons indicadores do desempenho reprodutivo dos machos apesar de não ser uma medida específica da capacidade fertilizante, pois apresentam diferenças entre as espécies e dentro dos indivíduos de uma mesma espécie (Kowalski & Cejko, 2019). A concentração é facilmente avaliada por diferentes técnicas, como contagem por microscopia utilizando câmaras hematimétricas, espectrofotometria, citometria de fluxo e determinação dos valores de espermatócrito (Alavi et al., 2008), sendo importante porque expressa a quantidade de células espermáticas por volume de sêmen. A contagem por microscopia, é o método mais utilizado e a forma mais barata de avaliar a concentração de espermatozoides. Além disso, a concentração espermátgica permite que seja otimizada a proporção adequada de espermatozoides e oócitos em processos de reprodução artificial de peixes (Rurangwa et al., 2004).

A motilidade (percentual, duração, vigor e velocidades) é o parâmetro mais comumente utilizado para avaliar a qualidade espermática (Rurangwa et al., 2004; Cosson et al., 2008a; Cosson et al., 2008b; Kowalski & Cejko, 2019). Diversos estudos comprovam que a porcentagem de motilidade está diretamente relacionada à fertilização (Boryshpolets et al., 2013; Sanches et al., 2013; Figueroa et al., 2016; Gallego et al., 2018; Kowalski & Cejko, 2019) e depende de vários aspectos, como o estado fisiológico das mitocôndrias, produção de ATP, integridade dos canais da

membrana plasmática e estrutura do flagelo (Figueroa et al., 2016). Em geral, espermatozoides de alta qualidade devem apresentar motilidade superior a 80%, porém, os valores deste parâmetro podem ser reduzidos e podem diferir significativamente dentro de indivíduos e entre espécies (Kowalski & Cejko, 2019). Sua relação com a morfologia e a cinética do movimento é importante para o aprimoramento das técnicas de reprodução artificial e de preservação dos gametas. A duração da motilidade espermática fornece uma indicação crucial sobre a janela de tempo prática que permite a manipulação correta em situações de inseminação artificial (Cosson, 2010). É avaliada para verificar se os espermatozoides estarão móveis por tempo suficiente para penetrar na micrópila e fertilizar os oócitos, já o vigor espermático representa a intensidade e tipo/padrão de movimento dos espermatozoides.

Inicialmente, a motilidade espermática era avaliada por meio de avaliação subjetiva por microscopia ótica, mas nas últimas décadas, os sistemas de Análise Espermática Assistida por Computador - CASA (*Computer-Assisted Sperm Analysis*) se tornaram uma ferramenta popular e útil para analisar a motilidade dos espermatozoides em várias espécies de peixes de forma objetiva (Kowalski & Cejko, 2019). A avaliação subjetiva permite apenas a avaliação em termos de porcentagem de espermatozoides móveis e duração da motilidade definida pelo período de tempo que leva à cessação de qualquer movimento progressivo (Fauvel et al., 2010). Além disso, esse tipo de avaliação dependerá da experiência do observador e de diversos aspectos, que podem causar leituras superestimadas ou subestimadas (Gallego et al., 2018). Apesar da análise CASA ser objetiva e livre de erros humanos, deve-se levar em consideração que diferentes sistemas CASA podem produzir diferenças nos valores medidos devido a razões técnicas (Boryshpolets et al., 2013). Além disso, o equipamento apresenta custo bastante elevado, o que ainda torna sua utilização pouco rotineira em alguns laboratórios.

Os softwares CASA tornaram possível estimar um número maior de parâmetros de movimento espermático (Gallego & Austuriano, 2019), porém deve ser padronizado de acordo com a biologia reprodutiva da espécie (Kowalski & Cejko, 2019). Eles integram as posições sucessivas das cabeças dos espermatozoides em movimento em quadros consecutivos de registros de vídeo para calcular as trajetórias e suas

características (Fauvel et al., 2010), fornecendo, além do percentual de motilidade, dados objetivos relacionados aos parâmetros cinéticos dos espermatozoides, ligados à velocidade e oscilação da cabeça. No entanto, a maioria destes sistemas são projetados para obter informações sobre o movimento da cabeça do espermatozoide, pois a resolução de imagem usada para obter os vídeos geralmente não permite obter informações sobre os flagelos (Cosson, 2010). Assim, a combinação destes resultados com as avaliações da morfologia espermática, conferem resultados mais precisos quanto à qualidade espermática e possíveis danos associados aos espermatozoides.

Os parâmetros de natação ligados à velocidade obtidos através da análise CASA são (Wilson-Leedy & Ingermann, 2007; Fauvel et al., 2010; Boryshpolets et al., 2013; Amann & Waberski, 2014; Lu et al., 2014; Kowalski & Cejko, 2019):

1) Velocidade curvilínear (VCL, μm. s^{-1}) - VCL é a velocidade média da cabeça do espermatozoide através de seu caminho real (ponto a ponto), e tem o maior valor numérico as velocidades;
2) Velocidade em linha reta (VSL, μm. s^{-1}) - correspondente à velocidade média da cabeça do espermatozoide através da linha reta do início ao fim do trajeto, e tem o menor valor numérico entre as velocidades;
3) Velocidade média da trajetória (VAP, μm. s^{-1}) - VAP mede a cabeça do espermatozoide ao longo de sua trajetória média espacial, ou seja, é a versão suavizada do VCL. Se a trajetória de movimento do espermatozoide for muito regular e linear, o VAP é quase idêntico ao VSL, mas para um espermatozoide de movimento irregular, o VAP é muito maior do que o VSL.
4) Espermatozoides móveis progressivos (PROG, %) – é a proporção de espermatozoides exibindo motilidade progressiva.

E os parâmetros ligados à oscilação da cabeça (Wilson-Leedy & Ingermann, 2007; Fauvel et al., 2010; Boryshpolets et al., 2013; Amann & Waberski, 2014; Lu et al., 2014; Kowalski & Cejko, 2019):

1) Linearidade do movimento (LIN, %) – é uma razão de velocidades, refere-se à linearidade de um caminho curvilíneo, ou seja, VSL/VCL;

2) Retilinearidade (STR, %) - é uma razão de velocidades, reflete a retidão de movimento, é um índice de progressividade, e é calculado como VSL/VAP x 100. STR e VSL são medidas de progressão para frente;
3) Índice de oscilação (WOB, %) - é uma razão de velocidades, é a oscilação do caminho real em torno do caminho médio, sendo a razão entre VAP e VCL;
4) Amplitude de deslocamento lateral da cabeça (ALH, μm) – é o desvio da cabeça em relação ao caminho médio. Está relacionada com a capacidade de penetração na zona pelúcida do oócito.
5) Frequência cruzada de batimento (BCF, Hz) - conta o número de vezes que a cabeça do espermatozoide cruza a direção do movimento (VAP), na qual o caminho curvilíneo cruza o caminho médio, avaliando o número de vezes que o batimento flagelar muda seu padrão de movimento/oscilação. Representa o número de ondas geradas a cada segundo. BCF juntamente com VCL são parâmetros indicativos do vigor espermático.

A morfologia espermática expressa o percentual de espermatozoides normais e com anormalidades morfológicas. A determinação destes defeitos tem servido de parâmetro para alertar sobre fatores que possam provocar alterações na fertilidade desde que foi discutida por Blom (1973) para bovinos, cuja classificação tem sido adotada ao longo dos anos, inclusive para peixes, mesmo que ainda seja questionável para esse espécime. A viabilidade dos espermatozoides e a capacidade de fertilização dependem da morfologia do espermatozoide e da integridade do DNA (Cabrita et al., 2014), e pode ser usada para prever a capacidade de um macho de fertilizar um óvulo (Butts et al., 2011). A análise morfológica dos espermatozoides pode auxiliar na caracterização dos espermatozoides e na verificação de seu potencial de fertilização, e é crítica para estudos avaliativos que buscam determinar a qualidade espermática (Rurangwa et al., 2004). Também pode ajudar a explicar a falta de sucesso reprodutivo em animais aparentemente com boa capacidade de fertilização com base na análise de motilidade espermática (Murgas et al., 2011). No entanto, para avaliar as alterações morfológicas, a morfologia e a morfometria normais dos espermatozoides devem ser conhecidas (Maria et al., 2010). Apesar da qualidade espermática em

peixes também ser determinada por sua morfologia, dados sobre a morfologia espermática de peixes são escassos devido a limitações metodológicas (Tuset et al., 2008). Além disso, a morfologia do espermatozoide é influenciada por componentes genéticos e ambientais experimentados pelo indivíduo durante seu desenvolvimento e por vários fatores aplicados aos espermatozoides durante a preparação da amostra (Butts et al., 2011).

A morfologia pode ser avaliada através de microscopia ótica, microscopia eletrônica de varredura, microscopia eletrônica de transmissão e através de análise de imagens por softwares especializados - ASMA (*Automated Sperm Morphology Analysis*). Até recentemente, a morfologia espermática era examinada e analisada exclusivamente usando técnicas manuais e subjetivas de microscopia eletrônica, varredura e transmissão, espectroscopia de dispersão de luz a laser e iluminação estroboscópica (Van Look & Kime, 2003). A análise automatizada de morfologia de espermatozoides (ASMA) é uma técnica relativamente nova, que possibilita uma avaliação rápida da morfologia dos espermatozoides e tem sido cada vez mais usada, porém assim como o sistema CASA, apresenta um custo mais elevado. Além disso, o ASMA gera medições do flagelo, cabeça e peça intermediária do espermatozoide, algumas das quais eram difíceis de obter com as técnicas anteriores (do espermatozoide) (Gallego et al., 2012). No entanto, apesar destes sistemas fornecerem maior poder de resolução do que um microscópio óptico movido a luz tradicional, existem algumas desvantagens (Butts et al., 2011), principalmente, no que diz respeito as técnicas necessárias de preparação e fixação das células, que podem facilmente provocar artefatos e possíveis distorções da morfologia dos espermatozoides (Fauvel et al., 2010; Gallego et al., 2012).

Em mamíferos, a determinação da morfometria da cabeça dos espermatozoides foi correlacionada com as taxas de fertilização (Ombelet et al., 1995; Aziz et al., 1998); em peixes, foi utilizada no desenvolvimento de protocolos de criopreservação (Asturiano et al., 2007), mostrando a importância de sua mensuração. Para avaliação da morfometria, existem técnicas de microscopia eletrônica de varredura e, mais recentemente, através de software de avaliação da morfologia espermática. No entanto, as possíveis distorções na morfologia espermática ocasionas pelo preparo prévio das amostras (técnicas de fixação e coloração), para

avaliação por microscopia eletrônica, varredura, transmissão e para os softwares de avaliação da morfologia espermática, podem influenciar também a morfometria (Gallego et al., 2012). Nesse sentido, avaliar e encontrar uma técnica ótima que produza a mínima variação na morfometria, assim como da morfologia, é um requisito importante para permitir a padronização de protocolos e comparações entre resultados.

Em um nível subcelular, a integridade de membrana pode ser utilizada como indicativo de qualidade espermática (Fauvel et al., 2010), e é frequentemente utilizada como indicador, principalmente em estudos que avaliam a criopreservação, visto que as membranas plasmáticas podem ser danificadas durante o processo, e a integridade de membrana prejudicada pode estar relacionada aos parâmetros de motilidade inferiores dos espermatozoides criopreservados (Xin et al., 2020). A membrana plasmática protege a célula e controla as trocas de íons e água entre o meio intra e extracelular que desencadeiam as flexões do axonema (Cosson et al., 2008a; Fauvel et al., 2010), sendo assim um indicador da qualidade dos espermatozoides. A integridade da membrana plasmática e sua permeabilidade seletiva são fundamentais para a sobrevivência celular (Figueroa et al., 2016). Além disso, este parâmetro pode ser correlacionado aos parâmetros de morfometria e morfologia, para evitar subestimar ou superestimar os valores reais da morfologia dos espermatozoides. Pode ser avaliada indiretamente pela avaliação de componentes citoplasmáticos cuja liberação no líquido seminal reflete alguma alteração na membrana (permeabilidade), ou pela medição da impermeabilidade dos corantes hidrofílicos, ou ainda, pela resistência da membrana ao choque osmótico (resistência mecânica e permeabilidade) (Bobe & Labbe, 2010). Os principais corantes utilizados são eosina / nigrosina, azul de tripano, ou com uso de marcadores fluorescentes de DNA, como iodeto de propídio (PI) (Fauvel et al., 2010). A porcentagem de membranas íntegras (viabilidade) é avaliada por contagem direta ou análise de imagem ao microscópio, por citometria de fluxo ou por meio de um contador automático (Figueroa et al., 2016). Também pode ser avaliada indiretamente por observação microscópica em alta ampliação da morfologia das células espermáticas (Fauvel et al., 2010). Outras avaliações quanto à integridade da célula podem ser: fluidez da membrana, proteínas estruturais e apoptose (Figueroa et al., 2016)

2.6 Criopreservação de sêmen de peixes de água doce

A criopreservação de sêmen de peixes é uma técnica valiosa que desempenha um papel importante na aquicultura, desde o transporte de material genético, como para minimizar a assincronia na maturação dos gametas e no uso seletivo de reprodutores, assim como para viabilizar a conservação da biodiversidade e proteção de espécies ameaçadas de extinção (Lahnsteiner et al., 2000; Cabrita et al., 2010; Cabrita et al., 2014; Dietrich et al., 2017; Gallego & Austuriano, 2019; Xin et al., 2020). A criopreservação é uma técnica de armazenamento de longo prazo utilizando temperaturas extremamente baixas (-196 ° C) para manutenção da viabilidade dos espermatozoides (Baust, 2008; Gallego & Austuriano, 2019). As técnicas de criopreservação envolvem a adição de diluidores e crioprotetores, congelamento e descongelamento de amostras de sêmen, que garantem a não ativação dos espermatozoides durante o armazenamento e protegem as células espermáticas dos danos celulares causados pela exposição a baixas temperaturas (Billard et al., 2004), sendo que todos estes processos podem resultar em algum dano aos espermatozoides, reduzindo o sucesso da fertilização (Kopeika et al, 2003). Estas substâncias ou agentes crioprotetores substituem a água presente no meio intracelular através de um gradiente osmótico, conferindo desidratação e redução do metabolismo, e consequentemente, maior proteção à célula (Pegg, 2007).

Durante o processo de criopreservação podem ocorrer danos de diferentes naturezas, que levam ao comprometimento da estrutura e função celular (Xin et al., 2020), implicando no comprometimento da viabilidade das células reprodutivas (Figueroa et al., 2016). Esses danos podem pode ocorrer em compartimentos celulares, núcleo, peça intermediária, flagelo, membrana plasmática, citosol, DNA, proteínas, fosfolipídios (Xin et al., 2020), devido à formação de gelo intracelular, efeito solução, toxicidade do crioprotetor, bem como por estresse osmótico e oxidativo, levando a alterações ultraestruturais, bioquímicas e fisiológicas que podem comprometer a qualidade espermática (Watson, 1995; Figueroa et al., 2016). O congelamento e descongelamento afetam a desidratação celular e a permeabilidade da membrana, assim como podem provocar a formação de gelo intracelular (Mazur,

1984). A osmolaridade pode causar mudanças na morfologia e capacidade de movimento (Cosson, 2010; Costa et al., 2019). A concentração e as propriedades físico-químicas dos crioprotetores podem causar toxicidade (Xin et al., 2020). E o aumento das espécies reativas de oxigênio (EROs) pode levar à peroxidação lipídica (LPO) (Sandoval-Vargas et al., 2021). Portanto, antes de se criopreservar os espermatozoides, é necessária uma avaliação completa das características dos espermatozoides e das soluções crioprotetoras a serem utilizadas para cada espécie de peixe (Yavas & Bozkurt, 2011).

Nas últimas décadas, os métodos de congelamento de espermatozoides progrediram, e novas técnicas estão surgindo para fornecer informações detalhadas sobre os efeitos negativos dos processos de congelamento-descongelamento (Gallego & Austuriano, 2019). No entanto, o desenvolvimento de técnicas de criopreservação para sêmen de peixes, que têm sido frequentemente utilizadas com base em testes espécie-específico, apresenta resultados heterogêneos, mesmo quando comparado os resultados obtidos entre indivíduos de uma mesma espécie (Viveiros & Godinho, 2009). Assim, estudos relacionados a criopreservação de espermatozoides de peixes, que objetivam estudar o impacto do processo de criopreservação à célula espermática, por meio novas ferramentas de análise da qualidade espermática mostram-se cada vez mais interessantes.

2.6.1. Danos causados pela criopreservação à célula espermática e sua morfologia

O processo de criopreservação espermática invariavelmente produzirá uma perda qualitativa em relação ao sêmen fresco (Costa et al., 2020). Estudos mostram que os espermatozoides de peixes de água doce criopreservado apresenta altos índices de injúrias celulares decorrentes do processo de congelamento e descongelamento ou da adição de soluções crioprotetoras (Kopeika et al., 2007). Estas injúrias podem ser um grande desafio para a aplicação da criopreservação de espermatozoides de peixes na aquicultura e programas de conservação de peixes.

Seguramente um dos principais problemas, que os espermatozoides com anormalidades espermáticas podem causar, é a redução da fertilização (Lassen et al.,

2021). Kavamoto et al. (1999) atribuíram as anormalidades morfológicas da peça intermediária e do flagelo como responsáveis pelas alterações progressivas da motilidade, aumentando o número de espermatozoides com movimentos circulares ou oscilatórios, reduzindo assim a taxa de fertilização. Considerando o grande número de anormalidades, observadas em maior frequência após a criopreservação (Streit et al., 2006; Streit et al., 2009; Galo et al., 2011; Costa et al., 2019; Galo et al., 2019; Costa et al., 2020), a compreensão sobre os processos causadores destes danos se faz extremamente necessários para que se possa estabelecer protocolos mais eficazes na mitigação dos agentes causadores destas anormalidades e seus reais efeitos sobre a fertilização.

