NAIARA CRISTINA MOTTA

CRIOPRESERVAÇÃO ESPERMÁTICA EM *Prochilodus lineatus* E *Chirostoma estor (Pisces, Prochilontidae e Atherinopsidae)*

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não-Ruminantes, para obtenção do título de Doutor.

Prof. Dr. Luis David Solis Murgas
Orientador

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CRIOPRESERVAÇÃO ESPERMÁTICA EM *Prochilodus lineatus* E *Chirostoma estor (Pisces, Prochilontidae e Atherinopsidae)*

SPERMATIC CRYOPRESERVATION IN *Prochilodus lineatus* AND *Chirostoma estor (Pisces, Prochilontidae e Atherinopsidae)*

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não-Ruminantes, para obtenção do título de Doutor.

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RESUMO

As construções de barragens, pesca predatória, poluição do habitat e introdução de espécies exóticas são fatores que afetam a reprodução de algumas espécies de peixes e, consequentemente a redução da população selvagem no ambiente. Dessa forma, dois experimentos foram conduzidos para avaliar os efeitos da criopreservação no sêmen de uma espécie modelo animal (*Prochilodus lineatus*) e de uma espécie ameaçada de extinção (*Chirostoma estor)*. O experimento 1 foi conduzido para avaliar os efeitos da suplementação de diferentes doses de melatonina na criopreservação do sêmen de *Prochilodus lineatus*. Foram utilizados 18 machos (6 pools de sêmen x 3 machos) e 2 fêmeas de *P. lineatus*. O meio crioprotetor foi suplementado com 2,00; 2,75; 3,50 e 4,25 mM de melatonina (MEL) e adicionalmente um grupo controle (sem adição de melatonina), totalizando 5 tratamentos e 10 replicatas por tratamentos. Amostras criopreservadas com 2,00 mM MEL exibiram maior taxa de motilidade (93%), em relação aos outros tratamentos com adição de melatonina (64–69%). O tratamento controle e 2,00 mM MEL resultaram em maior integridade de membrana (78%) se comparado a 4,25 mM MEL (65%). Em relação ao estresse oxidativo, menor peroxidação lipídica ocorreu nas amostras criopreservadas com 2,75 mM e 3,50 mM MEL (0,72-0,81 µM de MDA equivalentes), em comparação com o controle (2,12 µM de MDA equivalentes). Enquanto maior atividade enzimática da catalase ocorreu no controle (3,24 U-CAT/mg de proteína). Maiores taxas de fertilização e eclosão ocorreram em 2,75 mM MEL (27% e 17%, respectivamente) em comparação com 4,25 mM MEL (5% e 6%, respectivamente). Por sua vez, no experimento 2, avaliaram-se os efeitos de diluidores e crioprotetores na criopreservação do sêmen de *Chirostoma estor*. Foram utilizados 42 machos (6 pools de sêmen x 7 machos) e 3 fêmeas de *C. estor*. Os diluidores comerciais testados foram: BTS™, MIII™ e Androstar Plus™ e os crioprotetores testados foram: dimetilsulfóxido (DMSO) e metil glicol (MG), constituindo em um experimento fatorial de 3 x 2 (3 diluidores x 2 crioprotetores), totalizando 6 tratamentos e 3 replicatas. Os tratamentos MIII™+MG (40%) e Androstar Plus™+MG (48%) apresentaram as maiores taxas de motilidade (20–30%) que os demais tratamentos. Não foram observadas diferenças quanto a duração de motilidade (114–222 s) e integridade de membrana (54–60%). Somente para a taxa de fertilização houve interação significativa entre o diluidor e crioprotetor. O crioprotetor MG permitiu taxas de fertilização independente do diluidor (12–20%), e apenas em amostras criopreservadas em Androstar Plus™+DMSO (14%) foi possível obter taxas de fertilização. Em conclusão, para obter maior qualidade do esperma após o descongelamento, 2,00 mM de melatonina pode ser suplementada ao meio de criopreservação de *Prochilodus lineatus,* enquanto o sêmen de *Chirostoma estor* pode ser criopreservado nos diluidores comerciais MIII™ e Androstar Plus™, associado ao crioprotetor metil glicol.

Palavras-chave: Peixes. Espermatozoides. Qualidade Espermática. Antioxidante. Crioprotetores.

ABSTRACT

The construction of dams, overfishing, habitat pollution, and the introduction of exotic species are factors that affect the reproduction of some fish species and, consequently, the reduction of the wild population in the environment. Thus, two experiments were carried out to evaluate the effects of cryopreservation in the sperm of an animal model species (*Prochilodus lineatus*) and an endangered species (*Chirostoma estor*). Experiment 1 was carried out to evaluate the effects of supplementation of different doses of melatonin on cryopreservation of *Prochilodus lineatus* sperm. Eighteen males (6 sperm pools x 3 males) and 2 females of *P. lineatus* were used. The cryoprotective medium was supplemented with 2.00; 2.75; 3.50 and 4.25 mM of melatonin (MEL) and additionally a control group (without the addition of melatonin), totaling 5 treatments and 10 replicates per treatment. Samples cryopreserved with 2.00 mM MEL exhibited a higher motility rate (93%), compared to other treatments with the addition of melatonin (64-69%). The control treatment and 2.00 mM MEL resulted in greater membrane integrity (78%) compared to 4.25 mM MEL (65%). Regarding oxidative stress, lower lipid peroxidation occurred in samples cryopreserved with 2.75 mM and 3.50 mM MEL (0.72-0.81 µM MDA equivalents), compared to the control (2.12 µM MDA equivalents). While the higher enzymatic activity of catalase occurred in the control (3.24 U-CAT/mg protein). Higher fertilization and hatching rates occurred at 2.75 mM MEL (27% and 17%, respectively) compared to 4.25 mM MEL (5% and 6%, respectively). On the Other hand, in experiment 2, the effects of extenders and cryoprotectants on the sperm cryopreservation of *Chirostoma estor* were evaluated. Forty-two males (6 semen pools x 7 males) and 3 females of *C. estor* were used. The commercial extenders tested were: BTS™, MIII™, and Androstar Plus™ and the cryoprotectants tested were: dimethylsulfoxide (DMSO) and methyl glycol (MG), constituting a 3 x 2 factorial experiment (3 extenders x 2 cryoprotectants), totaling 6 treatments and 3 replicates. Treatments MIII™+MG (40%) and Androstar Plus™+MG (48%) had the highest motility rates (20-30%) than the other treatments. No differences were observed in motility duration (114-222 s) and membrane integrity (54-60%). Only for the fertilization rate, there was a significant interaction between extender and cryoprotectant. The MG cryoprotectant allowed dilution rates independent of the extender (12-20%), and only in samples cryopreserved in Androstar Plus™+DMSO (14%), it was possible to obtain fertilization rates. In conclusion, to obtain a higher sperm quality after thawing, 2.00 mM of melatonin can be supplemented to the cryopreservation medium of *Prochilodus lineatus*, while the semen of *Chirostoma estor* can be cryopreserved in commercial extenders MIII™ and Androstar Plus™ associated with cryoprotectant methyl glycol.

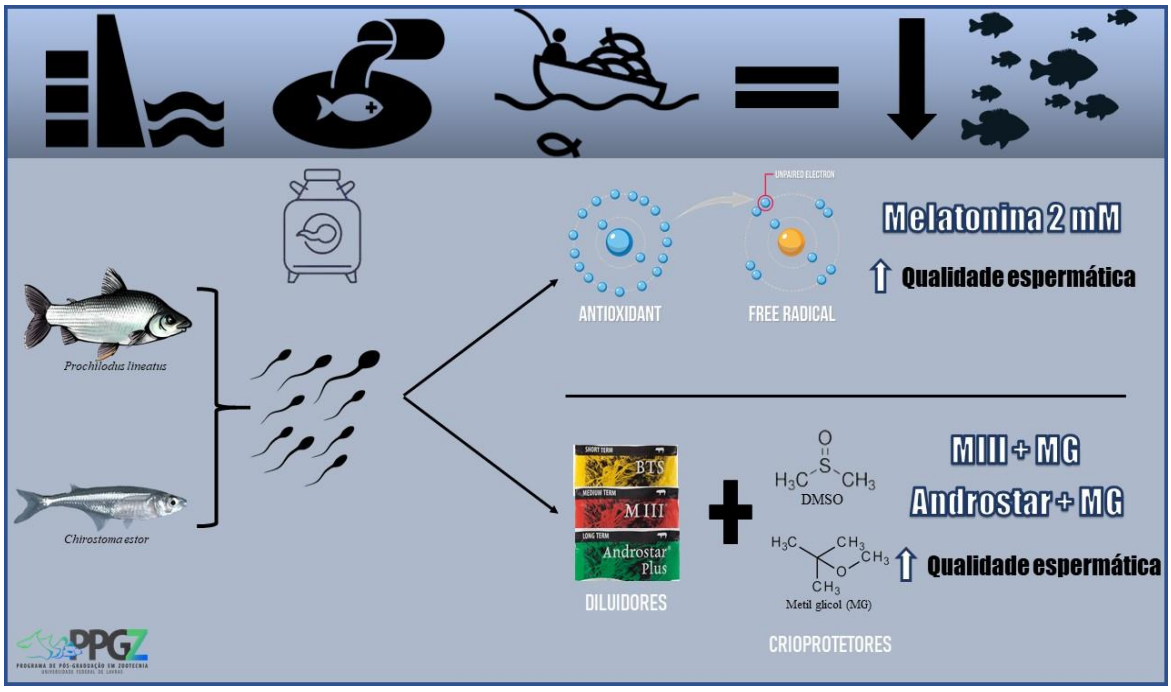
Keywords: Fish. Spermatozoon. Sperm Quality. Antioxidant. Crioprotectants.