A criopreservação envolve congelamento e descongelamento dos espermatozoides, que podem resultar em alterações letais e sub letais da célula, mesmo quando se usam as melhores técnicas disponíveis, causando injúrias oxidativas, alterações na membrana do espermatozoide e lesões no DNA (Xin et al., 2020). A lesão sub letal pode ocorrer em compartimentos celulares, incluindo núcleo, peça intermediária, flagelo, membrana plasmática e citosol, levando ao comprometimento da estrutura e função celular, incluindo alterações na integridade do DNA, dano mitocondrial e deficiência metabólica, redução na motilidade e velocidade dos espermatozoides, desintegração da membrana plasmática e vazamento de proteína (Gwo & Arnold, 1992; Lahnsteiner et al., 1992; Dietrich et al., 2015; Nynca et al., 2015; Xin et al., 2020).

As mudanças de temperatura causadas pela criopreservação podem causar danos tanto durante o congelamento, quanto no descongelamento. A taxa de congelamento pode afetar o grau de desidratação e a formação de cristais de gelo no corpo celular (Mazur, 1984), afetando a sobrevivência e morfologia dos espermatozoides pós-descongelamento. Assim, anteriormente ao congelamento, é necessária a desidratação da célula para evitar a formação de cristais de gelo no meio intracelular (Mazur, 1984). Isso é possível com a utilização de crioprotetores, visando alterar as condições desfavoráveis para a sobrevivência dos espermatozoides. No entanto, é importante destacar que, no que diz respeito aos crioprotetores, vários fatores podem afetar sua efetividade na proteção da célula, como o tipo e nível de toxicidade (concentração e tempo de exposição). O maior potencial para eventos ou

reações adversas é relacionado, principalmente, aos crioprotetores permeáveis, uma vez que estes são capazes de agir diretamente no meio intracelular em atividades celulares, como processos enzimáticos, mecanismos de transporte, trocas iônicas (Elliott et al., 2017). A combinação de crioprotetores também é utilizada, com o objetivo de reduzir a toxicidade (Elliott et al., 2017) e obter efeitos aditivos de cada um dos crioprotetores. A toxicidade crioprotetora pode ser induzida osmoticamente ou pela concentração insuficiente de crioprotetor no interior da célula que permite a formação de cristais de gelo intracelular (Xin et al., 2020), e seu efeito e toxicidade podem estar associados às particularidades de cada espécie. Da mesma forma, a taxa de descongelamento pode ser importante em termos de viabilidade de espermatozoides criopreservados, podendo causar a recristalização e afetar a reativação das atividades enzimáticas (Mazur, 1984).

A formação de cristais de gelo fora da célula é o principal fator que afeta fisicamente a morfologia celular, pois concentra a matriz circundante rapidamente, deixando as células em fluidos contendo alto teor de soluto (Ozkavukcu et al., 2008). Além disso, a troca de água entre a célula e o crioprotetor durante os estágios iniciais do procedimento causa inchaços e encolhimentos celulares que podem ser intoleráveis para a maioria das organelas (Medeiros et al., 2002).

O estresse oxidativo é associado a certos tipos de danos, como produção excessiva de espécies reativas de oxigênio, peroxidação lipídica (LPO), indução de danos oxidativos no DNA, formação de adutos proteicos, envolvendo vazamento de elétrons das mitocôndrias do espermatozoide e comprometimento da cadeia de transporte de elétrons (Cabrita et al., 2014). AS EROs desempenham um papel importante em muitas vias celulares reguladas por eventos redox, porém, são moléculas altamente reativas que podem interagir com proteínas, lipídios, DNA e RNA, promovendo lesão celular em vários níveis (Cabrita et al., 2014). O desequilíbrio entre a produção de EROs e o sistema de defesa antioxidante da célula e do plasma seminal, causam estresse oxidativo, que pode provocar a LPO (Sandoval-Vargas et al., 2021). Além disso, durante a criopreservação a barreira antioxidante fornecida pelo plasma seminal é extremamente enfraquecida, devido à diluição (Cabrita et al., 2014). O resultado final da geração de EROs mitocondriais é o dano dessas organelas e o início de uma cascata apoptótica intrínseca e, como consequência, os

espermatozoides perdem motilidade, integridade do DNA e vitalidade (Cabrita et al., 2014). A LPO, pode desencadear a perda de fluidez e integridade da membrana plasmática, que comprometem a motilidade e viabilidade celular (Ball, 2008). Danos à membrana plasmática podem resultar no vazamento de proteínas intracelulares envolvidas no metabolismo, sinalização, regulação biológica e organização do citoesqueleto (Nynca et al., 2015; Dietrich et al., 2017; Xin et al., 2020).

Umas das principais razões da baixa qualidade espermática pós-descongelamento é a alteração das estruturas mitocondriais e da membrana da peça intermediária, causando a diminuição da atividade metabólica (Figueroa et al., 2017; Xin et al., 2020) e do movimento flagelar (Zhang et al., 2003), tanto por dano mecânico como por redução das reservas energéticas para realização do movimento. A deformação estrutural que ocorre durante esse processo muitas vezes gera dilatação severa ou rompimento da membrana da peça intermediária e a cauda quebra. No estudo de Ozkayukcu et al. (2008), com humanos, os autores observaram duas questões interessantes. Primeiro, que os cristais de gelo que se formam durante o congelamento dos fluidos extracelulares provocam o deslocamento das duas estruturas que compõem os espermatozoides (cabeça e flagelo), resultando em sua separação estruturas; e segundo, que a alteração da osmolaridade durante o processo de criopreservação, pode resultar na deformação estrutural da membrana e consequentemente alterando da morfologia espermática.

A exposição dos espermatozoides a condições osmóticas extremas leva a mudanças na morfologia e capacidade de movimento (Cosson, 2010). Assim, a baixa osmolaridade, por exemplo, pode induzir a ondulação do flagelo (Ahmadi & Ng, 1997; Ramu & Jeyendran, 2013) e o inchaço da cabeça, podendo causar o rompimento da membrana plasmática (Poupard et al., 1997), afetando a capacidade de fertilização. Outros danos podem ser causados às proteínas de membrana e do citoesqueleto do espermatozoide (Xin et al., 2020). Estas proteínas são sensíveis ao choque térmico e osmótico, e são responsáveis por manter a estrutura da membrana e dos microtúbulos no flagelo e a forma celular normal do espermatozoide, atuando também no movimento e motilidade celular, bem como, para regulação adequada do volume celular (Nynca et al., 2015; Xin et al., 2020). Postula-se, então, uma conexão direta entre a regulação do volume celular, a morfologia flagelar e o citoesqueleto no dano

subletal que ocorre devido ao estresse osmótico durante a criopreservação (Correa et al., 2007; Nynca et al., 2015). Estes relatos podem explicar a ocorrência de várias anormalidades, como, cauda quebrada, cauda degenerada, cauda enrolada, cauda curta, cauda dobrada, cauda e cabeça solta, cabeça degenerada, macrocefalia e peça intermediária degenera, após a criopreservação.

2.6.2. Criopreservação e bancos de germoplasma de espécies nativas sul-americanas

Os estudos avaliando a criopreservação em sêmen de espécies sul-americanas iniciaram na década de 80. Em 1984, Coser e colaboradores, descreveram o processo para as espécies *Prochilodus scrofa* e *Salminus maxillosus*. Desde então, estudos para criopreservação espermática de espécies sul-americanas em diferentes foram publicados, como: *Leporinus silvestri* (Coser et al., 1987), *Piaractus mesopotamicus* (Carosfeld et al, 1990, Silveira et al., 1990). Nos últimos anos, diversos estudos avaliando a criopreservação em espécies sul americanas foram publicados, muitos destes mencionados em revisão publicada por Vivieros & Godinho em 2009, que avaliaram o conhecimento sobre a biologia espermática e criopreservação espermática de espécies brasileiras. Após a publicação desta revisão muitos outros estudos foram publicados, principalmente na última década, avaliando protocolos, taxas de congelamento e descongelamento, crioprotetores e diluentes, parâmetros de avaliação pós-descongelamento, capacidade de fertilização e danos causados pelo processo: *Prochilodus lineatus* (Órfão et al., 2010; Felizardo et al., 2011; Miliorini et al., 2011; Vasconcelos et al., 2015; Viveiros et al. 2015), *Brycon opalinus* (Órfão et al., 2011), *Piaractus brachypomus* (Ramirez-Merlano et al., 2011), *Brycon insignis* (Viveiros et al., 2011), *Brycon orbignyanus* (Galo et al., 2011; Viveiros et al., 2015), *Brycon nattereri* (Viveiros et al., 2012), *Colossoma macropomum* (Carneiro et al., 2012; Garcia et al., 2015b; Varela Junior et al., 2012; Varela Junior et al., 2015), *Prochilodus magdalenae* (Atencio-Garcia et al., 2013), *Brycon moorei* (Atencio-Garcia et al., 2017), *Brycon henni* (Restrepo-Betancur et al., 2017), *Rhamdia quelen* (Costa et al., 2019), *P. mesopotamicus* (Galo et al., 2019), *Prochilodus brevis* (Nunes et al.,

2019), *Steindachneridion scriptum* (Pereira et al., 2019), *Leiarius marmoratus* (Borges et al., 2020).

O banco de genes tem um papel importante no desenvolvimento de programas de melhoramento de espécies nativas (Viveiros e Godinho, 2009), mas outro objetivo importante do desenvolvimento de protocolos de criopreservação de sêmen de peixes para bancos de germoplasma, é a sua aplicação em programas de repovoamento e conservação (Martínez-Páramo et al., 2017). Estudos para a conservação e para avaliar o efeito da criopreservação na diversidade genética de algumas espécies têm sido desenvolvidos, fornecendo uma base para a aplicação dos métodos desenvolvidos em ações de conservação (Martínez-Páramo et al., 2017). No entanto, as informações sobre a aplicação de protocolos de criopreservação em programas de conservação ainda são limitadas. Segundo Viveiros e Godinho (2009), é reconhecida a importância dos bancos de sêmen criopreservado em países com uma fauna de peixes de água doce tão diversificada como o Brasil.

Porém, estudos empíricos tradicionais testando taxas de congelamento, crioprotetores ou diluentes ainda são conduzidos a fim de desenvolver protocolos de criopreservação para as espécies mais estudadas. Dados sobre viabilidade espermática pós-descongelamento são altamente heterogêneas mesmo para uma mesma espécie; e, se considerarmos que geralmente apenas resultados positivos são publicados, a verdadeira variabilidade dos resultados permanece desconhecida (Viveiros & Godinho, 2009). Por serem protocolos específicos para determinada espécie ou grupo de espécies, ainda faltam resultados confiáveis voltados para a maioria das espécies nativas sul-americanas.

2.7 Revisão sistemática

A revisão sistemática (RS) sintetiza os resultados de estudos originais, avaliando-os criticamente em sua metodologia, através estratégias e metodologias, explícitas e detalhados, para reduzir a ocorrência de erros aleatórios, sistemáticos e possíveis vieses, fornecendo resultados mais confiáveis, que permitem uma interpretação mais clara dos resultados e oferecendo sugestões imparciais, a partir das quais conclusões podem ser tiradas e decisões tomadas (Higgins et al., 2019).

Desta forma, utiliza estratégias e critérios de elegibilidade pré-especificados para diminuir a ocorrência de erros aleatórios e sistemáticos (Berwanger et al., 2007), reduzindo possíveis vieses que ocorreriam em uma revisão bibliográfica tradicional.

A condução de uma RS compreende seis etapas principais: definição de uma questão de pesquisa clara e estratégia de busca na literatura, seleção dos estudos relevantes, avaliação da qualidade metodológica ou risco de viés dos estudos, extração dados relevantes, síntese dos resultados e relatos achados (Moher et al., 2009). Para evitar viés de análise na RS, os métodos de seleção e análise dos dados são estabelecidos antes de a revisão ser conduzida, num processo rigoroso e bem definido. Para obter conclusões confiáveis, os autores da revisão precisam considerar cuidadosamente as limitações potenciais dos estudos incluídos (Higgins et al., 2019). Falhas no desenho, condução, análise e relato de estudos podem fazer com que os resultados sejam subestimados ou superestimados. Devido à grande variabilidade das pesquisas utilizando diferentes tipos de protocolos de criopreservação para diferentes espécies de peixes de água doce, a compilação de resultados, através de uma RS pode fornecer uma avaliação importante e imparcial dos efeitos observados sobre a morfologia espermática.

3. HIPÓTESE E OBJETIVOS

3.1. Hipóteses

As técnicas de criopreservação têm influência na qualidade dos espermatozoides de peixes de água doce.

As técnicas de criopreservação têm influência na morfologia espermática de peixes de água doce.

As anormalidades espermáticas devem ser consideradas na avaliação qualitativa do sêmen fresco e criopreservado das espécies de peixes.

A caracterização espermática das espécies de peixes tem importância para avaliação da qualidade espermática e para o desenvolvimento de protocolos padronizados de conservação de sêmen.

3.2. Objetivos

3.2.1. Objetivo Geral

Avaliar os danos, principalmente morfológicos, causados pela criopreservação à célula espermática, discutindo a importância da avaliação da morfologia. Além de descrever e caracterizar os parâmetros de qualidade espermática, quanto aos padrões de movimento e morfologia, de uma espécie nativa sul-americana para contribuir com o desenvolvimento de protocolos padronizados e mais eficientes de conservação de sêmen.

3.2.2. Objetivo específicos

I. Investigar e avaliar os danos causados pela criopreservação à morfologia espermática de zebrafish (*Danio rerio*).

II. Realizar uma revisão sistemática sobre os efeitos da criopreservação na morfologia e anormalidades espermáticas de peixes de água doce, a fim de

categorizar os danos morfológicos causados pela criopreservação aos espermatozoides.

III. Descrever e caracterizar os parâmetros espermáticos qualitativos de *Brycon hilarii*, e suas correlações.

IV. Discutir a importância da avaliação da morfologia espermática, e as implicações das anormalidades morfológicas à qualidade espermática.

CAPÍTULO II[1]

Morphological abnormalities in zebrafish cryopreserved sperm

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Morphological abnormalities in zebrafish cryopreserved sperm

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Abstract

The aim of the present study was to evaluate the effect of cryopreservation on the morphology of zebrafish sperm (*Danio rerio*). Sperm from 30 males were collected and divided in two treatments: fresh and cryopreserved semen. The following were measured sperm morphology, motility and membrane integrity. Cryopreservation reduced motility, the number of normal cells and the membrane integrity, as well as increased the percentage of sperm abnormalities. The most frequent types of morphological changes found in cryopreserved semen were macrocephaly, loose head, degenerated head, proximal gout, curled tail and short tail. This study opens the way for further investigations on morphological changes and for a new classification of these changes in fish semen due to cryopreservation.

Keywords: cryopreservation; *Danio rerio*; fish sperm; sperm morphology

Zebrafish (*Danio rerio*) is one of the most commonly used experimental model organism in biomedical, embryology and genetic research [10]. The species has characteristics that favor its choice as an experimental model, such as high fertility, small size, easy cultivation, fast time between generations, optical transparency during embryogenesis and genetic homology with humans [10]. This scenario has led researchers to produce several zebrafish strains and for this a solution widely adopted in other species is the sperm cryopreservation.

The cryopreservation protocols available for zebrafish semen are limited in its applicability, due to inconsistent rates of sperm motility as well as low post-thaw fertilization rates [10]. In a study by Yang et al. [17], methanol at a concentration of 8% showed greater motility and fertility after thawing. Regardless of the adopted cryopreservation protocols, the main damages can be caused by formation of intracellular ice, effect of the cryoprotective solution, toxicity of cryoprotective agents and oxidative stress, which in turn imply morphological, biochemical and physiological changes that can compromise quality sperm [7].

The sperm morphological classification adopted for South American fish follows that adopted by Kavamoto et al. [8] for *Prochilodus scrofa* (*Prochilodus lineatus)*, adapted from Blom et al. [3]. This classification was revised by Streit et al. [15] and these authors suggested that the spermatozoa morphological abnormalities previously classified as primary by Kavamoto et al. [8] was due to the cryopreservation processes in *Piaractus mesopotamicus*. Recently, in a study with *Rhamdia quelen*, Da Costa et al. [6] strongly suggested the need for an adjusted reclassification and consistent with the abnormalities arising from the sperm cryopreservation process, due to the increase in the abnormalities classified as primary.

Thus, the aim of this study was to investigate the sperm abnormalities found in zebrafish sperm after cryopreservation.

All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were previously approved by the Committee for Animal Care and Use from Universidade Federal do Rio Grande do Sul (protocol number 36140). The experiment was performed in the Aquaculture Laboratory at UFRGS, Porto Alegre, Rio Grande do Sul, Brazil.

Thirty specimens of zebrafish (*Danio rerio*), weighting 0.470 ± 0.106g were used. For semen sampling, animals were euthanized with a lethal dose of tricaine methane sulfate (0.6 mg / mL; 27°C ± 0.5; pH 7.0 to 7.4) [12]. The sperm was collected after surgical removal of the testicles. Adherent tissue was dissected and the testicles was placed in a 1.5 ml microtube and then weighed. The Ginsburg extender was added to the microtube (NaCl 6.50g, KCl 0.250g, $CaCl_2 2H_2O$ 0.350g and $NaHCO_3$ 0.20g, in 1000mL of distilled water, adjusting the osmolality to 300 mOsm / kg and the pH to 7.5) 1:50 ratio of gonad and diluting solution [16]. The semen was suspended in the solution by washing with a micropipette.

Six sperm pools (five animals/pool) were analyzed under two different conditions: (T1) semen collected and diluted in Ginsburg solution and (T2) semen diluted in Ginsburg solution + cryoprotective solution (Methanol 8% + powdered milk 15 %) and cryopreserved. The experimental design was completely randomized with six replicates for each pool by analyzing the slides for morphology, motility and membrane integrity per treatment. To compose the six pools, semen from all males that showed motility greater than 80% were used.

The six sperm pools diluted in the T2 treatment were frozen. Samples of 20µL were placed in (six) cryotubes (2mL). Then, the cryotubes with the samples were placed in a tube (15 mL), and then the falcon tube containing the cryotubes with the samples was closed and placed on crushed dry ice for 20 min. This procedure generated a cooling rate of 14-16ºC/min. Subsequently, cryotubes were removed from the falcon tube and transferred to liquid nitrogen (-196ºC) for storage [13]. After seven days of storage, samples were heated in a water bath at 38ºC for 10 s and subsequently analyzed for motility, sperm morphology and membrane integrity [13].

Motility was assessed on a histological slide, where the semen was diluted and activated in distilled water at a ratio of 1:10µL, and then a volume of 2 µl of this solution was placed between slide and cover slip for evaluation under an optical microscope (40x).

To analyze the sperm morphology of both treatments, the sperm was pre-fixed in 10% buffered formaldehyde. Subsequently, the sperm was stained with Bengal Rose. A Bengal Rose solution (4%) was diluted 1:10 (v/v) with pre-fixed sperm buffered formaldehyde. After dilution, 20 µL of stained sperm were placed on a slide to make the smear. The slides were dried and analyzed under an optical microscope (100x) and sperm (N = 200 spermatozoa/slide) were evaluated for sperm morphology, adapted from Blom [3]: broken tail, curled tail, short tail, folded tail, distal and proximal gout; loose head, degenerated head, microcephaly and macrocephaly.

The percentage of cells with intact membranes was assessed using the eosin-nigrosine dye protocol. Briefly, twenty microliters of diluted sperm were homogenized with 20 µL of the eosin-nigrosine dye and a smear was prepared. One hundred sperm were evaluated in each sample and the number of intact (colorless) and non-intact (colored head) cells was expressed as a percentage.

The data were expressed as mean and standard deviation were analyzed for normality (by Kolmogorov-Smirnov's and D'Agostino & Pearson's tests) and for variance homogeneity (Levene's test). When necessary, the data was LOG_{10} transformed.

Analysis of variance was applied to the data that showed normal distribution, followed by the Tukey test. For data that did not show a normal distribution, the data were analyzed using Kruskal-Wallis analysis, followed by Dunn's test.

Cryopreservation caused a reduction in the number of normal cells, from 71.00 ± 7.15% to 29.33 ± 4.93%. A similar result was observed for sperm motility and membrane integrity. Motility decreased from 76.40±6.88% in fresh sperm to 22.40±6.03% in cryopreserved sperm. The membrane integrity decreased from 93.80 ± 4.77% in fresh sperm to 55.00 ± 8.25% in cryopreserved sperm.

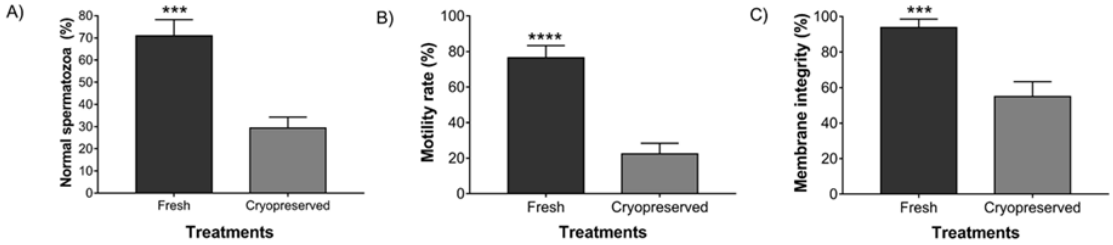

Fig. 1. A) Sperm with normal morphology (%), B) Motility rate (%) and C) Membrane integrity (%) of zebrafish fresh and cryopreserved sperm. Paired Student's t test. ****p<0.0001; ***p<0.001; **p<0.01.

In the fresh sperm, the most frequent sperm abnormality was distal gout. After cryopreservation, the high frequency of abnormalities was recorded for loose head, proximal gout, macrocephaly, degenerated head, curled tail and short tail.

Table 1

Results of the morphological evaluation of zebrafish fresh and cryopreserved sperm.

Variable (%)	T1-Fresh Sperm	T2-Cryopreserved sperm	p Value
Macrocephaly	2.00±1.55b	9.67±1.75a	0.0020
Microcephaly	3.50±1.05	4.50±1.87	0.2292
Degenerate head	2.00±1.27b	9.33±2.73a	0.0027
Loose head	4.00±2.97b	13.50±3.08a	0.0047
Distal Gout	5.33±2.73a	1.67±1.03b	0.0117
Proximal Gout	3.18±1.72b	12.33±1.21a	<0.0001
Broken tail	3.83±1.47	2.67±1.86	0.2387
Short tail	1.50±0.84b	5.17±1.72a	0.0060
Curled tail	1.00±0.89b	8.67±2.73a	0.0017
Folded tail	2.67±1.37	3.17±1.84	0.7015

*Values of mean and standard deviation followed by different letters on the same line indicate difference by the paired Student's T test.

The seminal cryobiology process will invariably produce a qualitative loss in relation to fresh semen. The most likely reason is related to the physical-chemical

environment that the sperm is exposed [14]. Motility is one of the most used parameters to assess sperm quality after cryopreservation and is generally well correlated with fertility [9]. Cryopreserved sperm normally have a lower percentage of sperm motility when compared to fresh semen. In zebrafish, motility behavior was recorded in the studies by Bai et al. [1], Wang et al. [16] and more recently by Matthews et al. [13] and the impairment of sperm motility after cryopreservation process is related to the increase in the percentage of sperm abnormalities in *Rhamdia quelen* [6]. Our results corroborate this reports, since we observed a reduction of 70.7% in motility and an increase of 59% in morphological abnormalities after freezing and cryopreservation.

Morphological changes have been attributed to the sperm response to changes in osmotic conditions [5]. This observation was confirmed by our study, from the increase in sperm abnormalities, as the increase macrocephaly and degenerated head became markedly evident after the freezing process. This fact becomes more explicit when we associate the membrane integrity analysis. In this case, the sperm cells in the T1 group suffered a lower percentage of membrane rupture than in the treatment that underwent the freezing process (T2). It is evident that the cryopreservation process was severe and caused changes in the plasma membrane of sperm.

Another relevant result that we observed after cryopreservation was a 4-fold increase in the incidence of proximal gout abnormality than in fresh semen. The appearance of proximal gout does not make sense in the semen after cryopreservation, as is an exclusive characteristic of immature sperm [11]. Therefore, it would be an abnormality associated with gametogenesis. As the sperm cell matures, the cytoplasmic gout moves from the proximal to the distal part, so the more proximal (head) the less mature the sperm are [2]. In order to explain the appearance of the proximal cytoplasmic gout in the cryopreserved semen of the zebrafish in our study, we hypothesized the rupture of microtubules that make up the axoneme of the sperm flagellum, forming a kind of envelope around the flagellum. This would characterize a structural fracture, that is, small amounts of cytoplasm that are adhered to the proximal portion of the head, coming from the intercellular bridge that connects the germ cells during spermatogenesis.

An important fact related to cytoplasmic gout, however distal (classified as an abnormality arising from gametogenesis), was a higher percentage in fresh sperm than in cryopreserved. Chatiza et al. [5] explained this, where the maturation process will continue to occur even during the cryopreservation process as well as thawing, promoting

the distal migration of cytoplasmic droplets. Thus, a lower percentage of this abnormality in fresh semen compared to cryopreserved is normal.

The loose head abnormality is a recurring problem after the sperm cryopreservation process. The significant increase in this abnormality was recently observed by Costa et al. [6] in *R. quelen* catfish. An explanation for the increased detachment of the flagellum from the sperm head, which results in the loose head abnormality, after cryopreservation was reported by Ozkayukcu et al. [14]. In the study developed by these researchers with humans, combining light and scanning microscopy, the ice crystals that form during the freezing of extracellular fluids, cause the displacement of the two structures that make up the sperm (head and flagellum), resulting in their separation structures.

The two abnormalities related to the sperm flagellum that increased the incidence after cryopreservation (short and curled tail) in the study, are certainly related to the severe process of cryopreservation or exposure in the extensor medium [4]. This was detailed in the study by Ozkayukcu et al. [14] who related as a reason for changing the sperm morphology, the osmolarity change due to the uncontrolled influx of liquids in the sperm when subjected to the cryopreservation process, resulting in the structural deformation of the membrane and consequently changing its morphology.

The increase in short tail abnormality after cryopreservation in zebrafish semen is a matter of discussion, and therefore a hypothesis. Blom [3] classified the short tail abnormality as primary, presumptive to spermatogenesis. The correct thing then would be that there was no significant increase in this (primary) abnormality after cryopreservation of zebrafish semen. In the study by Costa et al. [6], after cryopreservation, there was also an increase in short tail abnormality, as in our study. It is noteworthy that this abnormality, being classified as primary, originating in spermatogenesis, makes the sperm potentially inappropriate for fertilization. From this fact, we assume that the short tail abnormalities found, after cryopreservation, could be reclassified in future studies. In our opinion, they could be reclassified as a curled tail or partial loss of the tail due to mechanical rupture, based on the hypothesis previously discussed in this article, regarding the abnormality of proximal cytoplasmic gout. This would lead to the future need to propose a new classification of sperm abnormalities for cryopreserved fish semen, taking into account that they are caused by the physical-chemical action of the cryopreservation process and not in the gametogenic origin.

We concluded in this study that there was an increase in abnormalities classified as primary after the cryopreservation of zebrafish sperm, short tail, degenerated head, macrocephaly and proximal gout. This fact requires new studies with fish species must be developed, in order to arrive at a general classification of the morphology of fish semen.

Conflicts of interest

The authors have no potential conflicts of interest.

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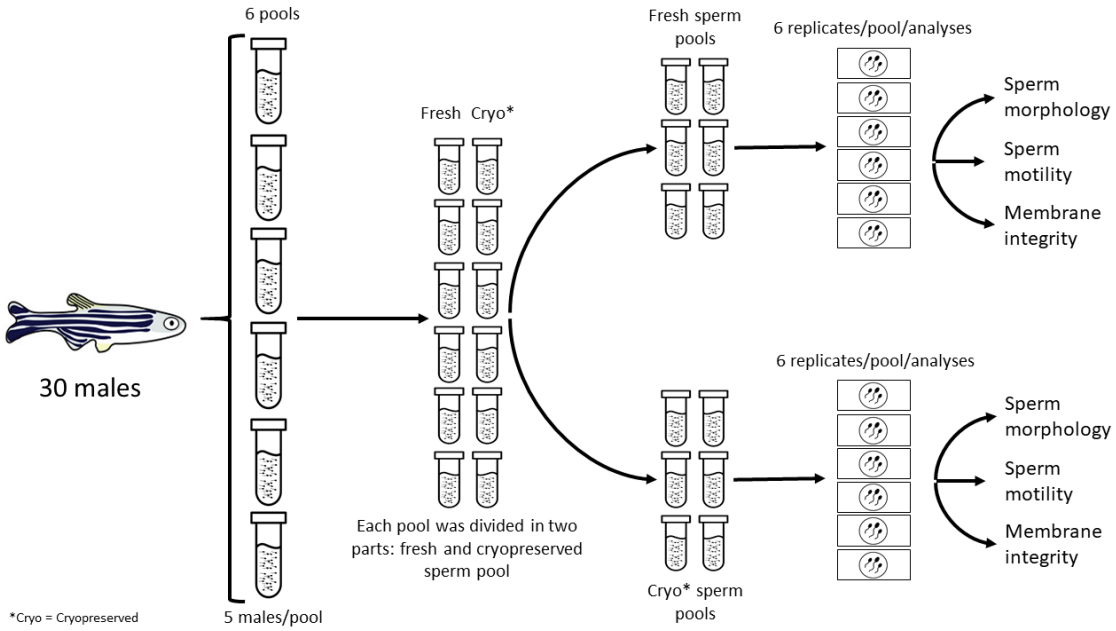

Supplementary figure 1.

CAPÍTULO III[1]

Cryopreservation-induced morphological changes in freshwater fish sperm - a systematic review

[1] Artigo elaborado conforme as normas do periódico *Reviews in Fisheries Science & Aquaculture.*

Cryopreservation-induced morphological changes in freshwater fish sperm - a systematic review

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Cryopreservation-induced morphological changes in freshwater fish sperm - a systematic review

A systematic review (SR) was performed to summarize the scientific evidence of the effects of cryopreservation on sperm morphology and abnormalities in freshwater fish. The search strategy was applied to four electronic databases (CAB Direct, Pub Med, Scopus, and ISI Web of Science). The main inclusion criteria involved studies on semen from freshwater fish subjected to the cryopreservation process, evaluating sperm quality through morphology. Risk of bias was assessed with respect to randomization, allocation concealment, blinding, incomplete outcome data, and selective reporting. A total of eight publications reporting sperm cryopreservation from five species (203 fish) were included in the SR. A high variability among studies was observed owing to the species-specific protocols and diversity of freshwater fish species studied. In general, all included studies reported negative effects of cryopreservation on sperm quality, especially morphology, highlighting the increase in incidence sperm abnormalities. However, only five studies statistically compared abnormalities between groups (fresh and cryopreserved sperm). Our results suggest the need to elaborate on a new morphological classification of fish spermatozoa through more in-depth studies on spermatogenesis, sperm characterization, and damage caused by different processes, by considering the structure and physiology of fish sperm.

Keywords: sperm quality, fish gametes, sperm morphology, sperm abnormalities, morphological classification

Introduction

Germplasm banks are important tools for the conservation of genetic material (sperm, oocytes, embryos, primordial cells, spermatogonia) of species of economic and ecological importance, endangered animals, or genetically superior males that require optimized management (Lubzens et al., 1997; Tiersch, 2008; Martínes-Páramo et al., 2017). In aquaculture, the conservation of genetic resources is important for the reproductive control of different species, mainly to minimize the difficulties associated

with the synchronization of gamete availability (Cabrita et al., 2010).

Fish sperm cryopreservation is an indisputable technique that can be applied in several research and production fields (Cabrita et al., 2010). However, this technique can cause cellular damage, which affects sperm morphology, compromises sperm quality, and reduces fertilization success (Da Costa et al., 2019). Thus, the success of semen cryopreservation depends on mitigating the damage caused to sperm cells during the process. These damages can be caused by freezing and thawing, which affect cellular dehydration and membrane permeability, as well as cause intracellular ice formation (Mazur, 1984) by osmolality; in turn, this causes changes in morphology and movement capacity (Da Costa et al., 2019), owing to the concentration and physicochemical properties of cryoprotectants that can cause toxicity (Xin et al., 2020) as well as the increase in reactive oxygen species (ROS) levels that can lead to lipid peroxidation (LPO). All these factors cause damage to both the structure and composition of membranes and the cytoskeleton, which compromises sperm quality and viability.

Sperm quality is essential for any process that requires the use of reproductive cells, whether it is in vitro reproduction or setting up a germplasm bank. In this case, sperm morphology can also be used as an indicator of sperm quality (Galo et al., 2011; Da Costa et al., 2019). However, fish sperm morphology can be affected by various biological factors (nutrition, age, and stage of gonadal maturation) resulting from sample handling (slide preparation and sperm staining methods) (Miliorini et al., 2011; Da Costa et al., 2019) and by reducing the temperature during the cryopreservation process (Miliorini et al., 2011; Da Costa et al., 2019; Galo et al., 2019), the osmolality of the medium (Galo et al., 2011; Da Costa et al., 2019; Da Costa et al., 2020), and the cryoprotective agents (Galo et al., 2019).

Owing to the great variability of research using different types of protocols for different species of freshwater fish, the compilation of results through an systematic review (SR) can provide an important and unbiased assessment of the observed effects on sperm morphology. Importantly, no SR on this topic is currently available. Thus, in this study, we sought to identify, summarize, and critically evaluate the evidence on the effects of cryopreservation on the sperm morphology and abnormalities of freshwater fish using a systematic approach.

Material and methods

Search strategy and data sources

Electronic searches were performed in April 2020 according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, in the CAB Direct (Springer Nature, 1973–2019), Pub Med (MEDLINE, 1940–2019), Scopus (Elsevier, 1960–2019), and ISI Web of Science (Thomson Reuters, 1900–2019) databases, with controlled vocabulary used when available. The search was limited to publications in English, Spanish, and Portuguese.

The research strategy was designed to identify relevant studies that address the effects of cryopreservation on sperm morphology and abnormalities in freshwater fish. The following search terms were used: (fish*) AND (cryopreserv* OR cryopreserv* OR freeze* OR cool* OR frost* OR froz* OR chill* OR vitrific*) AND (sperm* OR semen* OR morpholog*).

The population, intervention, comparison, and outcome (PICO) question was defined as: the population (P) studied consisted of freshwater fish species; the intervention (I) was the use of cryopreservation in sperm samples; the results (O) of interest were those evaluating sperm morphology and abnormalities. The comparison group (C) consisted of fresh sperm (control) subjected to the same evaluations. Only original research articles were selected.

The search results were exported to a bibliographic manager (Mendeley, Elsevier), and duplicate citations were removed.

Selection of studies

The generated list of articles was selected by title and abstract by the first two authors, and then the relevant full articles were examined. Articles with titles and abstracts that did not meet the eligibility criteria, as well as technical opinions/reports, review articles, event abstracts, guidelines, and letters to the editors were excluded.

To be considered eligible for this review, studies should: (1) be published in English, Portuguese, or Spanish; (2) research freshwater fish sperm; (3) assess the effect of cryopreservation on sperm quality; (4) investigate sperm morphology and abnormalities; (5) present results on fresh sperm.

Studies were excluded if both reviewers agreed that the article did not meet one or more of these criteria. In the event of conflicts, these were resolved by consensus between the two reviewers. If not resolved, the citation was sent to a third reviewer.