Resumo Interpretativo e Resumo Gráfico

Criopreservação espermática em *Prochilodus lineatus* e *Chirostoma estor* (*Pisces, Prochilontidae e Atherinopsidae*)

Elaborado por **Naiara Cristina Motta** e orientado por **Luis David Solis Murgas**

As construções de barragens, pesca predatória, poluição do habitat e introdução de espécies exóticas são fatores que afetam a reprodução de algumas espécies de peixes. Consequentemente ocorre a redução da população selvagem no ambiente, uma das formas de minimizar esse problema é a aplicação de biotecnologias reprodutivas. Dentre estas biotecnologias, encontra-se a preservação de sêmen, com o intuito de formar criobancos de sêmen. Para a espécie *Prochilodus lineatus* encontra-se definida a solução de criopreservação do sêmen. Atualmente o protocolo para essa espécie deve ser aperfeiçoado para obtenção de um sêmen com maior qualidade espermática após descongelamento. A adição de antioxidantes ao meio crioprotetor tem por função a redução do estresse oxidativo causado pelo processo de criopreservação. Na presente pesquisa, a suplementação de 2 mM de melatonina como um antioxidante ao sêmen de *P. lineatus* permitiu uma maior qualidade espermática após descongelamento, em relação a não suplementação de melatonina. A espécie *Chirostoma estor* encontra-se ameaçada de extinção e um protocolo de criopreservação para essa espécie não havia sido determinado. Um meio de criopreservação espermática deve conter um diluidor e um crioprotetor permeável. Para o esperma de *C. estor* os diluidores comerciais MIII™ e Androstar Plus™ combinado com o crioprotetor metilglicol, permitem uma maior qualidade espermática após o descongelamento. Pesquisas que envolvem protocolos de criopreservação de sêmen em peixes, apresentam grande importância devido à diversidade de espécies brasileiras, bem como a diversidade mundial.



Criopreservação espermática de *Prochilodus lineatus* suplementado com diferentes concentrações de melatonina como um antioxidante, e de sêmen de *Chirostoma estor* criopreservado em diferentes diluidores comerciais e crioprotetores.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A criopreservação é uma técnica que envolve o armazenamento por longo tempo de células vivas individuais e tecidos biológicos em temperaturas muito baixas, como a temperatura do nitrogênio líquido, geralmente a -196 °C (MINS et al., 2000). O processo de criopreservação permite que as atividades celulares sejam inibidas/reduzidas e as células podem ser mantidas geneticamente estáveis por um longo tempo até serem descongeladas (AFREN; UÇAK, 2020).

O primeiro protocolo de criopreservação de sêmen de peixes, foi realizado por Blaxter em 1953, sendo a espécie de estudo o arenque do atlântico (*Clupea harengus*). O processo de criopreservação de sêmen em peixes é uma biotecnologia reprodutiva que apresenta amplas vantagens como: a recuperação dos estoques silvestres para as espécies ameaçadas de extinção; redução no número de reprodutores, diminuindo os custos com a manutenção do plantel; eliminação da assincronia gonadal entre reprodutores; facilidade para transportar e trocar material genético; manutenção de banco de germoplasma em programas de melhoramento genético e conservação das espécies em extinção.

Apesar das aplicabilidades do processo de criopreservação reportados acima, existe uma grande biodiversidade de peixes de água doce e marinhos. Essa biodiversidade gera diferentes protocolos de criopreservação, devido às diferenças biológicas e morfológicas dos espermatozoides. Durante as etapas do processo de criopreservação (resfriamento, congelamento e descongelamento), alguns processos biofísicos e químicos, como mudanças na osmolalidade, desidratação e reidratação, mudanças de volume celular, formação de cristais de gelo, concentração iônica dos diluidores, toxicidade de crioprotetores, entre outros, podem ocorrer mais agressivamente em algumas espécies (CABRITA et al., 2014).

Apesar dos 68 anos do primeiro protocolo de criopreservação de sêmen de peixes, diversas pesquisas ainda vem sendo realizadas todos os anos. Esse fato ocorre, pois muitas espécies ainda não possuem um protocolo de criopreservação definido, como no caso da espécie ameaçada de extinção *Chirostoma estor*, ou em espécies que possuem o protocolo definido, como *Prochilodus lineatus*. Dessa forma, torna-se necessário pesquisas que visam melhorar os protocolos de criopreservação de sêmen de peixes para promover a

propagação de espécies de alto valor genético e a preservação daquelas ameaçadas de extinção.

2 REFERENCIAL TEÓRICO

2.1 Processo de Criopreservação

A criopreservação de espermatozoides de peixes pode ser aplicada na melhoria do manejo de reprodutores, preservação de linhagens geneticamente selecionadas resultantes de programas de melhoramento genético, auxilio com espécies com problemas reprodutivos como falta de sincronização entre machos e fêmeas ou com aqueles com baixa produção espermática (ASTURIANO; CABRITA; HORVÁTH, 2017).

O processo de criopreservação expõe as células ao estresse induzido por baixa temperatura e desequilíbrios osmóticos, gerando mudanças conformacionais irreversíveis (SIEME; OLDENHOF; WOLKERS, 2016). Segundo Chian (2010), durante o processo físico de congelamento, a água tende a cristalizar, enquanto os solutos dissolvidos ou suspensos se concentram no líquido restante. Em um processo relativamente lento de congelamento, o gelo começa a se formar no fluido que circunda as células, e a concentração de solutos dissolvidos no líquido restante aumenta. Um gradiente de concentração é estabelecido através da membrana celular, e a água se move para fora da célula em resposta à pressão osmótica. Enquanto o congelamento continua, a célula fica relativamente desidratada, que pode afetar a organização estrutural da célula e também os processos moleculares. Finalmente, à medida que os cristais de gelo aumentam, a membrana celular pode ser rompida pelos próprios cristais ou pelo alto gradiente de concentração imposto na membrana celular.

Se o processo de resfriamento ocorre rapidamente, não irá ocorrer a perda de água do meio intracelular, e dessa forma, o meio intracelular irá congelar formando cristais de gelo, durante o processo de descongelamento esses cristais de gelo formados anteriormente podem se recristalizar em cristais maiores, rompendo a membrana plasmática (VIVEIROS; ORFÃO; LEAL, 2014). No entanto, se as células são resfriadas muito lentamente, elas sofrerão uma severa desidratação e exposição a longo prazo a altas concentrações de soluto, cujo aumento de concentração de solutos e a desidratação celular tem efeitos deletérios sobre os complexos lipídico-proteicos das membranas celulares, enfraquecendo-as e aumentando

as perdas lipídicas e fosfolipídicas (GAO; CRITSER, 2000). Assim, a taxa de congelamento deve ser suficientemente lenta para permitir a correta desidratação, evitando a formação de gelo intracelular, e suficientemente rápida para impedir que as células fiquem expostas por longo prazo a altas concentrações do soluto (BENSON et al., 2012). Usualmente o sêmen de peixes pode ser criopreservado em botijão de vapor de nitrogênio líquido, onde a taxa de congelamento reportada é de -35,6 °C por minuto, entre 21 °C a -170 °C (MARIA et al., 2006).

2.2 Soluções de Criopreservação

O sucesso da criopreservação de sêmen de peixes está relacionado à adequação da solução de criopreservação para determinada espécie. Basicamente, a solução de criopreservação deve ser composta de um diluidor, que possui a função de nutrir a célula e, um crioprotetor permeável, que possui a função de proteger a célula das crioinjúrias (SALMITO et al., 2016).

Os espermatozoides de peixes são imóveis no testículo e no plasma seminal, dessa forma o principal requisito do diluidor é ser isosmótico ao plasma seminal. O plasma seminal de peixes de água doce apresenta uma osmolalidade de 230 a 346 mOsm/kg, logo diluidores que possuem menor osmolalidade ativam os espermatozoides, exaurindo sua fonte energética para motilidade após descongelamento (DZYUBA; COSSON, 2014). É importante, que o diluidor não apresente toxicidade e nem agentes contaminantes como microrganismos, e nutra a célula (JAWAHAR; BETSY,2020). Devido ao grande número de espécies e as diferenças na composição iônica do plasma seminal, diversos diluidores são reportados na literatura segundo a espécie.

As soluções utilizadas para a diluição de sêmen são basicamente compostas de sais (NaCl, KCl, $NaHCO_3$), açucares (glicose e frutose) ou combinação de sais/açucares, formando soluções mais complexas (TABELA 1). Os diluidores formulados existentes, muitas vezes estão relacionados a composição do plasma seminal da espécie ou soluções comerciais, como exemplo diluidores comerciais de sêmen de suínos utilizados em algumas espécies de peixes (MURGAS et al., 2014).

Os crioprotetores são adicionados ao meio de congelamento para proteger os espermatozoides através de diversos mecanismos, como a diminuição do ponto de congelamento do conteúdo intracelular e da água extracelular, penetrando e interagindo com

componentes citoplasmáticos, bem como formando uma camada protetora ao redor das membranas de espermatozoides, sendo dessa forma, classificados em dois grupos, intracelulares e extracelulares. (HEZAVEHEI et al., 2018).