Data extraction

Printed copies of all selected articles were obtained and read in full. The two independent reviewers extracted predetermined sets of data related to the methods, samples, intervention, and control groups (use of samples before and after the intervention) and outcome measures. If publications reported more than one study, data from each study were collected separately.

Study details included population, intervention, outcome measures, outcomes, and manuscript information, such as title, author(s), year of publication, and original language. Population characteristics were species, number of animals, hormonal induction method (pituitary carp extraction or other), and whether pools or individual samples were used to obtain aliquots. The data obtained for the control and treated

groups referred to the number of replicates/replicates, cryoprotectant and concentration, extender and concentration, technique (vitrification or slow freezing), measures evaluated, methodology used to evaluate sperm morphology, and classification of sperm abnormalities. Fresh and cryopreserved sperm samples were used in the control and treated groups, respectively.

For each result, we sought to gather the following information: mean, standard deviation (s.d.), or any available measure of dispersion, unit of measure, p-value, and number of animals in the control and treatment groups. If this was not available, measures of association with standard errors or 95% confidence intervals were collected. All results for normal cells and abnormalities were collected as percentages. When the results were presented as graphs, the corresponding author was contacted via e-mail and asked to provide summary statistics. If information was not provided, the article was excluded.

For results mentioned for the control and treated groups with standard error of the mean (SEMρ), the following formula was used in the transformation to s.d. (Mederos et al., 2012; Canozzi et al., 2017):

$$S\rho = SEM\rho \times \sqrt{n\rho}$$

where Sρ is the calculated s.d., and nρ is the number of samples used in the treated and control groups.

The selected articles were subjected to data extraction, including information on authors, year of publication, study design, animal characteristics (species and sample size), type of evaluation, and main outcomes.

Risk of bias assessment

The Cochrane risk of bias was applied to assess the quality of the eight included studies (Higgins et al., 2011), modified to include an assessment of randomization reporting (in

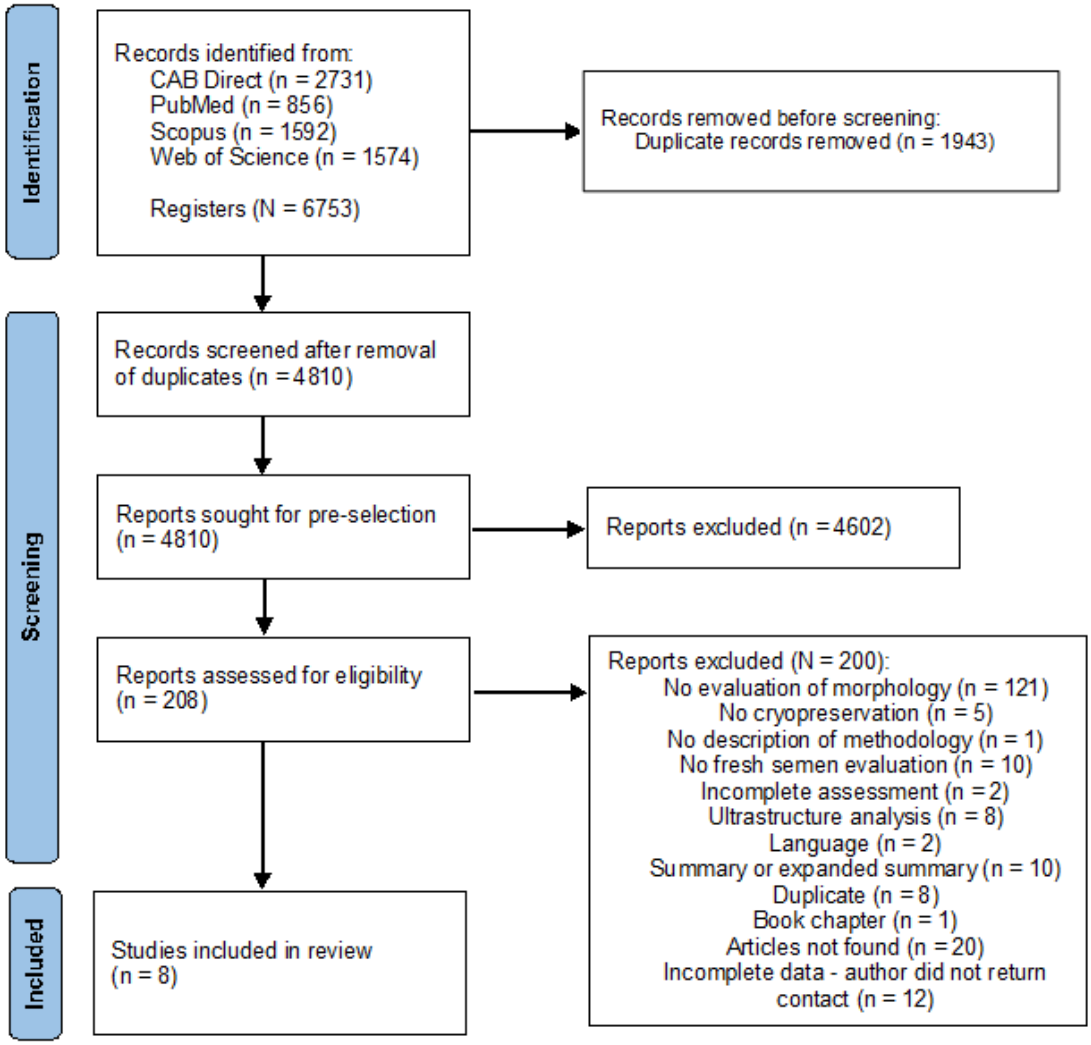
addition to random sequence generation). All outcomes were manually assessed by the first and second authors. Differences in opinion between the reviewers were resolved through discussion.

Results

Identification of studies and data extraction

A total of 4,810 non-duplicate abstracts were identified. After examining the titles and abstracts, 208 articles were selected for full-text reading. Finally, eight articles were included in the review (Figure 1). Of the 14 authors contacted who presented their results graphically or without sufficient data in the manuscripts, two provided us with numerical data, two reported that they no longer had data pertaining to their studies, while the rest did not reply.

Originally, we intended to carry out a formal meta-analysis assuming that we obtained an adequate number of studies. However, this was not possible owing to the great diversity of freshwater fish species studied, the low number of studies included, and the great variability in the protocols that are species-specific.



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158 Figure 1. Flowchart describing the screening process for the systematic review of the

159 effects of cryopreservation on the morphology and morphological changes of freshwater

160 fish sperm. Adapted from PRISMA guidelines (Page et al., 2021).

161 ***Characteristics of the selected studies***

162 The treatment evaluated in the SR included sperm abnormalities (n = 8). Of these, five

163 studies classified morphological abnormalities as primary (spermatogenesis) and

164 secondary (environmental factors and reproductive management), and three studies did

165 not use any classification regarding morphological abnormalities. A total of 203 fish

were used in these studies: *Brycon orbignyanus* (one study), *Danio rerio* (two studies), *Piaractus mesopotamicus* (three studies), *Prochilodus lineatus* (one study), and *Rhamdia quelen* (one study). All studies included in the SR were carried out in Brazil, but only two were published in Portuguese (Streit et al., 2006; Streit et al., 2009), while the others were published in English. Table 1 presents the main characteristics of the included studies.

In all studies, the effects of cryopreservation were evaluated, and in three of these studies, the effect of cryoprotectants and diluent solution was also investigated. In five studies, sperm was obtained after hormonal induction with carp pituitary extract (Streit et al., 2006; Streit et al., 2009; Miliorini et al., 2011; Da Costa et al., 2019; Galo et al., 2019). Analyses of sperm quality were performed in all studies, and fertility tests were performed in two studies. Sperm motility was investigated in seven studies, sperm vigor in four studies, motility duration and membrane integrity in two studies, and sperm viability, DNA damage, and biochemical parameters of oxidative stress in only one study.

In seven studies, the cytology technique using the Rose bengal dye was used to evaluate sperm morphology, and in only one study, formalin-citrate solution was used. In addition, all studies evaluated fresh sperm; however, in only six studies, fresh sperm was used as a control group to compare with the cryopreserved sperm. Additionally, in only five studies, the morphological changes between groups (fresh and cryopreserved) were statistically compared.

187 Table 1. General characteristics of the studies included in the systematic review on the effects of cryopreservation on the morphology and
188 morphological changes of freshwater fish sperm.

Study	Population (sample number)	Purpose of cryopreservation	Cryoprotective solution	Thawing	Evaluated morphological abnormalities	Additional assays
Da Costa et al. (2020)	*Danio rerio* (30)	Investigate sperm abnormalities found after cryopreservation	Methanol + powdered milk	38 °C/10 s	Broken tail, curled tail, short tail, folded tail, distal and proximal gout, loose head, degenerated head, microcephaly, and macrocephaly	Motility (%) and membrane integrity
Rodrigues et al. (2020)[3]	*Danio rerio* (120)	Compare the combination and interaction of different extenders with permeable and non-permeable cryoprotectants; analyze the effects of cryopreservation on several variables	HBSS + Me_2SO HBSS + methanol HBSS + Me_2SO + powdered milk HBSS + methanol + powdered milk Ginsburg + Me_2SO Ginsburg + methanol Ginsburg + Me_2SO + powdered milk Ginsburg + methanol + powdered milk	38 °C/10 s	Broken tail, curled tail, short tail, folded tail, distal and proximal gout, loose head, degenerated head, microcephaly. and macrocephaly	Motility (% and time), membrane integrity, viability, comet, and biochemicals (TBARS, DCF, SOD, CAT)
Da Costa et al. (2019)	*Rhamdia quelen* (5)	Assess sperm morphology after cryopreservation	Skimmed milk powder + fructose + methanol	25 °C/10 s	Broken tail, strongly curled tail, distally curled tail, short tail, folded tail, distal and proximal gout, loose head, degenerated head, microcephaly, and macrocephaly	Motility (%)
Galo et al. (2019)[1]	*Piaractus mesopotamicus* (6)	Evaluate fresh and post-cryopreservation seminal parameters, with emphasis on variables that affect fertilization and hatching rates	Egg yolk + glucose + Me_2SO	45 °C/5 s	Broken tail, curled tail, degenerated tail, folded tail[(5)], loose head[(6)], and loose tail[(7)]	Motility (% and time), vigor, fertilization, and hatching (percentages of non-viable, normal, abnormal, dead, dead, non-hatched,

						and live, non-hatched larvae)
Galo et al. (2011)[1]	*Brycon orbignyanus* (10)	Check the presence of changes in semen after cryopreservation	Egg yolk + glucose + Me_2SO	45 °C/5 s	Broken tail, curled tail[(8)], folded tail[(5)], degenerated tail, corrugated tail, loose tail[(7)], and loose head[(6)]	Motility (%) and vigor
Miliorini et al. (2011)[1,2,4]	*Prochilodus lineatus* (9)	Investigate the main abnormalities that occur in semen after cryopreservation and determine the best cryosolution	BTS + methanol BTS + Me_2SO	60 °C/8 s	Broken tail[(9)], tail stump[(10)], strongly coiled tail[(11)], loose head[(6)], folded tail[(5)], distal and proximal gout[(12)], head degeneration, microcephaly, macrocephaly, and midpiece degeneration	Fertilization rate (%)
Streit et al. (2009)[1]	*Piaractus mesopotamicus* (11)	Assess the motility, vigor, and morphology of sperm cells	Egg yolk + glucose + Me_2SO	45 °C/5 s	Broken tail, curled tail, short tail, degenerated tail, corrugated tail, folded tail, loose tail, proximal and distal gout, loose head, microcephaly, and macrocephaly	Motility (%) and vigor
Streit et al. (2006)[1,3]	*Piaractus mesopotamicus* (12)	Compare four extenders in the conservation of cryopreserved semen	Egg yolk + glucose + Me_2SO BTS + glycerol ZOR + glycerol BTZOR + glycerol Andro-Hepes + glycerol	45 °C/5 s	Folded tail, folded tail at the end, broken tail at the beginning, broken tail in the middle, broken tail at the end, broken tail close to the head, loose tail, curled tail, corrugated tail, short tail, and loose head	Motility (%) and vigor

(1) Classification of primary and secondary sperm abnormalities. (2) Fresh sperm was not used as a control group to compare it with cryopreserved sperm. (3) Fresh sperm was used as a control group to compare it with cryopreserved sperm, but the abnormalities between groups (fresh and cryopreserved) were not compared statistically. (4) Formaldehyde-citrate solution. (5) For formalization and standardization, folded tail is equivalent to the bent tail abnormality, present in the studies included in this SR. (6) For formalization and standardization, detached and free head is equivalent to the loose head

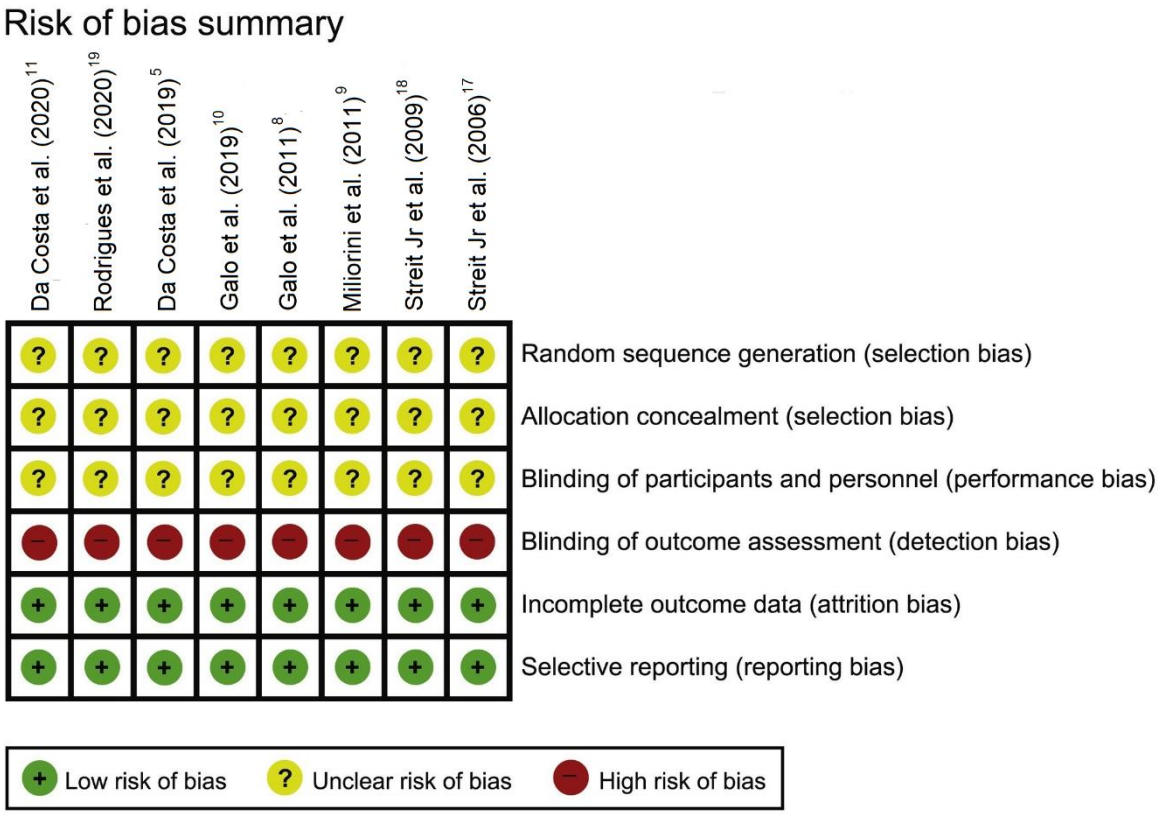
abnormality, present in the studies included in this SR. (7) For formalization and standardization, detached and free tail is equivalent to the loose tail abnormality, present in the studies included in this SR. (8) For formalization and standardization, curled tail is equivalent to the coiled tail abnormality, present in the studies included in this SR. (9) For formalization and standardization, broken tail is equivalent to the fractured tail abnormality, present in the studies included in this SR. (10) For formalization and standardization, short tail is equivalent to the tail stump abnormality, present in the studies included in this SR. (11) For formalization and standardization, strongly curled tail is equivalent to the strongly coiled tail abnormality, present in the studies included in this SR. (12) For formalization and standardization, distal and proximal gout is equivalent to the distal and proximal droplet abnormalities, respectively, present in the studies included in this SR. HBSS, Hank's balanced saline solution; Me2SO, dimethylsulfoxide; BTS, Beltsville Thawing Solution; ZOR, Modified Zorlesque; BTZOR, Medium developed in the Laboratory of the State University of Maringá (UEM).

Risk of bias

Table 2. Summary of methodological assessment and/or notification of publications reporting the studies included in this systematic review on the effects of cryopreservation on the morphology and morphological changes of freshwater fish sperm.

Variable	Evaluation	Number of studies
Was the sample size justified?	Yes	0
	No	8
How were samples distributed in the treatments?	Randomization	0
	Cited randomization	0
	Systematically	0
	Convenience or not reported	8
Has the intervention protocol been described in sufficient detail to be replicated?	Yes	0
	No	0
	Reference quote	8
Did the author report blindness when evaluating the results?	Yes	0
	No	8
Have confounding factors been identified, controlled, or tested?	Yes	0
	No	8
	Not applicable	0
Has the statistical analysis been properly described so that it can be reproduced?	Yes	8
	No	0
	References cited	0

The studies included in the SR were deficient in providing sufficiently detailed descriptions to assess the potential risk of bias. Risk of bias was assessed using the Cochrane Collaboration criteria (Table 2), and the analyses of the authors' judgments on each risk of bias item (Figure 2) were evaluated. Performance bias was not clear in 100% of the studies that analysed morphological changes, and the approach to blinding the outcome assessor was not reported, thereby increasing the risk of detection bias for both. The risk of attrition bias was low in all the included studies.



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214 Figure 2. Risk of bias summary: review of the authors' judgments on each risk of bias item
215 for each study for the systematic review of the effects of cryopreservation on the morphology
216 and morphological changes of freshwater fish sperm.

217 ***Effect of cryopreservation on sperm morphology***

218 All studies included in this SR (Streit et al., 2006; Streit et al., 2009; Galo et al., 2011; Da
219 Costa et al., 2019; Galo et al., 2019; Da Costa et al., 2020) reported a reduction in the values
220 of qualitative parameters of the evaluated sperm and an increase in incidence sperm
221 abnormalities after cryopreservation. Among the studies that classified morphological
222 changes into primary and secondary abnormalities, Streit et al. (2006) and Galo et al. (2019)
223 observed an increase in abnormalities classified as primary and a reduction in secondary
224 abnormalities after cryopreservation. In contrast, Galo et al. (2011) observed an increase in
225 sperm morphological alterations, classified as secondary abnormalities, and Streit et al. (2009)

observed an increase in primary abnormalities after cryopreservation.

The main morphological changes observed in fresh sperm were broken tails (Streit et al., 2009; Galo et al., 2011; Da Costa et al., 2019), distally curled tails (Da Costa et al., 2019), distal gouts (Da Costa et al., 2020; Rodrigues et al., 2020), curled tails (Streit et al., 2009; Galo et al., 2019), degenerated tails (Galo et al., 2011), and folded tails, loose heads, and loose tails (Streit et al., 2006). In cryopreserved sperm, the main abnormalities observed were strongly curled tails and distally curled tails (Da Costa et al., 2019), folded tails (Streit et al., 2006; Streit et al., 2009; Da Costa et al., 2019), short tails (Streit et al., 2006), curled tails (Streit et al., 2006; Streit et al., 2009; Da Costa et al., 2020; Rodrigues et al., 2020), broken tails (Streit et al., 2009; Galo et al., 2019), degenerated tails (Galo et al., 2011; Galo et al., 2019), proximal gouts (Da Costa et al., 2020; Rodrigues et al., 2020), distal gouts (Rodrigues et al., 2020), macrocephaly (Da Costa et al., 2019; Da Costa et al., 2020), microcephaly (Rodrigues et al., 2020), loose heads (Streit et al., 2006; Galo et al., 2011; Da Costa et al., 2019; Da Costa et al., 2020; Rodrigues et al., 2020), and degenerated heads (Miliorini et al., 2011; Da Costa et al., 2020; Rodrigues et al., 2020).

The main differences in the types of morphological changes observed, were increases in strongly curled tails (Da Costa et al., 2019), folded tails (Streit et al., 2009; Da Costa et al., 2019), short tails (Da Costa et al., 2019; Da Costa et al., 2020), curled tails (Da Costa et al., 2020), broken tails (Streit et al., 2009; Galo et al., 2019), degenerated tails (Galo et al., 2011; Galo et al., 2019), proximal gouts (Da Costa et al., 2020), macrocephaly (Da Costa et al., 2019; Da Costa et al., 2020), loose heads (Galo et al., 2011; Da Costa et al., 2019; Galo et al., 2019; Da Costa et al., 2020), and degenerated heads (Da Costa et al., 2020). In some studies, reductions in loose tails (Streit et al., 2009), curled tails (Galo et al., 2019), and distal gouts (Da Costa et al., 2020) were observed.

Discussion

This is the first SR that examines the available literature on the influence of cryopreservation on the morphology of freshwater fish sperm and investigates the types of morphological changes caused by this process. An SR employs explicit and detailed methods, allowing for a clear interpretation of the results and offering unbiased suggestions (Higgins et al., 2019). The results of our SR provide suggestive evidence of the effects of cryopreservation on sperm quality and the types of damage caused by this process on sperm morphology. On the other hand, this review intends to provide a better understanding of the effects of cryopreservation on fish sperm. The total number of studies and the heterogeneity among them, the diversity of fish species, and the average methodological quality of the studies, did not allow us to draw definitive conclusions; however, it was possible to extract information that will serve as a foundation for studies that will elucidate the effects of cryopreservation on the sperm morphology of freshwater fish.

Few rigorous trials have tested the effects of cryopreservation on the morphological parameters of freshwater fish sperm. The challenges of studies that evaluate cryopreservation for the conservation of freshwater fish species are to provide detailed information on the methodology, standardize the development of their research, and present complete and detailed results. As expected, our results highlighted the fundamental importance of sperm quality in artificial reproduction, especially when sperm is used for a process as severe as cryopreservation. Naturally, these findings corroborate those of previous studies that point to the use of sperm morphology assessment, based on the ease of use of the technique and its association with low percentages of motility (Galo et al., 2011; Da Costa et al., 2019) as well as declining fertilization rates (Kavamoto et al., 1999). Morphological analysis is frequently used in mammals as an indicator of sperm quality because of its low cost and ease of performance (Lassen et al., 2021). Therefore, considering standardizing the analysis of

changes in the sperm morphology of fish would not only be relevant but also fundamental. Our results show that most of the included studies (six) that used the morphological evaluation of spermatozoa as an indicator of the sperm quality of fish, used South American species as study models. The development of a cryopreservation protocol is not a simple task, as it depends on different factors, such as the ideal composition of the diluent, permeability characteristics and concentration of cryoprotectants, equilibrium time of the cryoprotectant(s), freezing rate, and thawing, in addition to the interaction among these factors (Butts et al., 2010). It is also influenced by factors such as nutrition, genetics, reproductive management, gonadal maturation, and sanitary management, which, according to Miliorini et al. (2011), may influence fish sperm motility and morphology.

Exposure to low temperatures, cryoprotectants, and extenders may be one of the possible causes of the increase in the occurrence of adverse reproductive outcomes after cryopreservation. In line with this assumption, some authors have explored the relationship between cryoprotectant types and concentrations and extender osmolarity. It is important to note that, with regard to cryoprotectants, several factors can affect the level of toxicity, such as concentration and exposure time. Cryoprotective toxicity can be induced osmotically or by insufficient concentrations of cryoprotectants inside the cells (Xin et al., 2020). In addition to the physicochemical properties and concentration of cryoprotectants, their effect and toxicity may be associated with the particularities of each species, which makes it difficult to correlate the protocols in the different species studied. Although several types of cryoprotectants are available for use in the cryopreservation of sperm from freshwater fish, it is not possible to determine which is the most efficient, given the particularities of the species and experiments used in each study. A significant effect was found between a non-permeable cryoprotectant and an extender (diluent) in relation to the sperm morphology of *D. rerio*, while the results of the principal component analysis showed that the synergy between the compositions provided

support to the sperm cell, thereby decreasing the damage caused by the freezing process (Rodrigues et al., 2020).

Other issues to consider are related to the temperature changes caused by cryopreservation. The freezing rate can affect the degree of dehydration and the formation of ice crystals in the cell body (Mazur, 1984), affecting post-thawing sperm survival and morphology. Likewise, the thawing rate may be very important in terms of the viability of cryopreserved sperm, as it can cause recrystallization and affect the reactivation of enzymatic activities (Mazur, 1984). However, little data are available on the optimal freezing rates and thawing conditions required for processing freshwater fish sperm. In the studies included in this SR, great variability was observed in terms of freezing and thawing rates, in part owing to the particularities among the protocols used for different species.

In two articles, the authors studied the correlation between sperm quality parameters after cryopreservation, including cells with normal morphology (Da Costa et al., 2019; Galo et al., 2011). These authors showed that sperm quality parameters showed significantly lower values after cryopreservation and were correlated with one another. Da Costa et al. (2019) observed a positive correlation between motility and the percentage of normal cells and negative correlations between motility and strongly curled tail, macrocephaly, loose head, and folded tail abnormalities. In contrast, Galo et al. (2011) found that secondary abnormalities were negatively correlated with sperm motility and sperm vigor. The compilation of these data reveals that abnormalities such as a strongly curled tail, macrocephaly, loose head, and folded tail are closely linked to reduced motility and sperm vigor. Motility is an important and widely used parameter in the evaluation of seminal quality (both the motility rate and the type of movement), as it is closely related to fertility in teleosts and depends on the physiological and structural state of the sperm cell. Additionally, membrane integrity is often used as an indicator of sperm quality, especially in studies that evaluate cryopreservation, as plasma

membranes can be damaged during the process. Xin et al. (2020) suggested that the reduction in motility and membrane integrity after thawing may occur owing to cryoinjury on membrane and cytoskeleton proteins. According to these authors, these proteins are sensitive to heat shock and are responsible for maintaining the structure of the sperm membrane and microtubules in the flagellum, while they also act on cell movement and motility. LPO, caused by an increase in ROS levels during cryopreservation, can trigger the loss of fluidity and integrity of the plasma membrane, which compromises cell motility and viability (Ball, 2008), and is necessary for fusion events associated with fertilization (Ball, 2008; Shiva et al., 2011). Damage to the plasma membrane can result in the leakage of intracellular proteins involved in metabolism, signaling, biological regulation, and cytoskeletal organization (Nynca et al., 2015; Dietrich et al., 2017; Xin et al., 2020).

Motility was not assessed in the studies included in this SR. Of the seven studies that assessed motility, only one did not statistically compare the differences between fresh and cryopreserved sperm. In other studies, a considerable reduction in motility after cryopreservation was observed. In *D. rerio* sperm, there was a reduction in motility from 76.40% in fresh sperm to 22.40% in cryopreserved sperm (Da Costa et al., 2020); in *R. quelen*, the reduction from fresh to cryopreserved sperm was from 80.00% to 35.00% (Da Costa et al., 2019). In the studies by Galo et al. (2019 and 2011), the reduction was even greater, from 90.83% in fresh sperm to 11.83% in cryopreserved sperm in *P. mesopotamicus* and from 90.02% in fresh sperm to 14.87% in cryopreserved sperm in *B. orbignyanus*. In two other studies involving *P. mesopotamicus* sperm (Streit et al., 2009 and 2006), the authors found that motility decreased from 40.21% to 31.50% and from 75.00% to 16.12%, respectively, after cryopreservation. This was also true for sperm vigor, which was evaluated in four studies.

Evidently, the cryogenic process is aggressive to the structure of a reproductive cell. The main reason for poor post-thawing sperm quality is the alteration of mitochondrial structures and midpiece membrane, causing decreased metabolic activity (Figueroa et al., 2017; Xin et al., 2020) and flagellar movement (Zhang et al., 2003), both owing to mechanical damage caused by the reduction of energy reserves to perform the movement. The structural deformation that occurs during this process often leads to the severe dilation or disruption of the midpiece membrane and breakage of the flagellum. Da Costa et al. (2020) suggested that the rupture of the microtubules that make up the flagellar axoneme would form an envelope around the flagellum, characterizing a structural fracture, to explain the appearance of the proximal cytoplasmic gout in cryopreserved sperm of the zebrafish, as this is an exclusive feature of immature sperm.

The differences between the frequencies of morphological alterations observed in the studies, evaluated in different species after cryopreservation as well as the percentage of normal sperm had great variability. On the other hand, regardless of the fish species, percentage of sperm with normal morphology after cryopreservation always suffered reduction in relation to that of fresh sperm [from 71.00% to 29.33% in *D. rerio* (Da Costa et al., 2020), from 67.35% to 21.48% in *R. quelen* (Da costa et al., 2019), and from 62.2% to 54.60% in *B. orbignyanus* (Galo et al., 2011)]. In these studies, the morphological changes with the highest incidence after cryopreservation, when compared to fresh sperm, were loose head and proximal gout in *D. rerio* (Da Costa et al., 2020), macrocephaly, strongly curled tail, folded tail, and loose head in *R. quelen* (Da Costa et al., 2019), and loose head and degenerated tail in *B. orbignyanus* (Galo et al., 2011). When evaluating studies with the same species, a variation in the morphological indices of spermatozoa was observed. In three studies, the sperm morphology of *P. mesopotamicus* was evaluated (Streit et al., 2006; Streit et al., 2009; Galo et al., 2019). As in other studies, the percentage of sperm with normal

morphology was reduced after cryopreservation. As for the highest-incidence abnormalities reported for the species after cryopreservation, these were degenerated tails (Galo et al., 2019) and folded and broken tails (Streit et al., 2009).

In a study by Ozkayukcu et al. (2008) on humans, the authors observed that the ice crystals that form during the freezing of extracellular fluids cause the displacement of the two structures that make up the spermatozoa (head and flagellum), resulting in their separation, and that the alteration of osmolarity during the cryopreservation process can result in the structural deformation of the membrane and consequently alter sperm morphology. A direct consequence of low osmolality, for example, can be the induction of flagellar undulation (Ahmadi and Ng, 1997) and head swelling, which can cause the plasma membrane to rupture (Poupard et al., 1997). These reports may explain the occurrence of several abnormalities in the flagellum, which are classified as broken, degenerated, curled, short, folded, and loose tail, and head abnormalities, such as loose or degenerated head, macrocephaly, and degenerated midpiece, after cryopreservation.

One of the main problems caused by abnormal sperm is reduced fertilization. Kavamoto et al. (1999) attributed the morphological abnormalities of the midpiece and flagellum as being responsible for the progressive changes in motility, increasing the number of spermatozoa with circular or oscillatory movements, thus reducing the fertilization rate. According to Da Costa et al. (2019), a lot of cryoinjury in sperm cells owing to inefficient cryopreservation processes can make fertilization ineffective. Considering the large number of abnormalities observed more frequently after cryopreservation, an understanding of the processes that cause these damages is essential to establish effective protocols that will help mitigate the agents that cause these abnormalities.

Some studies considered in this review (Streit et al., 2006; Streit et al., 2009; Galo et al., 2019) adopted the traditional classification for mammals disseminated by Bloom (1973),

399 who grouped sperm abnormalities according to their origin: primary (spermatogenesis) and
400 secondary (environmental and management factors). However, the authors of these studies
401 recorded an increase in the frequency of abnormalities considered to be primary after
402 cryopreservation, in contrast to Bloom's (1973) premise, which attributes the emergence of
403 these abnormalities to spermatogenesis. In the studies by Da Costa et al. (2020 and 2019), the
404 authors, even though not classifying the abnormalities in this manner, also observed an
405 increase in the incidence of abnormalities after cryopreservation, which, if classified
406 according to the assumption for mammals, would be considered primary. If we consider that
407 primary abnormalities are produced during spermatogenesis, they could not have originated
408 during or after cryopreservation. Based on this observation, Da Costa et al. (2020 and 2019)
409 mentioned the need for a new specific classification for fish sperm. For this objective, further
410 studies are needed on spermatogenesis, sperm characterization, and damage caused by
411 different processes during animal handling, environmental factors, and reproductive
412 techniques and how these issues can influence sperm cell morphology and cause
413 morphological changes. Such studies will allow for the objective assessment of the damage to
414 sperm morphology produced by cryopreservation. It is important to emphasize that the use of
415 a non-adapted and non-standardized classification for fish sperm may produce conflicting and
416 low reliability results. Therefore, the findings must be evaluated considering the structure and
417 physiology of fish sperm.

418 The approaches used to present results and methodological descriptions in studies
419 such as the incomplete description of methodologies and outcome measures, the presentation
420 of results in the form of graphs, and the lack of standardization in the evaluations and
421 morphological classifications, generally limit our ability to summarize and analyze the data.
422 We excluded 10 publications, initially selected to be read in full, which contained data on the
423 evaluation of morphological abnormalities, owing to the absence of data from the control

treatment and detailed data regarding the morphological abnormalities evaluated and/or the absence of a detailed methodological description, after failed attempts to contact the researchers. Three other studies included in this SR did not include statistical tests to identify significant differences in sperm morphology between fresh and cryopreserved sperm and/or statistical tests to identify significant differences between the means of each abnormality individually in fresh and cryopreserved sperm.

Although our SR showed the potential adverse effects of cryopreservation on freshwater fish sperm, it has certain limitations. The first limitation could be considered the great heterogeneity in the retrieved studies, which made it impossible to carry out a meta-analysis. The second limitation is that most of the publications included in this SR evaluated several parameters, but not all studies evaluated the same parameters and used the same methodology, thereby making it difficult to draw solid conclusions. Third, despite our extensive bibliographic research in the databases, we cannot be absolutely certain that all relevant articles were detected, as several failures in the indexing of studies can make them inaccessible in systematic research. Other limitations include the scarcity of data and suboptimal methodological quality of the primary data. Future and more rigorous research evaluate the effects of cryopreservation and its variables, such as cryoprotectants, extenders, and cooling rate, should be carried out. Additionally, basic research on sperm characterization and the damage that management and cryopreservation processes cause to fish sperm should also be conducted. Several study design issues regarding protocol standardization, analyses, and sample size should be considered, as these have a major impact on the final results of a study.

The low standardization of results in our SR was already expected, mainly owing to the great genetic plasticity of the freshwater fish species studied, the low number of studies included, and the great variability in sperm cryopreservation protocols that are species-

449 specific. However, the information collected in this review allows us to summarize studies on
450 cryopreserved sperm from freshwater fish, thereby setting the foundation for in-depth
451 discussions on the difficulties of standardizing sperm cryopreservation protocols. In any case,
452 all studies included in this SR associated the negative effects of cryopreservation with
453 qualitative sperm indices, including those related to sperm motility, as well as structural
454 indices (membrane rupture of the head or flagellum of the sperm and sperm morphological
455 changes). Collectively, the evidence from the included studies suggests that further studies
456 evaluating key points in cryopreservation protocols may be effective in improving the
457 qualitative indices of cryopreserved sperm.

458 In conclusion, the reduction of percentage of sperm with normal morphology showed
459 that the cryopreservation process causes morphological changes in sperm, with a direct
460 consequence on sperm motility and fertilization rate. However, the total number of studies
461 and the heterogeneity among them, the diversity of species, and the average methodological
462 quality of the studies were too limited to draw firm conclusions regarding the specifics of the
463 damages and their impacts on morphological changes. Therefore, in relation to the initial
464 question (effects of cryopreservation on the sperm morphology and abnormalities of
465 freshwater fish), the results of our review strongly suggest a discussion regarding the
466 elaboration of a classification for the morphological alterations specific to fish spermatozoa,
467 in order to better assess the phenomena occurring after cryopreservation.