Os agentes crioprotetores intracelulares são geralmente moléculas não iônicas e osmoticamente inativas, porque são igualmente distribuídas nos espaços intracelulares e extracelulares (SIEME; OLDENHOF; WOLKERS, 2016). Basicamente o crioprotetor intracelular atua reduzindo o ponto de congelamento do meio extracelular, atenuando os efeitos deletérios causados pela formação dos cristais de gelo, regulando a velocidade de desidratação das células e reduzindo os danos causados pela alta concentração dos solutos (VIVEIROS; ORFÃO; LEAL, 2014). Os crioprotetores comumente utilizados para peixes são o dimetil sulfóxido (DMSO), propilenoglicol, metil glicol, etilenoglicol, metanol e glicerol, destacando a utilização do metil glicol (TABELA 1); (AUSTURIANO; CABRITA; HORVÁTH, 2017).

As soluções crioprotetoras extracelulares são substâncias de alto peso molecular e podem ser divididas em moléculas osmoticamente ativas, como mono e dissacarídeos (sacarose, trealose e glicose) e compostos osmóticos inativos incluindo polissacarídeos (hidroxietilamido, maltodextrina) e proteínas (albumina, polivinilpirrolidona), recobrindo a superfície da célula e estabilizando a membrana, além de restituir os fosfolipídios perdidos pelos espermatozoides durante o choque térmico (SALMITO-VANDERLEY et al., 2012; VIVEIROS; ORFÃO; LEAL, 2014; SIEME; OLDENHOF; WOLKERS, 2016; ELLIOTT; WANG; FULLER, 2017).

TABELA 1 - Diluidores e crioprotetores de sêmen utilizados para peixes neotropicais.

Espécie	**Diluidor**	**Crioprotetor**	**Autor**
Prochilodus lineatus	BTS™ 5%	DMSO 10%	PAULA et al., 2019
	Glicose 5%	Metil glicol 10%	DI CHIACCHIO et al., 2017
	Glicose (325mOsm/Kg)	Metil glicol 10%	VIVEIROS et al., 2017
	BTS™ 5%	DMSO 8%	ASSIS et al., 2019
Prochilodus vimboides	Glicose 5%	Metil glicol 10%	FRANÇA et al., 2020
Prochilodus brevis	ACP-104	DMSO 10%	NASCIMENTO et al., 2017
Brycon orbignyanus	NaCl (325 mOsm/Kg)	Metil glicol 10%	DI CHIACCHIO et al., 2017
	BTS™ 5%	Metil glicol 10%	FRANÇA et al., 2020
	BTS™ 5%	Metil glicol 10%	PALHARES et al., 2020
Brycon insignis	BTS™ 5%	Metil glicol 10%	VIVEIROS et al., 2019
Colossoma macropomum	BTS™ 5%	DMSO 7.5%	GALO et al., 2020
	Glicose 5%	Gema de ovo 5% + Metil glicol 10%	GALLEGO et al., 2017
Piaractus mesopotamicus	Rafinose 100 mM	DMSO 10%	PIRES et al., 2018
Rhamdia quelen	D-frutose 5%	Leite em pó 5% + Metanol 10%	KRAUSE et al., 2021
	Glicose 5%	Leite em pó 5% + Metanol 10%	GOES et al., 2017
	Frutose 5%	Leite em pó 5% + Metanol 10%	COSTA et al., 2020
Leiarius marmoratus	BTS™ 5%	Trealose 100 mM	GHELLER et al., 2019
Austrolebias minuano	BTS™ (380 mosm/Kg)	Metil glicol 10%	FERNANDES et al., 2019
Steindachneridion scriptum	BTS™	Metil glicol 7.5%	PEREIRA et al., 2019

2.3 Crioinjúrias

Apesar dos benefícios do processo de criopreservação em espermatozoides é inevitável os que ocorra danos à estrutura e fisiologia celular, o que altera o funcionamento dos espermatozoides devido as crioinjúrias durante o congelamento e descongelamento (FIGUEROA te al., 2015). O espermatozoide em sua totalidade é passível de sofrer os danos criogênicos, sendo que as principais estruturas afetadas são a membrana plasmática, peça intermediaria e flagelo (CABRITA et al., 2014).

O principal dano que ocorre na membrana plasmática está associado a formação de espécies reativas ao oxigênio (ERO's), que induz a peroxidação lipídica, gerando degradação das proteínas membranares, extravasamento de elementos intracelulares, e perda da fluidez e integridade da membrana (XIN et al., 2020). Em relação à peça intermediária o principal dano ocorre ao nível mitocondrial, onde a ruptura da membrana da mitocôndria leva à liberação de componentes mitocondriais, resultando na inativação de enzimas que estão relacionadas a produção de energia para a motilidade espermática (SANDOVAL-VARGAS et al., 2021). Além disso, danos à membrana mitocondrial prejudica a eficiência respiratória por promover a liberação de ERO's que aumenta o estresse oxidativo sofrido pelos espermatozoides (CABRITA et al., 2014). Por sua vez, os danos que ocorrem no flagelo estão relacionados a mudanças no perfil das proteínas flagelares e alterações causados por danos mecânicos, gerando distúrbios de motilidade que afetam a capacidade de fertilização do espermatozoide (XIN et al., 2020).

O DNA e RNA dos espermatozoides também podem ser danificados durante o processo de criopreservação, estudos reportam que a expressão de genes e proteínas, a estabilidade do mRNA e o conteúdo epigenético dos espermatozoides são modulados durante o processo de congelamento e descongelamento (HEZAVEHEI et al., 2018). As ERO's também estão associadas a danos no DNA como fragmentação da cromatina, oxidação das bases nitrogenadas, redução do tamanho do telômero, substituição das bases nitrogenadas e modificações no perfil de metilação, enquanto no RNA os danos estão associados em sua desestabilização e oxidação (CABRITA et al., 2014). Entretanto, para *Oncorhynchus mykiss* o processo de criopreservação aumentou a fragmentação do DNA, mas não foi observada a presença de bases oxidadas, sugerindo que outros mecanismos além do estresse oxidativo poderiam estar envolvidos na fragmentação do DNA promovida pelo congelamento (PÉREZ-CEREZALES et al., 2009). Independente se a fragmentação do

DNA e RNA ocorrer do processo de criopreservação ou da ação das ERO's sobre a célula espermática, deve-se atentar para essa crioinjuria, pois essas danos estão associados com a redução as taxas de fertilização e o desenvolvimento embrionário das larvas (XIN et al., 2020).

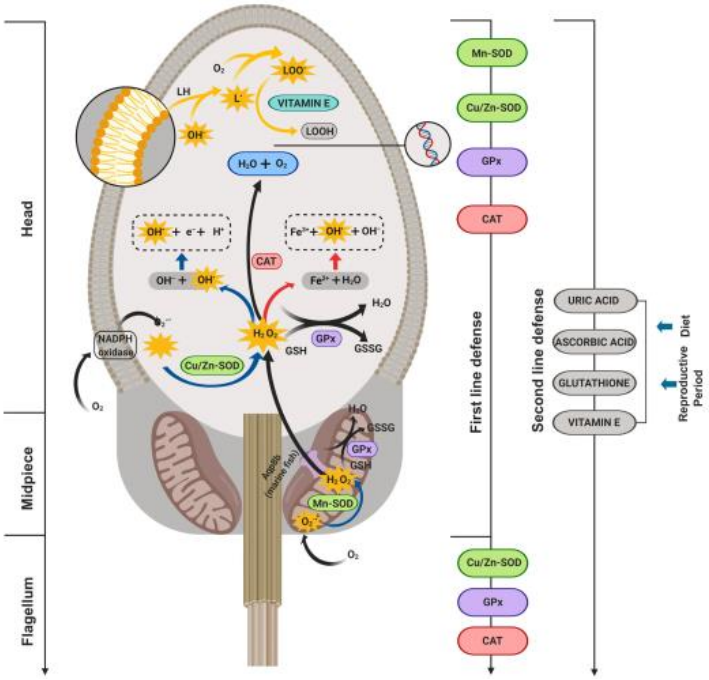
2.4 Estresse Oxidativo

As ERO's são encontradas em todos os sistemas biológicos, em condições fisiológicas do metabolismo celular aeróbio, onde o oxigênio (O_2) sofre redução tetravalente, com aceitação de quatro elétrons, resultando na formação de água (H_2O), e nos espermatozoides, as ERO's comumente geradas são o ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (OH^-) (FIGURA 1) (FIGUEROA et al., 2018; SANDOVAL-VARGAS et al., 2021). Quando produzidas em pequenas quantidades, as ERO's são funcionalmente importantes na condução das cascatas de fosforilação da tirosina associadas à capacitação espermática (GUTHRIE; WELCH, 2012).

Os espermatozoides são particularmente vulneráveis ao estresse oxidativo devido ao reduzido citoplasma, da composição de sua membrana plasmática rica em ácidos graxos polinsaturados e particularmente devido ao ambiente não fisiológico que são expostos durante o processo de criopreservação/reprodução artificial (FÉLIX; OLIVEIRA; CABRITA, 2021). Para neutralizar as ERO's o sêmen apresenta uma linha de defesa composta inicialmente pelas enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx). A SOD é responsável por catalisar a dismutação de O_2^- em H_2O_2 e O_2, seguida pela CAT que catalisa a redução H_2O_2 para H_2O e O_2 enquanto, a GPx decompõe H_2O_2 em água na mitocondrial, podendo competir com a catalase no citosol (SANDOVAL-VARGAS et al., 2021). Como uma segunda linha de defesa contra as ERO's, o sêmen possui antioxidantes não-enzimáticos que atuam como removedores de radicais, inibindo as reações oxidativas (LAHNSTEINER; MANSOUR et al., 2010).