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471 **Conflict of interest**

472 The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CAPÍTULO IV[1]

Characterization of sperm quality and morphological abnormalities in *Brycon hilarii* for devising artificial reproduction strategies

[1] Artigo elaborado conforme as normas do periódico *Reproductive Biology*.

Characterization of sperm quality and morphological abnormalities in *Brycon hilarii* for devising artificial reproduction strategies

Short Title: Spermatic characterization of *Brycon hilarii*

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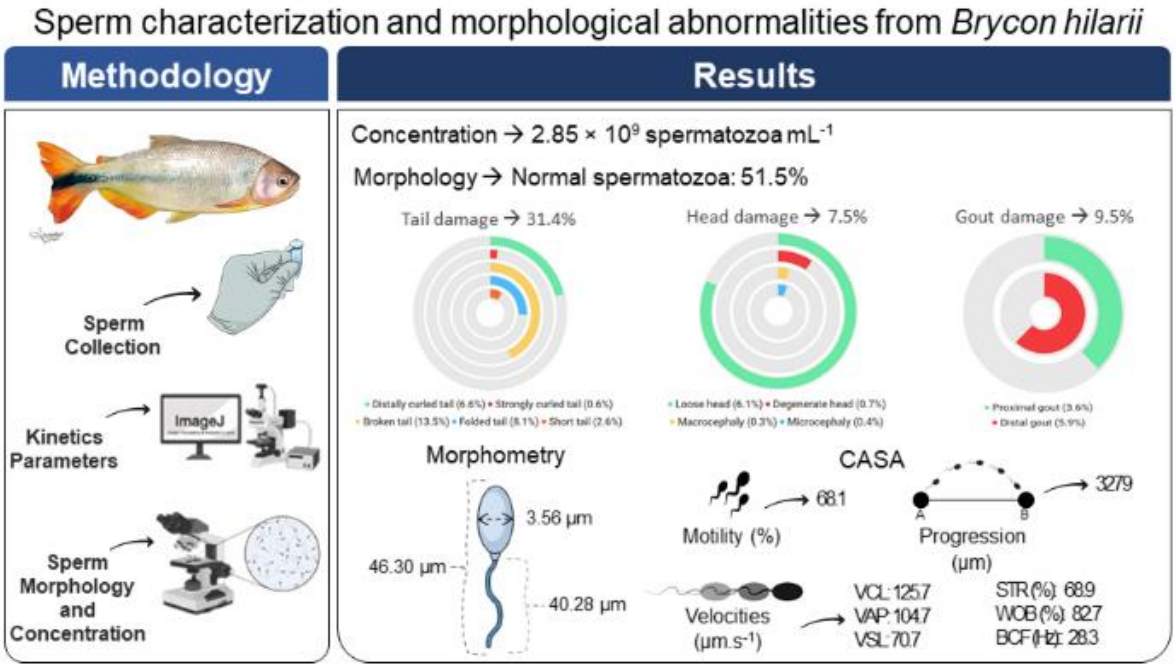
ABSTRACT

Brycon hilarii is a neotropical fish whose population is being threatened by overfishing, construction of hydroelectric dams, transposition of riverbeds, and pollution. Fundamental knowledge of the reproductive characteristics of this species is necessary to enable its breeding in captivity, for commercial purposes as well as for repopulation. The aim of this study was to investigate and describe the sperm parameters of this species, their correlations, and relevant factors. Sperm was collected after hormonal induction using carp pituitary extract. The following sperm parameters were evaluated: 1) concentration (2.85×10^9 sperms mL^{-1}), 2) total length (40.28 μm); 3) motility (68.1%), and 4) Percentage of normal cells (51.6%). The description of sperm characteristics and the characterization of spermatozoa are crucial for the artificial reproduction of the fish and represents a significant contribution to the knowledge of its reproductive biology. This information will facilitate the reproduction of the species in captivity.

Key words: Artificial reproduction; Sperm abnormality; Sperm kinetics; Sperm morphology; Sperm motility.



1. Introduction

Piraputanga (*Brycon hilarii*) is a native species belonging to the genus *Brycon*, which is distributed from Central to South America, and in the main Brazilian river basins, such as the Amazon, Paraná, Paraguay, and Araguaia-Tocantins [1]. *B. hilarii* is a migratory species native to the La Plata basin and is found in the middle Paraná and throughout the Paraguay River Basin; it reproduces between spring and summer [2]. Despite the dearth of information related to its biology and production, this species can be found in fish farms in several regions of Brazil because of the recognized quality of its meat [2,1] and its rapid growth and weight gain. The species is also highly appreciated in commercial and sport fishing [3]. However, *B. hilarii* populations have been decreasing, probably because of overfishing and human activities such as hydroelectric dam construction, riverbed transposition, and pollution [4], which threaten local biological cycles and negatively affect reproduction [5]. Thus, restocking programs can not only help rebuild populations but also minimize the impacts of the reduction of wild fish stocks.

As the reproductive success of a fish in the laboratory depends on the improvement of artificial reproduction techniques, knowledge of the quality of gametes is essential in induced reproduction procedures [6]. In males, gamete quality can be measured using different techniques such as the definition of movement and morphological patterns of sperm cells — parameters that are essential for assessing the reproductive success of fish [7]; this information can help establish protocols for in vitro handling and sperm storage [8].

Despite several studies on fish sperm characterization, the knowledge of these characteristics in native species (such as *B. hilarii*) is still scant. The sperm quality of migratory neotropical species in captivity shows a high degree of variability within and between species, and it is affected by several factors such as age, size, time and season of the year, frequency of collection, hormonal induction, and nutritional status [9], which can cause morphological

changes in the sperm [10]. The analysis of sperm morphology is critical for studies that seek to evaluate sperm quality [11]. Motility is the most used parameter to assess sperm quality [12, 13], and its relationship with morphology and movement kinetics is important for improving artificial reproduction techniques and gamete preservation. It may also be important to investigate the lack of reproductive success in animals in which the motility analysis indicates good fertilization capacity (14, 15, 16]. The objective of this study was to investigate and describe the parameters of sperm concentration, viability, motility, kinetics, morphometry, and morphology of *Brycon hilarii* sperm, to contribute to the sperm characterization of this commercially important species.

2. Material and methods

All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were approved by the Committee for Animal Care and Use of the Federal University of Mato Grosso do Sul (protocol no. 1083/2019). The experiment was carried out in the spring of 2019, performed at the Laboratory of Juvenile Production of Neotropical South American Species in Terenos, Mato Grosso do Sul, and the analyses were performed at the Aquaculture Laboratory in Porto Alegre, Rio Grande do Sul, Brazil.

2.1. Experimental design and maintenance of males

The experimental design was completely randomized. The concentration, membrane integrity, morphology, morphometry, motility and sperm kinetics parameters data were obtained in duplicate for each male. A total of 22 specimens of *B. hilarii* (weighing 500 ± 50 g) were used. Males were kept in a pond of 1,000 m^2 with continuous water renewal of 10% per day. They were fed extruded 6 mm pellet feed (Socil – Peixes Brasileiros 32®) once a day, with an amount equivalent to 1% of the biomass of each tank. Animals with evidenced reproductive characteristics were selected and transported (silicone transport bags containing

water) to the reproduction laboratory and placed in 500 L water tanks with continuous water renewal.

2.2. Hormonal induction and collection of sperm

Hormonal induction was performed according to standardized methodology [37, 39], with an intramuscular injection of a single 2.5 mg/kg dose of carp pituitary extract (CPE). After 6 h (water temperature 24 °C), the animals were manipulated for sperm collection. The animals were handled with wet towels, the region of the urogenital papilla was dried with paper towels, and the anteroposterior massage was performed in the abdominal region. Sperm was collected with a 1 mL syringe, and aliquots were transferred to a 1.5 mL microtube and immediately evaluated.

2.3. Sperm evaluation

Spermatozoa movement video capture was performed to measure the sperm motility and kinetic parameters. For the evaluation of concentration, morphometry, and morphology, the samples were fixed in 10% buffered formaldehyde solution at a 1:1000 dilution.

Sperm concentration: An aliquot (10 µL) of the fixed sample was pipetted into each counting field of a Neubauer hemocytometer chamber (Olen®, Kasvi), which was covered with a coverslip and left for 15 min for the cells to stabilize. Using a microscope at 400× magnification (Nikon® E200, Tokyo, Japan) and a manual counter, the gametes were quantified by counting five squares. After cell counting, sperm concentration was estimated using the following equation [17]:

$$Spermatozoa\ mL^{-1} = \left(\frac{\sum SPTZ}{5\ q.c}\right) x \left(\frac{25\ q.t\ x\ dilution\ x\ 1000}{chamber\ depth\ (mm)}\right),$$

where:

Spermatozoa mL^{-1}: Number of spermatozoa per milliliter of sperm,

∑SPTZ: Total number of spermatozoa counted.

5 q.c: Squares counted,

25 q.t: Total squares,

Dilution: Factor of dilution of the sperm by the fixative and BTS, and

Chamber depth: Normality 0.1 mm.

Membrane Integrity: The percentage of cells with intact membranes was assessed using the eosin-nigrosine dye protocol, according to the procedure adapted from [18]. Briefly, 20 μL of sperm was homogenized with 20 μL of the eosin-nigrosine dye, and a smear was prepared. One hundred sperms were evaluated in each sample, and the number of intact (colorless) and non-intact (colored head) cells was expressed as a percentage.

Sperm Kinetic Parameters: These parameters were evaluated using computer-assisted sperm analyzers (CASA), as reported by Wilson-Leedy and Ingermann [19] and Neumann et al. [20]. For these analyses, sperm samples were activated with distilled water (1 μL of sperm:500 μL of water) in 1.5 mL microtubes and 5 μL was immediately placed in a Neubauer hemocytometer (0.1 mm deep) and covered with a glass cover slip (24 × 24 mm). Analyses were performed using a Solaris Bel trinocular light microscope (100× magnification). Images were captured using a Basler camera (acA640-120gc) connected to a computer and configured to capture images at 100 frames per second.

Videos were recorded at the exact time of sperm activation using the Basler Pylon camera software. Each video was 60 s long. The videos were edited using VirtualDub 1.9.0 software (Virtualdub.org) and saved in a specific folder. They were evaluated using the CASA plugin in ImageJ software (imagej.nih.gov/ij), using the settings adapted for Brazilian neotropical fish by Neumann et al. [20]. The analyzed sperm parameters included motility rate (MOT, %), curvilinear velocity (VCL, μm s^{-1}), average path velocity (VAP, μm s^{-1}), straight line velocity (VSL, μm s^{-1}), straightness of sperm path (STR, %), wobble (WOB, %), progressive motility of sperm (PROG, μm), beat cross frequency (BCF, Hz), and sperm number

(SPTZs); considering as mobile, cells with values above 55, 20 and 15 μm.s-1 for VCL, VAP and VSL, respectively.

Sperm Morphology and morphometry: The sperm fixed in buffered formaldehyde was stained with 4% Rose Bengal [21] at a dilution of 1:10 in a 1.5 mL microtube. Three drops of 10 μL were placed on a glass slide for preparation, using the drained drop method (adapted from Sanches et al. [22]). The slides were dried and analyzed under an optical microscope at 100× magnification (Nikon® E200, Tokyo, Japan), and sperm samples (*n* = 200 spermatozoa/slide) were evaluated for sperm morphology for the following characters [23]: loose head, degenerated head, macrocephaly, microcephaly, distally curled tail, strongly curled tail, broken tail, folded tail, short tail, and proximal and distal gout. Sperm morphometry was evaluated using 20 images of sperm with normal morphology for each male, generated from each histological slide, using an optical microscope (100×, Nikon® E200, Tokyo, Japan). Dimensions were obtained using the image analysis program ImageJ (imagej.nih.gov/ij). Head diameter, flagellum length, and total length of the sperm were determined (μm).

2.4. Statistical analysis

An exploratory analysis was performed because of the scarcity of information on the species analyzed in this study. A descriptive statistical analysis of the data was performed to select relevant data regarding normal morphology, sperm abnormalities, morphometry, and sperm kinetics parameters. The following data were calculated for descriptive statistics: number of values (*n*), minimum, maximum, median, mean, standard deviation, standard error of the mean, and coefficient of variation.

To assess the most prevalent sperm abnormality for each species, the abnormality data were subjected to normality analyses (Shapiro–Wilk, Kolmogorov–Smirnov, or D'Agostino–Pearson) and Levene's homogeneity test. After confirming compliance with statistical assumptions, the data were subjected to one-way analysis of variance (ANOVA), followed by

Tukey's test. To verify the correlation between the values of normal sperm and sperm abnormalities with the sperm kinetics parameters, Pearson's correlation analysis was performed.

3. Results

Optical microscopy images of sperm cells with normal morphology and the results of sperm morphometry for *B. hilarii* are shown in Figure 1. Sperm concentration of *B. hilarii* was $2.85 \pm 0.71 \times 10^9$ spermatozoa mL^{-1} and membrane integrity was 97.67 ± 1.99%.

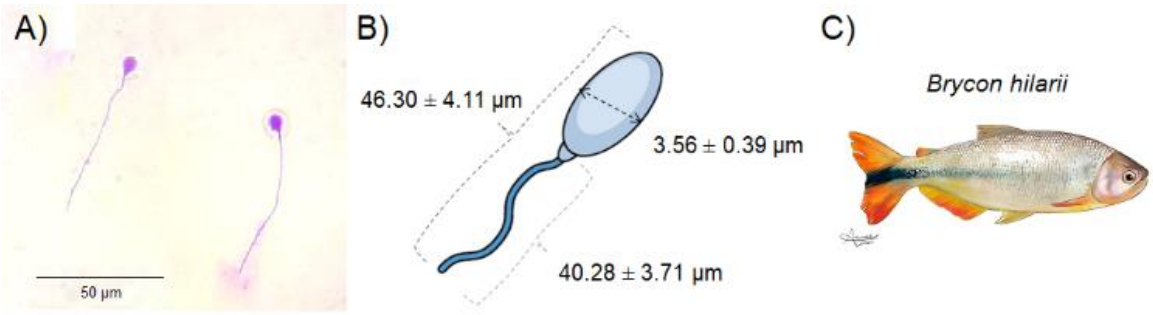


Fig. 1. A) Optical microscopy images of sperm cells with normal morphology (100×); B) Results of the analysis of sperm morphometry; and C) *Brycon hilarii*.

3.1. Descriptive statistics of B. hilarii sperm

Table 1 shows the descriptive statistics of sperm morphology and kinetic parameters of *B. hilarii* sperm at 10 s after sperm activation.

Table 1

Descriptive statistics of the sperm morphology and sperm kinetic parameters (CASA; 10s after sperm activation) of *Brycon hilarii.*

	Sperm morphology (%)											
Descriptive statistics	**NS**	**DCT**	**SCT**	**BT**	**FT**	**ST**	**LH**	**DH**	**MAC**	**MIC**	**PG**	**DG**
Number of values	16	16	16	16	16	16	16	16	16	16	16	16
Minimum	32	2.5	0	5	4	0.25	1	0	0	0	1.75	2

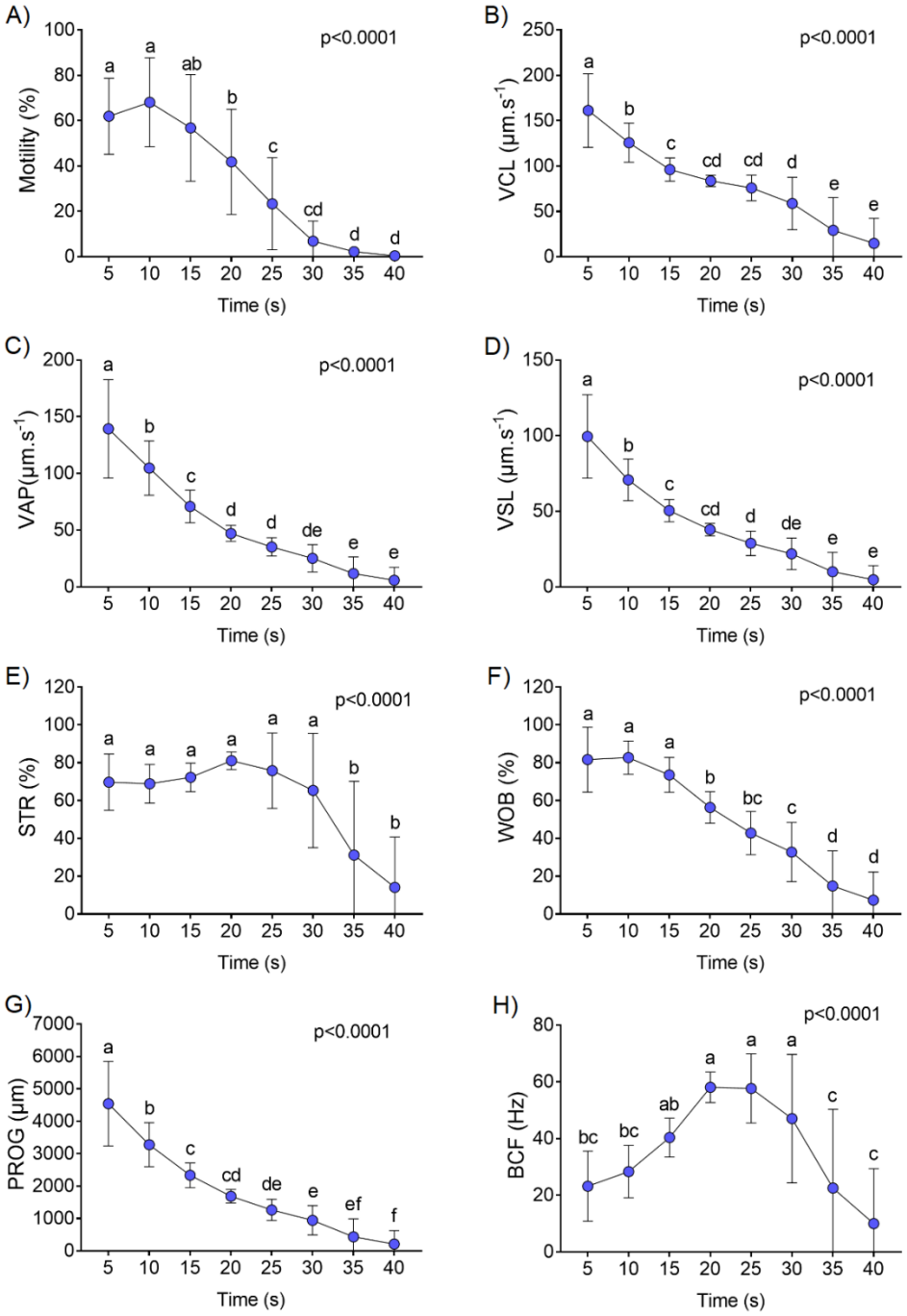
Median	53.9	5.4	0.5	14.4	7.5	2	5.8	0.6	0.3	0.3	3.6	5.3
Maximum	69.3	21	1.3	23.3	13.5	6.3	17.8	1.8	1.3	2	6.3	14.8
Mean	51.6	6.6	0.6	13.5	8.1	2.6	6.1	0.7	0.3	0.4	3.6	5.9
Std. Deviation	10.9	4.8	0.4	4.3	2.9	2.0	3.9	0.5	0.2	0.5	1.4	3.1
Std. Error of Mean	2.7	1.2	0.1	1.1	0.7	0.5	1.0	0.1	0.1	0.1	0.3	0.8
Coefficient of variation	21.2	72.1	65.3	32.3	35.9	76.9	65.3	73.3	120.4	122.8	37.8	51.2