Apesar do sêmen possuir seu próprio sistema antioxidante, durante o processo de criopreservação é realizada uma diluição prévia do sêmen, reduzindo a concentração dos antioxidantes presentes naturalmente e ocasionando desequilíbrio entre oxidantes e antioxidantes, e consequentemente o estresse oxidativo da célula.

Figura 1 - Mecanismo de produção de ROS, peroxidação lipídica e defesa antioxidante em espermatozóides de peixes.



Fonte: Sandoval-Vargas et al. (2021).

2.5 Antioxidantes no processo de criopreservação

Por definição os antioxidantes são substâncias capazes de prevenir ou diminuir a oxidação de outras moléculas e as formas de atuação dos antioxidantes se dão através do sequestro de radicais livres do meio, ligação com íons metálicos, inibição de enzimas produtores de radicais livres e ativação de enzimas antioxidantes (CAROCHO; MORALES; FERREIRA, 2018). Os antioxidantes utilizados no processo de criopreservação são divididos em duas classes: segundo o seu mecanismo de ação, antioxidantes enzimáticos (constituídos de enzimas) e os não-enzimáticos (constituídos de vitaminas, minerais, aminoácidos, ácidos graxos, carotenoides, carnitinas, compostos polifenólicos e antioxidantes de baixo peso molecular) (FÉLIX; OLIVEIRA; CABRITA, 2021).

Dentre os antioxidantes enzimáticos, as enzimas primárias SOD, CAT e GPx, atuam diretamente sobre os radicais livres, enquanto as demais enzimas atuam indiretamente e apresentam efeito sinérgico regenerando as enzimas primárias (FÉLIX; OLIVEIRA; CABRITA, 2021). Na literatura foi reportada a adição de antioxidantes enzimáticos ao meio de criopreservação em salmonidae (LAHNSTEINER; MANSOUR; KUNZ, 2011; KUTLUYER et al., 2014), acipenseridae (LI et al., 2018) e ciprinidae (MUTHMAINNAH

et al., 2018). Em relação à utilização de antioxidantes não-enzimáticos, em protocolos de criopreservação de sêmen de peixes observou-se o uso das vitaminas (C e E), minerais (selênio e zinco), aminoácidos (cisteína, metionina, taurina e triptofano) e como antioxidante de baixo peso molecular destaca-se a melatonina (FÉLIX; OLIVEIRA; CABRITA, 2021).

Pesquisas envolvendo a adição de antioxidantes ao meio de criopreservação do sêmen torna-se uma alternativa para reduzir o estresse oxidativo da célula espermática durante o processo de criopreservação, permitindo uma maior qualidade espermática após o descongelamento e consequentemente, maiores taxas de fertilização e eclosão. Entretanto, devido ao grande número de espécies de peixes, torna-se necessário mais pesquisas que envolvam diferentes categorias de antioxidantes, bem como a determinação da melhor concentração.

2.5.1 Melatonina

A melatonina, quimicamente identificada como N-acetil-5-metoxitriptamina, é originária do aminoácido triptofano, onde inicialmente o aminoácido é convertido em 5-hidroxitriptofano através da ação da enzima hidroxilase 5-triptofano, seguida pela ação da enzima 5-hidroxitriptofano descarboxilase originando a serotonina, a qual é convertida em N-acetilserotonina através da reação de acetilação e da enzima arilalquilamina N-acetiltransferase, no processo final de síntese, a N-acetilserotonina sofre ação da enzima 5-hidroxiindole-O-metiltransferase produzindo a melatonina (CARDINALI; PEVET,1998; CARPENTIERI et al., 2012). A síntese de melatonina descrita acima, ocorre na glândula pineal, entretanto, as enzimas envolvidas na síntese já foram reportadas nas demais estruturas produtoras de melatonina (PEVET; KLOSEN; FELDER-SCHMITTBUHL, 2017).

Em 1958, Lerner et al. conseguiram isolar o agente responsável pela agregação de melanóforos. No período de descoberta da melatonina acreditava-se que o composto era basicamente sintetizado pela glândula pineal e estava relacionado somente ao ciclo circadiano e reprodução sazonal (CARPENTIERI et al., 2012; PEVET; KLOSEN; FELDER-SCHMITTBUHL, 2017). Atualmente a melatonina é produzida por bactérias, seres unicelulares, espécies invertebradas e vertebradas (PANDI-PERUMAL et al., 2006; REITER et al., 2013). A produção de melatonina encontra-se em diversos órgãos como trato gastrointestinal, testículos, ovário, pele, fígado, rim, pâncreas, timo, epitélio respiratório,

baço, glândulas lacrimais e tireoidianas, cristalino, glóbulos vermelhos, plaquetas e células mononucleares (CRUZ et al., 2014; PEVET; KLOSEN; FELDER-SCHMITTBUHL, 2017). Consequentemente, diversas outras ações da melatonina foram descobertas, como de antioxidante, capacidade de redução na pressão sanguínea, estimulação imunológica, inibição inflamatória e ações oncostáticas/citoprotetora (PANDI-PERUMAL et al., 2006; CARPENTIERI et al., 2012; PANG et al., 2018).

Por se tratar de uma substância com propriedade anfifílica, ou seja, que possui uma região polar (hidrofílica) e uma região apolar (hidrofóbica), a melatonina possui a capacidade de se difundir em qualquer compartimento celular, reduzindo os danos oxidativos em toda célula (REITER et al., 2013; CRUZ et al., 2014). Dessa forma, toda a sua capacidade de remoção de radicais livres, ocorre nas membranas externas e internas, localizadas nos compartimentos celulares. Quando a melatonina passa pelas membranas celulares, sua localização é principalmente em posição superficial nas bicamadas lipídicas, próxima à cabeça polar dos fosfolipídios da membrana, atuando como um grande eliminador de moléculas reativas baseadas em oxigênio e nitrogênio (CARPENTIERI et al., 2012). Além da eliminação dos radicais livres, a melatonina promove uma maior resistência a membrana celular através da estabilização da fluidez da membrana (REITER, 2000).

A melatonina é uma indolamina, que possui um anel aromático rico em elétrons, atuando como um doador de elétrons, reduzindo e reparando radicais eletrofílicos. Dessa forma, a melatonina é oxidada por um radical livre, forma-se um radical de cátion indolil, seguida da reação desse radical com o radical ânion superóxido (O_2^-), eliminando o ânion O_2^- e formando uma molécula estável e não tóxica, a 5-metoxi-N-acetil-N-formil-quinurenamina. A melatonina também atua contra a peroxidação lipídica, através da eliminação do radical peroxila (LOO) e consequentemente ocorre uma estabilização da membrana celular causada pelo efeito antioxidante da melatonina (REITER, 1998). Na mitocôndria a melatonina estabiliza a membrana interna e atua na cadeia transportadora de elétrons, protegendo as proteínas da cadeia e o DNA mitocondrial dos danos causados pelas ERO's (CARPENTIERI et al., 2012). Através da doação de elétrons aos complexos I e IV da mitocôndria a melatonina pode aumentar a produção de ATP (LEON et al., 2004).

A melatonina possui a capacidade de atuar indiretamente na redução das ERO's, através do aumento dos níveis das enzimas antioxidantes SOD, GPx, glutationa redutase e CAT (ANISIMOV et al., 2006). Em sêmen criopreservado de coelho, a adição de 0,1 mM de melatonina foi responsável pelo aumento das enzimas SOD, GPx e CAT, sugerindo que

a melatonina protege os espermatozoides contra os danos das ERO's por meio da ativação da fosforilação de AMPK (proteína quinase ativada por adenosina monofosfato) aumentando as defesas antioxidantes (ZHU et al., 2019). Da mesma forma, a adição de 0,25 mg/ml de melatonina ao meio de criopreservação de sêmen de frangos foi responsável pelo aumento da atividade enzimática de SOD, GPx e CAT (APPIAH et al., 2019). Em sêmen de humanos, além da melatonina (0,1 mM) estimular a atividade das enzimas antioxidantes, observou-se o aumento da expressão do gene Nrf2 (Fator de transcrição NF-E2 relacionado ao fator 2) que está relacionado a indução da expressão de proteínas antioxidantes (DENG et al., 2017). Para a espécie *Capoeta trutta* a adição de 1 mM de melatonina também permite o aumento da atividade da SOD durante o processo de resfriamento do sêmen (ÖZGÜR et al., 2020).

Outra ação da melatonina é que ao remover os radicais livres, alguns dos produtos que são produzidos também atuam como antioxidantes eficientes, entre eles a N1-acetil-N2-formil-5-metoxiquinuramina (AFMK) e N-acetil-5-metoxiquinuramina (AMK) que removem o H_2O_2 (LEON et al., 2004). Dessa forma, a melatonina possui efeitos antioxidantes que podem ser diretos, através da eliminação de espécies reativas ao oxigênio e nitrogênio e atuando na cadeia transportadora de elétrons, e indiretos que estão relacionados ao aumento dos níveis de outras enzimas antioxidantes. Ambos efeitos, associados, atuam para manter a estabilidade das membranas externas e dos compartimentos internos da célula.

No processo de resfriamento (4 °C/72 h) de sêmen de *Polyodon spathula* e *Onychostoma macrolepis* a adição de 0,5 e 1,0 µM respectivamente, conseguiu aumentar a integridade de membrana, os parâmetros cinéticos, concentração de ATP e manter estáveis os níveis de ROS no espermatozoide (GAO et al., 2019; YANG et al., 2020). Para *Capoeta trutta* a suplementação de 0,1 e 1 mM de melatonina reduziu a toxicidade de nanopartículas de dióxido de titânio através da redução da peroxidação lipídica e aumento das velocidades espermáticas (ÖZGÜR et al., 2020).

No processo de criopreservação de sêmen de *Brycon orbignyanus*, a suplementação de 2 mM de melatonina aumentou a motilidade total e progressiva, duração da motilidade, integridade de membrana e as taxas de fertilização (PALHARES et al., 2020). Diferentemente para *Prochilodus lineatus*, a suplementação de 1 a 3 mM não apresentou efeitos sobre parâmetros cinéticos dos espermatozoides após descongelamento, entretanto observou-se um aumento de 14-19% nas taxas de fertilização em todos os tratamentos suplementados com melatonina (ASSIS et al., 2019). Diante disso, o efeito da suplementação

de melatonina ao sêmen é espécie-específica e poucos trabalhos com sêmen de peixes são reportados na literatura, tornando-se necessário pesquisar sobre a adição de melatonina e a concentração ideal para as espécies de peixes.

2 CONSIDERAÇÕES FINAIS

Apesar de o processo de criopreservação ser bem estabelecido para algumas espécies de peixes, ainda é necessário a realização de pesquisas básicas para um grande número de espécies que ainda não tem protocolo definido. Para essas pesquisas devem ser avaliados a combinação de diluidores e crioprotetores, bem como definir a taxa de diluição (diluidor) e concentração (crioprotetor) ideal para cada espécie. Para as espécies que possuem protocolo estabelecido, a inclusão de antioxidantes pode atuar melhorando a qualidade espermática após o descongelamento, se comparado ao protocolo previamente estabelecido sem a suplementação. Torna-se necessário realizar análises mais complexas como, por exemplo, as relacionas ao estresse oxidativo, com o intuito de explicar a melhoria da qualidade espermática devido aos parâmetros oxidativos espermáticos.

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SEGUNDA PARTE

Article 1 - Melatonin supplementation on the quality of cryopreserved sperm in the neotropical fish *Prochilodus lineatus*

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Abstract

The cryopreservation process causes damage to sperm structures and supplementation of the cryoprotective medium is an alternative to reduce these damages. The aim of this study was to determine the effect of melatonin supplementation in post-thaw sperm quality in *Prochilodus lineatus*. The cryoprotective medium was supplemented with 2.00, 2.75, 3.50, and 4.25 mM melatonin, and the control group (without melatonin). Post-thaw sperm was analyzed for its motility rate, velocities, beat cross frequency, membrane integrity, sperm morphology, oxidative stress, and fertilization capacity. Samples cryopreserved with 2.00 mM melatonin yielded higher sperm motility rate than other treatments with the addition of melatonin. Curvilinear velocity and average path velocity were higher in samples containing

2.00 mM melatonin. Samples from control and with 2.00 mM melatonin presented higher membrane integrity and morphological normality. Regarding oxidative stress, lower lipid peroxidation occurred in 2.75 and 3.50 mM melatonin, compared to control. While higher enzyme activity of catalase occurred in the control, no differences were observed in the activity of superoxide dismutase. Higher fertilization and hatching rates occurred at 2.75 mM melatonin compared to 4.25 mM. In order to obtain a higher sperm quality after thawing, melatonin can be supplemented at 2.00 mM to the cryopreservation medium of *Prochilodus lineatus* sperm.

Keywords: Antioxidant; Cryopreservation; Semen; Oxidative stress biomarkers; Neotropical fish.

1. Introduction

The streaked prochilod *Prochilodus lineatus* (Valenciennes, 1836), known as curimba is a neotropical Characiforme fish that presents distribution in South America hydrographic basins [1]. Despite being a migratory species, this fish displays good adaptation in captivity, thus, it is a model species used in research, including studies on cryopreservation of neotropical fish sperm [2-7]. The main advantages provided by the cryopreservation process include reduced gonadal asynchrony, sperm economy by optimizing the artificial inseminating dose, simplification of breeding management, facility to transport genetic material, and formation of cryobanks for genetic selection programs or conservation of species [8].

Despite the advantages reported above, sperm cryopreservation involves extreme freezing and thawing temperatures that can result in lethal and sublethal alterations to the spermatozoa [9]. The principal types of cryodamage occur in the spermatozoa plasma membrane, flagellum, midpiece/mitochondria, DNA, and RNA [10,11]. The damage that occurs during the cryopreservation process induces the increase in the generation of reactive

oxygen species (ROS), resulting in lipid peroxidation in the plasma membrane and mitochondria, as well as DNA and RNA fragmentation [10]. Although the sperm has a defense system against oxidizing agents, the cryopreservation process also generates a reduction in antioxidant agents due to the dilution process in the cryoprotective medium, causing an imbalance between oxidizers and antioxidant agents [12,13].

To reduce the oxidative stress caused by ROS, the addition of antioxidants to the sperm cryoprotective medium is an approach currently used in several fish species [13,14]. The use of this approach is related to increased sperm motility, velocities and membrane integrity, and reduced DNA fragmentation, lipid peroxidation, and ROS formation [15-23].

Among antioxidants, the hormone melatonin stands out for its amphiphilic property, acting not only at the level of the cell membrane, but also at the mitochondrial level [24,25]. Thus, melatonin is a highly effective scavenger of a variety of toxic radicals, as ROS and reactive nitrogen species (RNS). Furthermore, some metabolites that result from the interaction of melatonin with free radicals also have the ability to neutralize these radicals [26]. Additionally, melatonin stimulates several antioxidative enzymes including catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase, further increasing its antioxidant potential [27].

In fish sperm, the use of melatonin has already been reported for cryopreservation in *Prochilodus lineatus* [7] and *Brycon orbignyanus* [23], and for short-term storage in *Polyodon spathula* [28] and *Capoeta trutta* [29]. Only for post-thaw sperm in *P. lineatus*, melatonin did not show improvement in the kinetic parameters of motility, however, the effects were not evaluated in relation to the parameters associated to oxidative stress. Thus, the present work is the first to evaluate the antioxidant capacity of different concentrations of melatonin during sperm cryopreservation further investigating kinetic motility parameters and oxidative stress indices in post-thawed sperm of *Prochilodus lineatus*.

2. Materials and methods

2.1. Fish handling and sperm collection

This study was approved by the Ethical Committee of Animal Use at Federal University of Lavras-UFLA (CEUA Protocol N° 023/18), in the city of Lavras, Minas Gerais State, Brazil.

Prochilodus lineatus (males *n* = 18 and females *n* = 2) with approximately 4 years of age were selected from fish cultured in earthen ponds at the Fish Culture Station of the Agricultural Research Company of Minas Gerais (EPAMIG) in the city of Leopoldina (21°28'34"S; 42°43'17"W), located in the Zona da Mata region, Minas Gerais State, Brazil, during the spawning season in 2018/2019. The fish were fed a commercial extruded feed (28% crude protein) twice a day *ad libitum.* Males (306 ± 130 g of body weight, BW) releasing a few drops of sperm under soft abdominal pressure received a single intraperitoneal dose of carp pituitary extract (CPE; Argent Chemical Laboratories, Redmond, USA) at 4 mg/kg BW 8 h prior to sperm collection. Females (654 ± 76 g BW) with bulging of the celomathic cavity and a protruding and reddish urogenital papilla received two intraperitoneal doses of CPE at 0.5 and 5 mg/kg BW 20 h and 8 h prior to collection, respectively. These protocols are used at the Fish Culture Station in the reproduction routine to induce spermiation and oocyte extrusion.

Sperm collection was carried out at room temperature (~27 °C), and immediately after collection, the tubes containing sperm were maintained in a cooler containing chemical ice (~6-9 °C) for a maximum of 30 min. During sperm collection, contamination with water, urine, or feces was carefully avoided. After collection, each sperm sample was subjectively evaluated for motility rate (expressed as the percentage of motile sperm), motility quality score (assigned using an arbitrary grading system ranging from 0 to 5 – no movement to fast swimming sperm), and duration of sperm motility (seconds) [30]. Samples were analyzed

after activation in 150 mOsm/kg glucose solution [6], under a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×200 magnification. All samples achieved at least 90% motility rate and thus were used in the subsequent cryopreservation.

2.2. Sperm cryopreservation

Six sperm pools containing sperm samples from three randomly selected animals were used for sperm cryopreservation. The cryopreservation occurred within 45 min after collection following the methodology described by Viveiros et al. [3] with melatonin supplementation. The cryoprotective medium was composed of glucose 5% (325 mOsm/kg; pH 7.6) diluted in reverse osmosis water as an extender, and methyl glycol [$CH_3O(CH_2)_2OH$] as cryoprotectant agent. Chemicals were purchased from Vetec Química Fina Ltd. (Duque de Caxias, Rio de Janeiro, Brazil). Each sperm pool was individually diluted in the cryoprotective medium at a ratio of 1: 8: 1 (50 µL sperm (10% v/v) + 400 µL extender (80% v/v) + 50 µL methyl glycol (10% v/v)). Melatonin (*N*-acetyl-5 methoxytryptamine (M5250); molecular weight 232.28, Sigma-Aldrich, Missouri, USA) was dissolved in the cryoprotective medium (extender and methyl glycol), yielding four final different concentrations of melatonin 2.00 mM, 2.75 mM, 3.50 mM, and 4.25 mM. Control was composed of sperm and cryoprotective medium without melatonin. All six sperm pools were used in the cryopreservation protocols with each melatonin concentration and in control.