	Sperm Kinetics (CASA)								
Descriptive statistics	**MOT**	**VCL**	**VAP**	**VSL**	**STR**	**WOB**	**PROG**	**BCF**	**SPTz**
Number of values	16	16	16	16	16	16	16	16	16
Minimum	30.8	87.2	53.5	42.3	55.1	52.8	1858	17.9	16.5
Median	70	126.3	106.3	70.5	69.2	84.5	3221	27.2	29.5
Maximum	96.3	154.8	140.5	98.4	90.9	91.6	4616	56.8	61.5
Mean	68.1	125.7	104.7	70.7	68.9	82.7	3279	28.3	34.5
Std. Deviation	19.6	21.6	23.9	13.8	10.2	8.8	677.8	9.2	13.7
Std. Error of Mean	4.9	5.4	6	3.4	2.6	2.2	169.5	2.3	3.4
Coefficient of variation	28.8	17.1	22.8	19.5	14.9	10.7	20.7	32.7	39.8

Sperm morphology: NS: Normal spermatozoa; DCT: Distally curled tail; SCT: Strongly curled tail; BT: Broken tail; FT: Folded tail; ST: Short tail; LH: Loose head; DH: Degenerate head; MAC: Macrocephaly; MIC: Microcephaly; PG: Proximal gout; DG: Distal gout. **Sperm kinetics (CASA)**: MOT (motility; %), VCL (curvilinear velocity; $\mu m.s^{-1}$), VAP (average path velocity; $\mu m.s^{-1}$), VSL (straight line velocity; $\mu m.s^{-1}$), STR (straightness of sperm path; %), WOB (wobble; %), PROG (progressive motile sperm; µm), BCF (beat cross frequency; Hz), SPTz (number of sperm; *n*).

The motility rate increased between 5 and 10 s after activation, but not significantly. It significantly decreased 20 s after sperm activation (Figure 2A). The oscillation coefficient (Figure 2F) indicated an increase in the sinuosity of the movement 20 s after activation. The

184 motility ceased after 40 s. The velocities (VCL, VAP and VSL) and PROG (Figures 2B, C, D
185 and G, respectively) showed a similar pattern, significantly reducing 10 s after activation. STR
186 (Figure 2E) showed significant reduction only 35 s after activation. The BCF (Figure 2H)
187 increased between 20 and 30 s after activation, indicating that movement became more chaotic
188 in this period, and then reduced.



189

Fig. 2. Comparison between the results of sperm motility and kinetic parameters analysis (CASA) of *Brycon hilarii* over 40 s after the start of video recording (the moment of sperm activation). Different letters indicate difference between times calculated using Tukey's test.

Figure 3 shows a comparison of the sperm abnormalities of *B. hilarii*. The average value of spermatozoa with normal morphology (51.6 ± 6.6%; Figure 3A) and the morphological abnormalities were observed. When comparing the types of abnormalities, the tail abnormalities (31.4 ± 7.33%) had higher values than the head abnormalities (7.5 ± 4.34%) and cytoplasmic gouts (9.5 ± 3.28%) (Figure 3A). The most frequent and least frequent morphological changes were broken tail (13.5 ± 4.3%) and macrocephaly (0.3 ± 0.2%), respectively (Figure 3B).

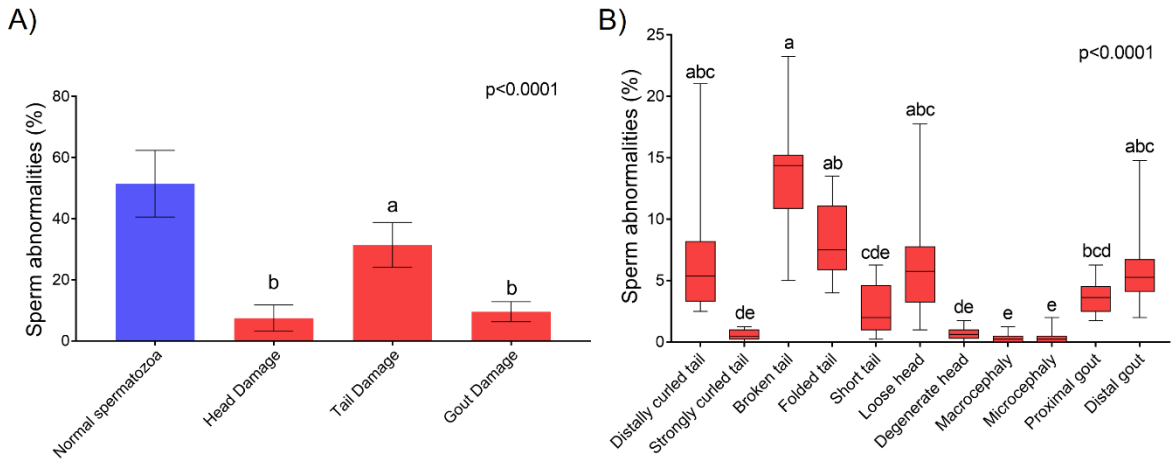


Fig. 3. A) Comparison of different types of sperm abnormalities in *Brycon hilarii*. B) Comparison of different sperm abnormalities in *B. hilarii*. Different letters indicate a difference between different sperm abnormalities.

3.2. Correlation between the incidence of sperm abnormalities and the sperm kinetic parameters

Figure 4 shows the correlation between abnormalities and sperm kinetic parameters for *B. hilarii*. A negative correlation was found between degenerated head and the motility variables VCL, VAP, VSL, WOB, and PROG (Figures 4A-F). Therefore, a higher percentage of degenerated heads in the sample decreased the responses of these CASA variables.

A negative correlation was found between macrocephaly and VSL and PROG (Figures 4H-J), and between microcephaly and WOB (Figure 4K). Therefore, a higher percentage of macrocephaly and microcephaly in the sample decreases the response to these kinetic parameters. In addition, a positive correlation was found between BCF and degenerated head and microcephaly abnormalities (Figure 4G and L), indicating that the increase in these abnormalities was directly correlated with an increase in BCF.

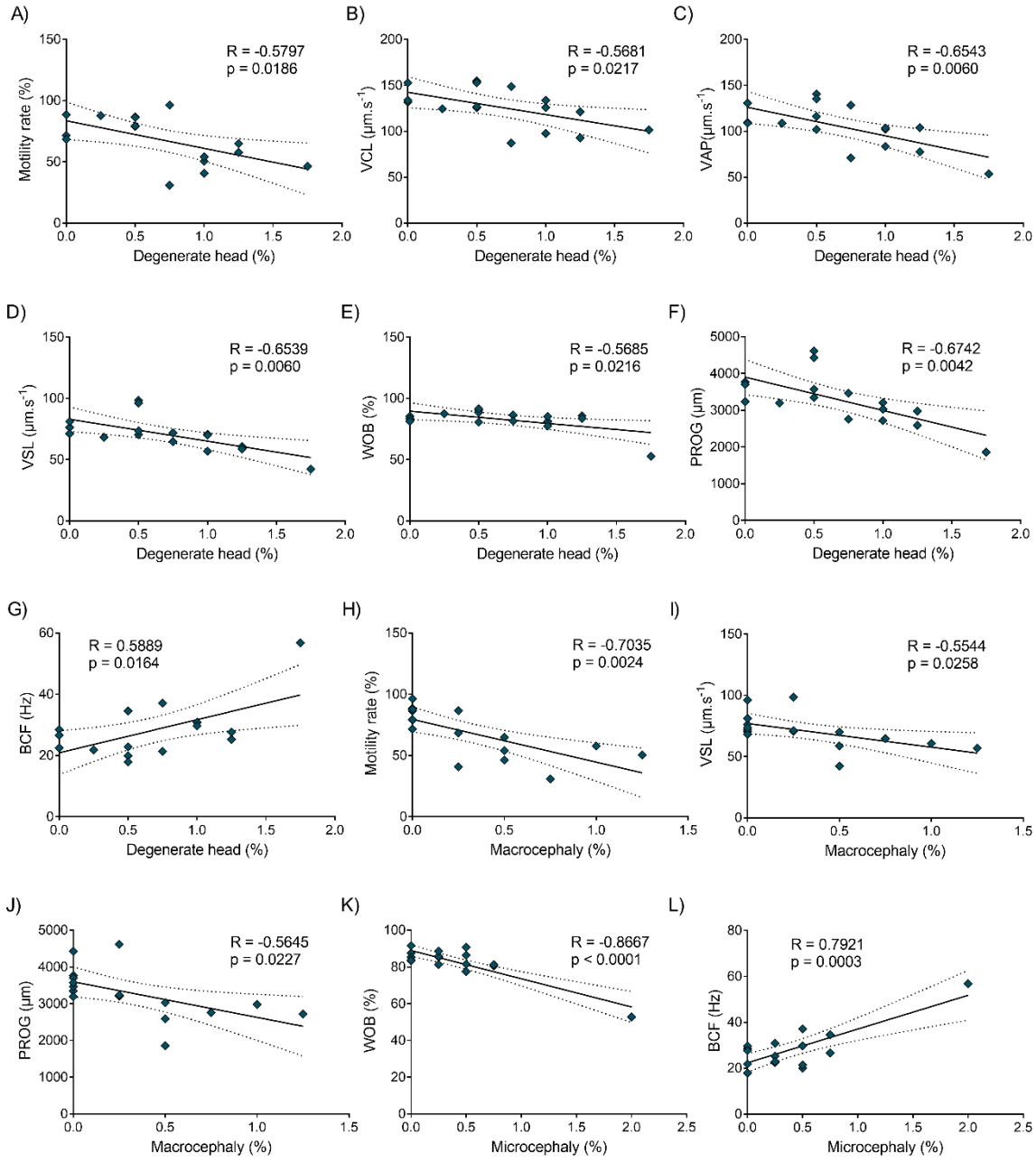


Fig. 4. Significant correlations between the sperm abnormalities and sperm kinetic parameters of *Brycon hilarii* sperm. A) Degenerate head (%) X Motility rate (%) (R=-0.5797; *P*=0.0186); B) Degenerate head (%) X VCL (μm s^{-1}) (R=-0.5681; *P*=0.0217); C) Degenerate head (%) X VAP (μm s^{-1}) (R=-0.6543; *P*=0.0060); D) Degenerate head (%) X VSL (μm s^{-1}) (R=-0.6539; *P*=0.0060); E) Degenerate head (%) X WOB (%) (R=-0.5685; *P*=0.0216); F) Degenerate head (%) X PROG (μm) (R=-0.6742; *P*=0.0042); G) Degenerate head (%) X BCF (Hz) (R=0.5889; *P*=0.0164); H) Macrocephaly (%) X Motility rate (%) (R=-0.7035; *P*=0.0024); I) Macrocephaly (%) X VSL (μm s^{-1}) (R=-0.5544; *P*=0.0258); J) Macrocephaly (%) X PROG (μm) (R=-0.5645; *P*=0.0227); K) Microcephaly (%) X WOB (%) (R=-0.8667; *P*<0.0001) and L) Microcephaly (%) X BCF (Hz) (R=0.7921; *P*=0.0003).

4. Discussion

The study of reproductive biology and sperm characterization of native South American species is extremely important in the control of reproduction in captivity because sperm quality directly affects successful fertilization [24]. In this study, the qualitative sperm indices of *B. hilarii* were analyzed for the first time using individuals kept in captivity, making it possible to morphologically characterize the sperm, in addition to measuring the kinetic parameters and observing the correlations between these indices. The quality of gametes, despite their importance for reproduction in captivity, is one of the limiting factors for reproductive success, as it is affected by several environmental variables [25, 26]. Therefore, the reproductive success of fish depends on improving artificial reproduction techniques and obtaining quality gametes.

Overall, the mean of the parameters observed for piraputanga was similar to that of other studies that evaluated the sperm profile of *Brycon*. An important reference was the high percentage of sperm cells with intact membranes, which was consistent with the values found in *B. orbignyanus* (94.2% and 89.7%; [27], [28], respectively); *B. opalinus* (96%; [29]) and *B. amazonicus* (91%; [30]).

241 Sperm concentration is essential for quantifying the minimum number of sperm needed
242 to fertilize oocytes, especially when fish are managed in the laboratory to produce juveniles.
243 The mean concentration observed in our study with *B. hilarii* was lower than that observed in
244 other species of the same genus, such as *B. orbignyanus* [31], *B. insignis* [32], *B. vermelha* [33],
245 *B. amazonicus* [30] and *B. siebenthalae* [34], for which the mean values observed varied from
246 3.4–13.9 × 10^9 spermatozoa mL^{-1}. When analyzing this parameter, we must also consider the
247 variation within and between species, age of the animal, time and season of the year, frequency
248 of collection, and hormonal protocol, among other variables for fish considered rheophilic [11],
249 such as those of the genus *Brycon*. In one neotropical rheophilic species (*Leiarius marmoratus*),
250 it is possible that sperm concentration dropped throughout the reproductive period, as
251 successive collections took place in captivity [35]. In another evaluated species,
252 *Steidachneridion parahybae*, sperm concentration varied throughout the reproductive period,
253 reaching maximum sperm production (associated with semen volume) after four months of
254 evaluation in captivity [36]. The use of a hormone induction protocol generally reduces sperm
255 concentration, as observed in *Pseudoplatystoma reticulatum* [37], *Colossoma macropomum*
256 [38], and *Salminus maxillosus* [39]. However, according to the study by Damasceno et al. [40]
257 on *Prochilodus britskii*, hormonal induction is associated with the reproductive period, because
258 at certain times, induction causes an increase in sperm concentration. Furthermore, a correct
259 assessment of the reproductive maturation stage may be a prerequisite for the success of
260 hormonal induction [41].

261 In our study, we measured *B. hilarii* sperm cells using optical phase contrast
262 microscopy, fixed them in saline formalin, and stained them with Rose Bengal, recommended
263 for Neotropical fish by Streit Jr. et al. [21]. Our objective, when measuring sperm using this
264 simple technique, was to ensure a practical, efficient, and cheap analysis, especially for juvenile
265 production laboratories. According to Gallego et al. [42], it is possible to use a simple method

to measure fish sperm heads for scientific applications on a commercial scale in aquaculture production, provided it meets the prerequisites mentioned by Rosenthal et al. [43] to decrease the coefficients of variation. The premise of Gallego et al. [42] can be observed when we associate the morphometry of the spermatozoa of *B. hilarii* with other analyses of kinetics, morphology, and membrane integrity. From this, we can understand and predict phenomena that were found when analyzing the sperm of males of this species kept in captivity.

The morphometric averages of *B. hilarii* spermatozoa were higher than those observed in studies on other species of the same genus. For example, Faustino et al. [33] recorded in *B. vermelha*, 32.4 μm, 29.6 μm, and 1.3 μm for total length, flagellum length, and head width, respectively. These measurements are similar to those observed by Ninhaus-Silveira et al. [44] for *B. cephalus* — 31.3 μm, 29.5 μm, and 1.7 μm for the same inferences (total length, flagellum length, and head width, respectively). Notably, the results recorded for the other species of the genus *Brycon* were obtained using the scanning electron microscopy technique, which is different from the light microscopy technique used in our study. This certainly explains the larger dimensions of *B. hilarii* spermatozoa observed in our study. However, light microscopy, using a suitable fixation and staining technique, provides a fast and adequate analysis at a much lower cost compared to electron microscopy [45].

Due to the growing number of studies on the importance of sperm morphometry, the technical debate regarding which methodology should be used is extensive in zootechnical species, unlike the methodology used for the evaluation of human spermatozoa, for which there are clear guidelines from the World Health Organization (WHO). For example, though scanning microscopy perfectly reveals the surface of a sperm cell, it requires a technical protocol that changes the true dimensions of the sperm cells. The fixation protocol and subsequent dehydrations with increasing concentrations of ethanol [46] strongly affect the morphometric variables of the sperm cells [45], resulting in shrinkage of the biological material [47]. In any

case, there is a consensus that the ideal method for optical microscopy should interfere as little as possible with the structure and size of the sperm, while clearly revealing the limits of its head, midpiece, and flagellum [48]. Finally, Czubaszek et al. [49] reported the need to establish and develop a staining technique that allows unambiguous and accurate analysis of sperm morphology and morphometry in different animal species.