Samples were loaded into 0.5 mL straws (total of 300 straws; 6 pools × 10 replicate straws × 5 treatments). Loaded straws were kept for 10 min at room temperature (~27 °C) during equilibration time [6] and placed on racks. The racks were transferred to a nitrogen vapor vessel (Dry Vapor Vessel YDH-8, Cryofarm, Itu, Brazil) and straws were frozen at –170 °C for 24 h (approximately –36 °C/min; [31]), and then transferred to a cryogenic tank (BioCane 34 Thermo Fisher Scientific, Dubuque, USA) at –196 °C for storage.

2.3. Post-thaw sperm analysis

2.3.1. Computer-Assisted Sperm Analysis (CASA)

Straws (*n* = 3 straws per sperm pool) were individually thawed in a water bath (Water-bath MA 127, Marconi, São Paulo, Brazil) at 60 °C for 8 s [3]. Post-thaw sperm features were measured using the Computer-Assisted Sperm Analysis (CASA) system, following the methodology described by França et al. [32]. Briefly, sperm motility was triggered in a 150 mOsm/kg glucose solution at a ratio of 1: 10 (1 µl post-thaw sperm: 10 µl activating solution) at approximately 27 °C directly in a Makler™ counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) placed under a phase-contrast microscope (Nikon™ Eclipse E200, Tokyo, Japan) at ×100 magnification, green filter and phase one position. The microscope was connected to a video camera (Basler Vision Technologies™ A780-54FC, Ahrensburg, Germany) generating 50 images/s; video recording started approximately 10 s post-activation. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™ 2013, Microptics, S.L. Version 5.4, Barcelona, Spain). Each straw was analyzed in duplicate. Motility rate, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), and beat-cross frequency (BCF) were considered for analysis. To determine these parameters, each spermatozoon (*n* = 1408 ± 133 sperm cells per straw) was individually followed throughout the recorded video images from which sperm trajectories were evaluated.

2.3.2 Membrane Integrity and Sperm Morphology

The assessment of membrane integrity was performed using the nigrosin-eosin staining technique. Sperm was stained with nigrosin-eosin (2 µL sperm: 4 µL nigrosin-eosin), and 200 cells were counted in distinct histological slide fields under light microscope (Eclipse E200, Nikon, Tokyo, Japan) at ×400 magnification. Two wet preparations per sperm pool were analyzed. White spermatozoa (unstained) were considered to have an intact

membrane and those with pink or red heads were considered to have damaged membranes. Membrane integrity was calculated as the percentage of unstained cells [33].

For morphological sperm analysis, the sperm was diluted at a ratio of 1: 1000 in citrate formaldehyde solution (2.9% sodium citrate, 4% commercial solution of formaldehyde 35% and distilled water; Vetec Química Fina Ltd., Duque de Caxias, Brazil). The diluted sample was stained with 4% Rose Bengal (3: 20; stain: sperm) and two wet preparations per sperm pool were analyzed [34]. Each sample was observed under a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×1000 magnification and the morphology of 200 sperm cells was evaluated. Primary (head degeneration, midpiece degeneration, tail stump, fractured tail, strongly coiled tail, macrocephaly, and microcephaly) and secondary (free normal head, simple bent tail, proximal, and distal droplet) damages were considered [35]. Data were recorded as the percentage of abnormal sperm cells.

2.3.3. Oxidative Stress Indices

Straws (n = 2 straws per sperm pool) were thawed in a polystyrene box containing crushed ice (5 ± 2 °C) and analyses were carried out on total sperm (seminal plasma was not separated). The results were normalized to the total protein content, determined by Bradford method [36].

For lipid peroxidation (LPO), malondialdehyde concentration was analyzed by QuantiChromTM TBARS Assay Kit (DTBA-100) (BioAssay Systems, Hayward, USA) according to the manufacturer's protocol. The absorbance was measured at 535 nm (Multiskan GO, Thermo Scientific, Waltham, USA). Each sample was processed in triplicate.

Evaluation of superoxide dismutase (SOD) activity in sperm was determined based on the autoxidation of pyrogallol by the methods of Madesh and Balasubramanian [37].

Samples (30 μL) were diluted in 99 μL of 50 mM phosphate buffer solution (pH 7.2), and 6 μL of MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide; Sigma-Aldrich, St. Louis, USA) and 15 μL of 1.25 mM pyrogallol (Sigma-Aldrich, St. Louis, USA) were added. The absorbance was read in triplicates using a microplate reader at 570 nm (Multiskan GO, Thermo Scientific, Waltham, USA). The total SOD activity was expressed in units per milligram of protein, where one unit of SOD activity is defined as the amount of the enzyme necessary to produce 50% dismutation of the superoxide radical per min.

The catalase (CAT) activity was measured according to Aebi [38]. The enzyme activity was determined by H_2O_2 consumption, seen as the decrease in absorbance at 240 nm during 60 s. Samples were assayed in triplicates in a microplate reader (Multiskan GO, Thermo Scientific, Waltham, USA). The specific activity of CAT was reported as units per milligram of protein (one unit is defined as 1 pmol of H_2O_2 consumed per min).

2.3.4. Fertilization Assays

For fertilization tests, 0.1 g oocytes (average 95 oocytes) were weighed into 50 mL disposable cups, and one straw per sperm pool from each treatment was thawed (60 °C for 8 s). Fresh sperm from two males were collected and used as control. To activate fertilization, 5 mL of 150 mOsm/kg glucose solution was added to the cups, circular motions were performed for 90 s, and the eggs were randomly transferred to experimental incubators [32]. The incubators were arranged in a tank with constant water renewal and oxygenation, where eggs remained in movement at approximately 27 °C.

The fertilization rate was determined 8 h after fertilization when the blastopore closure can be observed [39] by analyzing all eggs from each incubator using a trinocular stereomicroscope (Q7740SZ-T, Quimis, Diadema, Bazil) at ×10 magnification. The result was given by the formula: fertilization rate (%) = (number of fertilized eggs/ total number of eggs) × 100. The hatching rate was estimated 22 h [39] after fertilization and the result

was given by the formula: hatching rate (%) = (number of hatched larvae/ total number of eggs) × 100.

2.4. Statistical analyses

Data were expressed as mean ± standard deviation (SD). Data were tested for normal distribution using Shapiro–Wilk test and for significant differences using ANOVA, followed by Student–Newman–Keuls test, when applicable. Statistical analyses were conducted using the R software version 3.3.2 (R Development Core Team, 2016) and the level of significance for all statistical tests was set to 5% ($P < 0.05$).

3. Results

3.1. Fresh Sperm Motility

Each sperm pool was individually evaluated, and yielded mean values for motility rate and motility quality score of 90% and 4.7, respectively. Additionally, the mean duration of motility was 104.2 s.

3.2. Computer-Assisted Sperm Analysis

Samples cryopreserved with 2.00 mM melatonin showed a higher sperm motility rate (93%) than other treatments with the addition of melatonin (64-69%). Whereas the mean values of motility rate were similar among control and the treatments containing melatonin (Fig. 1). Additionally, sperm velocities VCL and VAP were higher in samples containing 2.00 mM melatonin ($P < 0.05$), and higher VSL was observed in samples containing 2.00 mM than other treatments containing melatonin (Table 1). There were no differences for BCF among treatments ($P > 0.05$).

3.3. Membrane Integrity and Sperm Morphology

The membrane integrity was higher in control and samples with 2.00 mM melatonin in relation to the dosage of 4.25 mM (Table 2). No morphological differences were observed for primary damages ($P > 0.05$) among treatments. Samples with 2.75 mM melatonin

exhibited higher percentage of secondary damages than control and samples with the dosage of 2.00 mM melatonin (Table 2). Also, the percentage of normal cells was lower in samples with 2.75 mM melatonin, compared to samples from control and with 2.00 mM melatonin.

3.4. Oxidative Stress Indices

Sperm cryopreserved with 2.75 mM and 3.50 mM melatonin yielded lower values of LPO if compared to control samples (Table 3). Regarding the enzymatic activity, no differences were observed among treatments for SOD ($P > 0.05$). Whereas an increase in the activity of CAT was observed in control samples in relation to the treatments with the addition of melatonin (Table 3).

3.5. Fertilization Assays

The samples containing 2.75 mM melatonin showed higher fertilization rate (27%) and hatching rate (17%) rates if compared to the treatment containing the highest concentration of melatonin (5% of fertilization and 3% of hatching rates) (Fig. 2). Fertilization and hatching rates did not differ among control and the treatments containing melatonin (13-23% for fertilization and 7-13% for hatching rate). The fresh sperm control yielded fertilization rate of 77% and hatching rate of 66%.

4. Discussion

It is known that supplementation of the cryoprotective medium with antioxidants is used as an alternative to reduce cryodamages caused during the sperm cryopreservation process. In the present study, melatonin supplementation in the cryoprotective medium of *Prochilodus lineatus* sperm allowed to improve the cryopreservation protocol for this species. Our findings showed that melatonin influences the sperm cryopreservation process and affects sperm quality and oxidative stress indices of post-thaw sperm.

The quality of fresh sperm is recognized to influence the quality of post-thawed sperm, thus, the evaluation of sperm before the cryopreservation process is crucial to ensure

samples of good sperm quality after thawing [8]. The motility, motility quality score, and duration of motility in the present study were within the range for fresh sperm of this species, presenting good quality [6,7,30,40].

Despite using fresh sperm showing good quality, a reduction in motility rates, speeds, membrane integrity, and increased morphological damages after the cryopreservation process are inevitable. The reduction in sperm quality after cryopreservation is related to the damage that occurs in the sperm cell that will cause an increase in oxidative stress [10]. Thus, supplementation of the cryoprotective medium with antioxidants aims to reduce the effects of cryoinjuries on sperm quality [14].

In the present study, the addition of the lowest melatonin (2.00 mM) concentration did not affect the motility rate in relation to the control, as reported in another study in *P. lineatus* cryopreserved sperm with 1 mM to 3 mM melatonin [7]. However, in the present study, this low melatonin dosage was capable to increase sperm curvilinear velocity, average path velocity, as well as straight line velocity. An increase in straight line velocity was also observed for short-term storage in *C. trutta* sperm, when supplemented with 0.1 mM and 1 mM melatonin, compared to the control [29]. The increase in these velocities may be related to the ability of melatonin to act in the mitochondria, increasing the activity of mitochondrial complexes I and IV, which consequently would increase the production of adenosine triphosphate (ATP) [28,41]. This hypothesis would also explain the increase in total motility, progressive motility, and post-thaw motility duration in *P. spathula* and *B. orbignyanus* sperm [23,28] since mitochondria are related to energy production and consequently sperm motility [42].

Membrane integrity is a parameter for determining sperm quality [33]. There were no differences in the membrane integrity of the post-thawed sperm between the control and the concentrations of 2.00 mM to 3.50 mM, the same occurred between the control and 1

mM melatonin in *B. orbignyanus* cryopreserved sperm [23]. However, in the present study, the dosage of 4.75 mM melatonin showed lower membrane integrity than the control. Concentrations of 1 µM and 5 µM melatonin also reduced membrane integrity, as well as concentrations of 0.1 µM and 0.5 µM after 72 hours of short-term storage in *P. spathula* sperm [28]. In the present study, this lack of difference among control and samples with 2.00 mM to 3.50 mM melatonin could be related to the effect of the glucose extender, which acts as an extracellular cryoprotectant and has the function of stabilizing the membrane [43]. The reduction in membrane integrity in a higher concentration of melatonin may be related to the imbalance between oxidizers and antioxidant agents. In this way, the melatonin present in the plasma membrane is in a superficial position in the lipid bilayers, acting as a great ROS eliminator [44,45].

Analysis of morphological damage in spermatozoa should also be used in the evaluation of sperm quality since morphological changes are related to fertilization ability [35]. For *P. lineatus* post-thawed sperm, the percentage of normal morphological cells ranged from 54% to 56% in the control and with 1 mM to 3 mM melatonin supplementation [7]. In contrast, in the present study, only the control and samples with 2.00 mM melatonin showed similar values of normal cells (57-59%), while cryopreserved samples with concentrations among 2.75 and 4.25 mM melatonin showed a percentage of normal cells below 50%. In *B. orbignyanus* sperm, an increase in the percentage of normal cells was observed after supplementation of 1 mM and 2 mM melatonin, compared to control [23]. The artificial reproduction of *P. lineatus* involves a high sperm:oocyte ratio in a controlled environment, and it is stated that the critical percentage of sperm abnormalities for fertilization is about 50% [35]. Thus, the concentrations ranging from 2.75 mM to 4.25 mM melatonin, could reduce the rates of fertilization, due to the higher morphological damages.

For evaluation of post-thaw sperm quality, research on oxidative stress analysis has been used to complement the analysis of sperm kinetics, membrane integrity, and sperm morphology [10]. During the cryopreservation process, an increase in ROS levels is observed, which induces oxidative damage due to the high content of polyunsaturated fatty acids present in fish sperm, resulting in lipid peroxidation [13,14]. In the present study, the potent effect of melatonin as an antioxidant was observed, since the sperm supplemented with 2.75 mM and 3.50 mM melatonin, presented a significantly lower LPO when compared to the control. The same was observed in *C. trutta* sperm, which presented lower LPO in samples supplemented with 0.01 mM, 0.1 mM, and 1 mM melatonin [29]. Also, lower levels of ROS were observed in *P. spathula* sperm supplemented with 0.1 µM to 5 µM melatonin [28]. The reduction in the level of ROS is possibly associated with the fact that products from the melatonin oxidation and reaction of melatonin with ROS, such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK), also act to eliminate free radicals [41].

Despite sperm dilution during the cryopreservation process, the sperm still presents an enzymatic system that acts to combat lipid peroxidation by removing ROS [12,13]. This main enzymatic system is composed of superoxide dismutase, catalase, and glutathione peroxidase [14]. Despite the well-established role of melatonin in stimulation the activity of antioxidant enzymes [27], in the present study, there were no differences among the treatments regarding the enzymatic activity SOD. On the other hand, CAT activity increased in the control, compared to those supplemented with melatonin. Differently, the supplementation with 1 mM melatonin in *C. trutta* sperm was reported to increase the activity of SOD in relation to the control [29]. In this study, the increase in CAT activity in control samples could be related to a higher LPO in this treatment, since catalase has an important function in degrading hydrogen peroxide into oxygen and water, preventing

hydroxyl radical damage [14]. In the present study, lower CAT activity in treatments with melatonin may be related to the antioxidant action of melatonin. This shows that melatonin was efficient in removing ROS making it not necessary the increase in the activity of catalase.

One of the indirect ways of assessing sperm quality is through its fertilization capacity however, the quality of oocytes also influences this analysis, affecting the results of fertilization [4]. In the present study, despite the good sperm motility rate and sperm velocities obtained after thawing, low fertilization rates were observed. Motility rate and sperm velocities are positively correlated to fertilization rate [4]. Possibly, the use of a higher number of oocytes and sperm samples during fertilization, could minimize the effect of the female and generate higher fertilization and hatching rates.

Despite the extensive benefits of adding melatonin as an antioxidant to the cryoprotective medium, it is observed that high concentrations can cause an excessive neutralization of ROS that are necessary for sperm function [46]. Several studies report that high concentrations of melatonin were related to reduced total and progressive motility, membrane integrity, mitochondrial membrane potential, and total antioxidant capacity, in bull, equine, rabbit, chicken, and human sperm [47-51]. These negative results occur due to the fact that physiological concentrations of ROS act by increasing the membrane fluidity, preserving the fertilization capacity of sperm and also have some beneficial effects on sperm hyperactivation, capacitation, and acrosome reaction [47].

In the present study, the use of high concentrations of melatonin was detrimental to *P. lineatus* cryopreserved sperm. In order to obtain a higher sperm quality after thawing, melatonin can be supplemented at 2.00 mM to the cryopreservation medium of *Prochilodus lineatus* sperm, since it provides good sperm motility and velocities due to melatonin antioxidant activity.

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Declaration of interest

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Table 1

Post-thaw curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), and beat-cross frequency (BCF) of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with different concentrations of melatonin (2.00-4.25 mM).

Treatment	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	BCF (Hz)
Control	69 ± 20^{b}	43 ± 11^{b}	25 ± 7^{ab}	9 ± 2
2.00 mM	84 ± 12^{a}	54 ± 10^{a}	31 ± 7^{a}	10 ± 1
2.75 mM	54 ± 9^{c}	35 ± 10^{bc}	22 ± 8^{b}	9 ± 3
3.50 mM	55 ± 21^{c}	36 ± 8^{bc}	22 ± 4^{b}	9 ± 2
4.25 mM	48 ± 6^{c}	33 ± 2^{c}	21 ± 2^{b}	9 ± 2

Data were expressed as mean ± standard deviation (SD); n = 6 sperm pools (3 males/pool) ^{a,b}Means followed by different superscript in the same column differ ($P < 0.05$, Student–Newman–Keuls test).

Table 2

Post-thaw membrane integrity and sperm morphology of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with different concentrations of melatonin (2.00-4.25 mM).

Treatment	Membrane Integrity (%)	Primary Damages (%)	Secondary Damages (%)	Normal Cells (%)
Control	78 ± 9^{a}	22 ± 3	21 ± 9^{a}	57 ± 12^{a}
2.00 mM	78 ± 3^{a}	22 ± 4	19 ± 3^{a}	59 ± 6^{a}
2.75 mM	75 ± 9^{ab}	29 ± 9	33 ± 13^{b}	38 ± 18^{b}
3.50 mM	75 ± 9^{ab}	25 ± 8	29 ± 5^{ab}	46 ± 12^{ab}
4.25 mM	65 ± 1^{b}	29 ± 14	24 ± 6^{ab}	47 ± 16^{ab}

Note Primary damages: head degeneration, midpiece degeneration, tail stump, fractured tail, strongly coiled tail, macrocephaly, and microcephaly; Secondary damages: free normal head, simple bent tail, proximal, and distal droplet. Data were expressed as mean ± standard deviation (SD); n = 6 sperm pools (3 males/pool). ^{a,b}Means followed by different superscript in the same column differ ($P < 0.05$, Student–Newman–Keuls test).

Table 3

Post-thaw oxidative stress indices of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with different concentrations of melatonin (2.00-4.25 mM).

Treatment	LPO (µM MDA equivalents)	SOD (U-SOD/mg of protein)	CAT (U-CAT/mg of protein)
Control	2.12 ± 1.25[b]	1.46 ± 0.44	3.24 ± 2.18[a]
2.00 mM	1.38 ± 0.54[ab]	1.38 ± 0.48	0.55 ± 0.23[b]
2.75 mM	0.81 ± 0.30[a]	1.33 ± 0.35	0.61 ± 0.55[b]
3.50 mM	0.72 ± 0.15[a]	1.31 ± 0.37	0.51 ± 0.49[b]
4.25 mM	1.39 ± 0.4[ab]	1.43 ± 0.43	0.70 ± 0.59[b]

Note: LPO: lipid peroxidation; MDA: malondialdehyde; SOD: superoxide dismutase enzyme; CAT: catalase enzyme. Data were expressed as mean ± standard deviation (SD); n = 6 sperm pools (3 males/pool). [a,b]Means followed by different superscript in the same column differ ($P < 0.05$, Student–Newman–Keuls test).