To characterize the sperm of *B. hilarii*, it was essential to discriminate the different parameters that translate to its kinetics, and later associate them with morphometric and morphological parameters. Thus, combining the results obtained for the kinetics of spermatic movement, we can characterize the spermatozoa of *B. hilarii* as medium progressive, slightly sinuous, not very fast, and quite vigorous, with chaotic movement and frequent changes in swimming pattern. In general, the highest means of the variables (MOT, VCL, VAP, VSL, STR, WOB, and PROG) occurred between 5 and 10 s after activation. Sperm motility, assessed 10 s after activation, was 68.1% — a value close to that reported by Seabra de Souza et al. [50] for *B. insignis* (64.57%). However, this was lower than the motility observed by Di Chiacchio et al. [31], which was 78% in *B. orbignyanus*. These differences may be due to difficulties in assessing motility in a CASA system, which is highly affected by system settings, but can also be affected by more trivial factors such as immobile cells not being measured as a result of being out of focus [51].

In terms of time, motility had a short duration, reaching higher rates between 5 and 15 s, similar to what occurs with sperm from other species of the genus *Brycon* [34, 30, 52, 33, 28]. As for its displacement over 40 s, it was possible to observe a more chaotic and slightly faster movement in the first 30 s after activation, and then a reduction in motility, which could be explained by the reduction in energy availability. Thus, at the end of the motility period, a slower, sinuous, and non-progressive movement pattern was observed. A very high initial velocity, due to the high frequency of flagellar beating, is characteristic of fish sperm, but this

316 “highly active” period lasts only a few seconds immediately after contact with the activating
317 medium, and the values of all parameters of motility decline rapidly after the onset of flagellar
318 movement [53]. Liao et al. [54] related water turbulence to sperm number, swimming speed,
319 and motility time. Thus, lotic breeders release a greater number of sperm and have slower sperm
320 and shorter motility time than lentic breeders do.

321 In other species, sperm with abnormalities are not able to reach the oocyte [55]. In
322 mammals, only a small proportion of the sperm population is competent for fertilization due to
323 differences in their integrity and/or the presence of morphological and genetic abnormalities
324 [56]. Galo et al. [57] established that for *Piaractus mesopotamicus* primary abnormalities were
325 the main variable affecting fertilization and hatching rates in fresh and thawed sperms.
326 According to Boryshpolets et al. [58], sperm motility and morphology are the most important
327 parameters in the reproductive biology of fish. However, for the evaluation of morphological
328 changes, the normal morphology and morphometry of spermatozoa must be known to provide
329 a reliable representation [59].

330 The analysis of sperm morphology in this study revealed that 48.5% of spermatozoa
331 showed some type of morphological abnormality. Based on the value of the sperm
332 concentration obtained (2.85×10^9 spermatozoa mL^{-1}), practically half of these cells would be
333 committed to fertilize the oocytes. Successful fertilization results from a sequence of several
334 events that lead to gamete fusion. Nevertheless, the short life span of sperm and eggs, as well
335 as environmental conditions, limits reproductive success. This scenario is exacerbated, for
336 almost all fish species, by the existence of only one place on the surface of the oocyte where
337 spermatozoa can enter, the micropyle [60]. If a large proportion of the sperm is compromised
338 by the presence of morphological abnormalities, fertilization can be drastically affected. Thus,
339 the result of the morphological analysis must be correlated with the sperm concentration to
340 determine the insemination dose in captive reproduction. Nonetheless, the resulting

concentration in this study is still higher than the ideal minimum observed by Seabra de Souza et al. [50] (1.6×10^5 spermatozoa mL^{-1}) to obtain a sperm:oocyte ratio of 152,172:1 for *B. insignis*. According to Hirano et al. [61], the use of morphometric and sperm morphology reference values can increase our knowledge of their capacity for natural and in vitro fertilization, as well as their quality and function after cryopreservation. Furthermore, some morphological abnormalities may be formed during gamete preservation techniques [62, 63].

The forward movement of sperm depends on the flagella's ability to generate waves that propagate from the head-tail junction towards the tip of the flagellum, resulting in forward movement, as most of the flagellar length is covered by these waves [64]. Thus, tail abnormalities can compromise cell progression and alter the kinetics of movement. Therefore, sperm with a folded flagellum will surely have less success in fertilization owing to their oscillation [65] and non-progressive circular movement. In *B. hilarii* sperm, tail abnormalities were more frequent than head and cytoplasmic gouts, with emphasis on broken tail, folded tail, and distally curled tail. We observed a high percentage of immature sperm, characterized by cytoplasmic gouts (proximal and distal). In a previous study, Da Costa et al. [23] observed a positive correlation ($R = 0.91$) between the percentage of normal sperm and motility, reinforcing the findings of Cosson et al. [66] that morphological abnormalities of the sperm are responsible for the decrease in motility.

While tail abnormalities were the most frequent abnormalities in *B. hilarii* sperm, correlation analyses showed that head abnormalities are closely related to practically all sperm kinetic parameters for *B. hilarii*, with the exception of STR. It is possible to make robust inferences in macroscopic analysis. For example, of the head abnormalities, loose head abnormality was the most frequent (6.1%), but it did not present significant correlations with the kinetic parameters as compared to the degenerated head and macrocephaly abnormalities, which, despite the low frequency, significantly impacted sperm mobility. A fundamental point

must be considered when analyzing loose and degenerated head abnormalities and associating them with sperm kinetics. Loose head originate from the fragility of the head attachment region, with the connecting piece derived from the interaction of the centrioles with the spermatid nucleus (67, 68). According to several studies [69, 70, 71, 72, 73, 74], the increased fragility of the head-tail connection becomes evident when sperm are subjected to mechanical forces. Therefore, CASA did not accurately count the loose heads, as their appearance may have been affected when the sperm of *B. hilarii* were subjected to the mechanical action of making the slides (even though the drained drop method, and not the smear method, was used). However, sperm head and tail separation can be caused by several adverse factors that affect spermiogenesis and sperm maturation [75]. Notably, the sperm head is mainly occupied by the nucleus and DNA. In humans, sperm with head abnormality cannot fertilize oocytes [76, 77]. Therefore, *B. hilarii* spermatozoa with both abnormalities (loose and degenerated head) would not be able to fertilize the oocytes.

The macrocephaly abnormality also showed a negative correlation with some kinetic parameters. There is a consensus among several authors that its origin in spermatogenesis in many species could be due to the incomplete partition of meiotic divisions and a failure in nuclear cleavage [78, 79, 80, 81, 82, 83]. Though the cause of macrocephaly is still unclear, an accepted theory is related to the presence of the recessive mutation in aurora kinase C (AURKC) in infertile men [84]. This abnormality is also related to infertility, which has already been proven in humans [85]. Infertility caused by macrocephalic abnormalities is directly related to the concept proposed by Humphries et al. [86]. According to these authors, the speed reached by the sperm is proportional to the balance between the drag force (head) and thrust (flagella). Thus, the ratio of head to flagellum size can be used as an indicator of sperm swimming speed. The observed correlations corroborate these definitions, as macrocephaly abnormalities alter the ratio between flagellum length and head size (thrust/drag force). An increase in this

abnormality can, therefore, negatively affect motility and movement kinetics (velocities and PROG). In a turkey sperm study, Du Plessis et al. [87] observed that low VAP means indicated that sperm with macrocephaly swim slower than those considered normal. This proves what had already been observed in other groups such as birds [88], and humans [89]. The negative correlation observed between microcephaly and WOB can also be explained by the change in the ratio between flagellum length and head size, with the change in the head affecting its hydrodynamics, reducing WOB and increasing the sinuosity/oscillation of movement.

Interestingly, in our study, we observed positive correlations between BCF and degenerate head and microcephaly abnormalities. Thus, an increase in BCF would occur as degenerated head and/or microcephaly abnormalities increase. In this case, the issue may seem less obvious, but as the sperm trajectory depends on flagellar movement, and the BCF assesses the number of times the flagellar beat changes its pattern [90], our results suggest that these abnormalities, in addition to negatively affecting motility and movement kinetics, modify the pattern of sperm cell movement and alter vigor.

Fitzpatrick [91] however, raised an important question. According to the author, morphology is rarely related to the outcome of competitive fertilization success, and the role of sperm competition in influencing sperm morphology remains unclear. However, if sperm morphology is linked to swimming speed in species with external fertilization, why is sperm morphology generally uninformative for predicting competitive fertilization success? These issues may be further elucidated as more peer-reviewed studies are conducted on the sperm and reproductive biology of more fish species.

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Author contribution statement

All authors were involved in data analysis, interpretation, statistical analyses and drafting the manuscript. All authors gave final approval for publication.

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Declaration of competing interest

The authors declare have no potential conflicts of interest.

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CAPÍTULO V

CONSIDERAÇÕES FINAIS

O processo de criopreservação causa alterações morfológicas nos espermatozoides, com consequência direta na motilidade espermática e na taxa de fertilização, o que mostra a importância da avaliação da morfologia espermática, e as implicações das anormalidades morfológicas à qualidade espermática. Além disso, o aumento das anormalidades espermáticas classificadas como primárias, após a criopreservação, abre espaço para a discussão sobre a elaboração de uma classificação para as alterações morfológicas específicas dos espermatozoides de peixes, a fim de proporcionar uma melhor caracterização espermática das espécies e melhor avaliar os fenômenos que ocorrem após a criopreservação. Concluímos também que há uma alta heterogeneidade entre os estudos que avaliam os efeitos da criopreservação na morfologia e anormalidades espermáticas de peixes de água doce, devido à grande diversidade de espécies, a qualidade metodológica média dos estudos e as dificuldades na padronização dos protocolos, que limitam as conclusões sobre as especificidades dos danos e seus impactos nas alterações morfológicas.

Nossos estudos mostram que a caracterização espermática das espécies de peixes é extremamente importante para avaliação da qualidade espermática e para o desenvolvimento de protocolos padronizados de conservação de sêmen, sendo um passo fundamental no estudo da biologia reprodutiva das espécies e trazendo informações de extrema importância no controle e na eficiência da reprodução em cativeiro. No entanto, ainda é um dos fatores limitantes para o sucesso da reprodução artificial e na avaliação e padronização dos processos de conservação de sêmen. Assim, estudos básicos de caracterização espermática, são fundamentais no desenvolvimento de técnicas e protocolos reprodutivos, e de extrema importância para avaliação dos danos que estes processos causam aos espermatozoides de peixes. Além de contribuir em estudos futuros para fins de conservação de espécies.

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ANEXOS

ANEXO A – Guia para autores Cryobiology

INTRODUCTION

The Official Journal of the Society for Cryobiology

Types of article
•Regular Papers
•Brief Communications
•Reviews
•Letters to the Editor

Regular papers will describe experimental findings, techniques, or theory. They will consist of an abstract that summarizes the objective of the study, the methods used, and the conclusions reached. Abstracts should not exceed 250 words and should be adequate for direct presentation to abstracting services. After the abstract a list of up to 10 keywords that will be useful for indexing or searching must be included. The Introduction will contain a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations. The Materials and Methods section must be presented in sufficient detail to enable other investigators to repeat the work. The Results should be concise and should avoid redundant tables and figures illustrating the same data. The Discussion should interpret the results, with minimal recapitulation of findings.
Brief Communications are concise reports of original findings, techniques or theory and include an abstract no longer than 150 words and a list of up to 10 keywords. They are not divided into sections. As a guideline it is suggested that there should be no more than 3 tables and/or figures and a maximum of 10 references. The total length, including references, should not exceed 2500 words.
Reviews should only be submitted after first discussing the article with the Editor or a member of the Editorial Board. As with regular papers and brief communications, reviews will be subject to peer review.
Letters to the Editor should concern matters of general interest to the readership of the journal or papers recently published in the journal. Authors of papers that are the subject of comment will be given an opportunity to reply. Letters may not exceed 1 printed page in length and if publication deadlines are pressing, proofs may not be provided. The Editor's decision will be final.

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Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

[2] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon. 2018;19:e00205. https://doi.org/10.1016/j.heliyon.2018.e00205

Reference to a book:

[3] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.

Reference to a chapter in an edited book:

[4] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.

Reference to a website:

[5] Cancer Research UK. Cancer statistics reports for the UK, http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/; 2003 [accessed 13 March 2003].

Reference to a dataset:

[dataset] [6] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. https://doi.org/10.17632/xwj98nb39r.1.

Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also Samples of Formatted References).

Reference style

Format the references in compliance with the recommended style for this journal:
Last name, initial, full paper title, journal full name, year, volume (number) first and last page.
Examples:
Reference to a journal publication:
[1] Paivio A, Jansen B, Becker LJ. Comparisons through the mind's eye. Cognition 1975;37(2):635–47.
[2] Ulbrich SE, Kettler A, Einspanier R. Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. J Steroid Biochem Mol Biol 2003;84(2–3):279–289.
Reference to a book:
[3] Letheridge S, Cannon CR, editors. Bilingual education: teaching English as a second language. New York: Praeger; 1980.
Reference to a chapter in an edited book:
[4] Strunk Jr W, White EB. The elements of style. 3rd ed. New York: MacMillan; 1979 [chapter 4].
Reference to a conference (only abstracts 2-page long may serve as references):
[5] Douglis F, Ball Th. Tracking and viewing changes on the web. In: Proc. 1996 USENIX technical conference; 1996.
Reference to a Dataset
[6] [dataset] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. http://dx.doi.org/10.17632/xwj98nb39r.1.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

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ANEXO D - Documento de aprovação da CEUA projeto número 36140

CEUA UFRGS

PRÓ-REITORIA DE PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CARTA DE APROVAÇÃO/ADENDO

Número: 36140

Título: CARACTERIZAÇÃO QUALITATIVA E BIOQUÍMICA DO SÊMEN DE ZEBRAFISH (DANIO RERIO) COMO FERRAMENTA NA FORMULAÇÃO DE UM MEIO PARA A CRIOPRESERVAÇÃO

Pesquisador Responsável: DANILO PEDRO STREIT JR

A Comissão De Ética No Uso De Animais aprovou o Adendo ao Projeto **36140** em reunião realizada em 15/04/2019 - Sala 223 do Prédio do Instituto de Ciências Básicas da Saúde – ICBS - Campus Centro - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 200 exemplares de zebrafish (Danio rerio) a serem adquiridos de fornecedor especializado (DelphisPet, Porto Alegre, RS- CNPJ: 00.187.830.0001/70); de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa. **Este documento revoga a Carta de Aprovação emitida anteriormente.**

Porto Alegre, 04 de abril de 2019.

Alexandre Tavares Duarte de Oliveira

Coordenador da CEUA/UFRGS

ANEXO E - Documento de aprovação da CEUA projeto número 1083

Serviço Público Federal
Ministério da Educação
Fundação Universidade Federal de Mato Grosso do Sul

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação reprodutiva de machos e fêmeas de espécies reofílicas: indutores hormonais, retorno reprodutivo e sistemas reprodutivos", registrada com o nº 1.083/2019, sob a responsabilidade de **Jayme Aparecido Povh** - que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA DA UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL/UFMS, na 7ª reunião ordinária do dia 26/09/2019.

FINALIDADE	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	01/11/2019 a 31/10/2023
Espécie/Linhagem/Raça	*Astyanax spp. / Lambari, Piaractus mesopotamicus / Pacu, Colossoma macropomum / Tambaqui, Prochilodus lienatus / Curimba, Brycon hilarii / Piraputanga, Leiarius marmoratus / Jundiá amazônico, Pseudoplatystoma corruscans / Pintado, Pseudoplatystoma reticulatum / Cachara*
Nº de animais	110 (55 machos + 55 fêmeas) de cada espécie
Peso/Idade	10 g / 1 ano, 3000 g / 3 anos, 6000 g / 3 anos, 1000 g / 2 anos, 1000 g / 2 anos, 4000 g / 3 anos, 6000 g / 3 anos, 4000 g / 3 anos
Sexo	Machos e Fêmeas
Origem	Estação Experimental de Piscicultura da UFMS (Campo Grande/MS); Setor de Piscicultura da UFMS (Terenos/MS); Piscicultura Piraí Terenos/MS).

Fábio José Carvalho Faria
Coordenador da CEUA/UFMS
Campo Grande, 30 de setembro de 2019.

Documento assinado eletronicamente por **Fabio Jose Carvalho Faria**, **Professor do Magisterio Superior**, em 30/09/2019, às 10:38, conforme horário oficial de Mato Grosso do Sul, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS
Av Costa e Silva, s/nº - Cidade Universitária
Fone:
CEP 79070-900 - Campo Grande - MS

VITA

Bruna Bitencourt da Costa, nascida no dia 06 de novembro de 1988, na cidade de Porto Alegre, filha de Telmo Dornelles da Costa e Solange Bitencourt da Costa. Cursou ensino fundamental no Colégio Cruzeiro do Sul e médio no Colégio Marista Assunção, em Porto Alegre, concluindo seus estudos em 2005. Ingressou no curso de Administração, da Universidade Estadual do Rio Grande do Sul em 2006, que cursou até 2008. Em 2007 ingressou no curso de Física (Licenciatura), da Universidade Federal do Rio Grande do Sul, até 2008. Ingressou no curso de Agronomia, da Universidade Federal do Rio Grande do Sul em 2010, onde estagiou nas áreas de nutrição animal, fitotecnia, biologia molecular e aquicultura; concluindo o curso em 2015. Mestre em Zootecnia pela Universidade Federal do Rio Grande do Sul (2018), ingressou em abril de 2018 no curso de Doutorado em Zootecnia pela Universidade Federal do Rio Grande do Sul, sob orientação do Prof. Danilo Pedro Streit Jr.