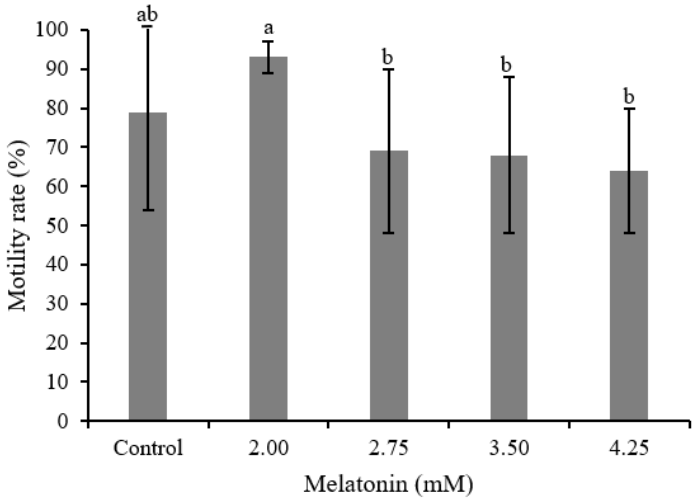

Fig. 1. Post-thaw motility rate of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with different concentrations of melatonin (2.00-4.25 mM). Bars indicate mean ± standard deviation; n = 6 sperm pools (3 males/pool). [a,b]Means followed by different superscript differ ($P < 0.05$, Student–Newman–Keuls test).

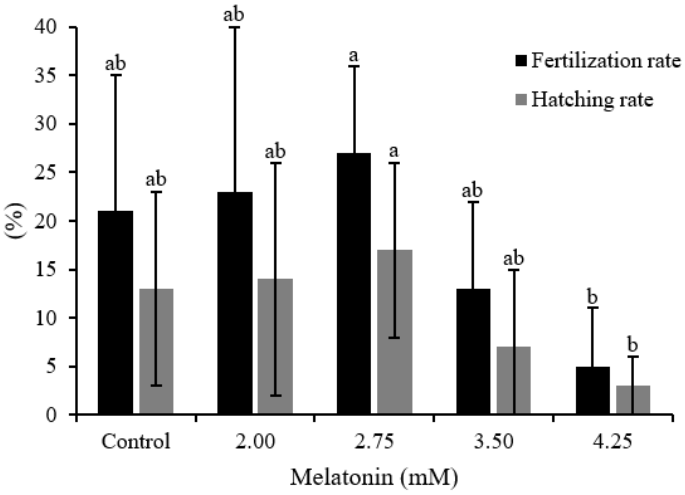

Fig. 2. Post-thaw fertilization and hatching rate of *Prochilodus lineatus* sperm cryopreserved in solutions without (control) and with different concentrations of melatonin (2.00-4.25 mM). Bars indicate mean ± standard deviation; n = 6 sperm pools (3 males/pool). [a,b]Means followed by different superscript differ ($P < 0.05$, Student–Newman–Keuls test).

Article 2 - Sperm characterization and cryopreservation of the endangered freshwater fish *Chirostoma estor* (Atheriniformes)

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Abstract

The knowledge of the physiology of sperm of an endangered species allows the implantation of reproductive biotechnologies that aim at conservation. The aim of this study was to characterize fresh sperm and evaluate different cryopreservation solutions for sperm in *Chirostoma estor*. The characterization of *Chirostoma estor* fresh sperm (n = 22 males) was performed through analyzes of sperm concentration, membrane integrity, sperm morphology, motility rate, motility quality score, and motility duration. For cryopreservation (n = 42 males), 3 extenders (BTS™, MIII™, or Androstar Plus™) in combination with 2 permeable cryoprotectants (dimethyl sulfoxide (DMSO) or methyl glycol (Methyl)) were used. Analyzes of post-thaw sperm were performed as described for fresh sperm and

additionally the fertilization rate analysis was performed. Fresh sperm presented a sperm concentration of 29.2 $x10^9$ spermatozoa/mL, membrane integrity of 82.4%, and morphologically normal cells of 53%. After glucose activation (150mM) a motility rate of 87.5%, sperm quality score of 5.0, and a duration of motility of 285 s were observed. For post-thaw sperm, MIII+Methyl and Androstar+Methyl solutions resulted in the highest motility rates of 40 -48%. No differences were observed for motility duration, membrane integrity, and sperm morphology. Samples cryopreserved in Methyl (12-20%) showed a higher fertilization rate than DMSO, independently of the extender. In conclusion, the fresh sperm collected artificially from *Chirostoma estor* presents a compatible quality to carry out fertilization and can be cryopreserved in the commercial extenders MIII™ and Androstar Plus™ together with the cryoprotectant Methyl glycol.

Keywords: Spermatozoa, Sperm quality, Activating agents, Commercial extenders, Cryoprotectants

Introduction

The Mexican Pike silverside (*Chirostoma estor* Jordan, 1880), is an endangered freshwater fish endemic to Lake Pátzcuaro, in the state of Michoacán, México, of great economic and cultural relevance [25,26,27,41]. This species presents a population decline due to overfishing and reduction in the quality of natural habitat, due to water pollution, predation, and/or competition from invasive fish alien species and is currently considered an endangered species [41].

Assisted reproductive techniques may offer new opportunities for the propagation of endangered species. Amongst the different biotechnologies available, the creation of gamete (mostly sperm) cryobanks, is the most widespread technique that has been applied successfully to several aquatic species [1]. However, the establishment of sperm cryobank is not used only for preservation of species. This technique has also been used to reduce

gonadal asynchrony, sperm economy by optimizing the artificial inseminating dose, simplification of breeding management, facility to transport genetic material, and formation of cryobanks for genetic selection programs [8].

Due to species-specific differences in sperm biochemical composition, cryopreservation protocols should be determined according to the species [4]. Among the different stages of the cryopreservation development process, the determination of the cryoprotective solution is extremely important in order to maintain sperm viability and fertilizing capacity after thawing [44]. In general, a cryopreservation solution must contain an extender, which provides an adequate osmotic and nutritional environment, and a permeable cryoprotectant responsible for protecting the sperm cell from cryodamage [39].

Sperm extenders usually are composed of salt, sugar, or by combining salts and sugars, as in commercial swine extenders that can be used to dilute fish sperm [31]. For fish sperm, the main intracellular cryoprotectants used are methanol, dimethyl sulfoxide, ethylene glycol, methyl glycol, and amide base, and for external cryoprotectants, sugars, polymers, and proteins are used, such as powdered milk and egg yolk [4,21].

Currently, there are a large number of studies describing cryopreservation protocols for several species of fish [4,8,21,39] and recently for *Chirostoma jordani*, which belongs to the same genus as the species of the present study [6]. The aim of the present study was to characterize fresh sperm and evaluate different cryopreservation solutions for sperm in *Chirostoma estor*.

Materials and Methods

Fish handling and sperm collection

Handling of animals was carried following the guidelines for animal experimentation described by Van Zutphen et al. [42]. All experimental procedures were performed according to the guidelines for the care and use of experimental animals and were approved

by local authorities, Aquaculture Biotechnology Laboratory of the Michoacana University San Nicolas de Hidalgo.

Three-year-old *Chirostoma estor* males were selected from a pool of fish cultured in captivity in 3-meter circular tanks at the Aquaculture Biotechnology Laboratory at the Institute of Agricultural and Forestry Research (IIAF-UMSNH) in the city of Morelia (19°41'22.5"N; 101°14'56.0"W), Michoacán, México, during the months of march to july of 2020. The fish were fed formulated dry feed (41% crude protein) eight times a day.

For sperm collection, the urogenital papilla was cleaned and dried carefully avoiding contamination with water, urine, feces, or blood. Sperm from each male was collected using a micropipette and transferred to graduated volume test tubes. The volume was recorded with the aid of a micropipette and the sample was stored in a cooler containing chemical ice (~6–9°C) for a maximum of 15 min.

Sperm characterization

A total of fresh sperm from 22 males (51.8 ± 8.7 g of body weight) was evaluated. Immediately after collection from each male, sperm samples were subjectively evaluated for motility rate (expressed as the percentage of motile sperm) and duration of sperm motility, in seconds [18]. The evaluation of the quality of spermatic movement was defined through the motility quality score, using an arbitrary grading system ranging from 0 to 5 [18]. The attributions of each score are: rectilinear and very fast progressive movement (score 5); rapid rectilinear progressive movement (score 4); intermediate movement (score 3); slow movement (score 2); oscillatory movement (score 1); and absence of movement (score 0) [10,18]. Samples were analyzed after activation with tank water and glucose solution at 150 mM, under a light microscope (Axioskop 40, Carl Zeiss, Oberkochen, Germany) at ×100 magnification [17,47]. Sperm were evaluated at a dilution rate of 1µL of sperm : 250µL of activating agent (tank water or glucose 150 mM) at room temperature of 23°C.

The assessment of membrane integrity was performed by the nigrosin-eosin staining technique. The sperm were stained with nigrosin-eosin (1 µL sperm: 6 µL nigrosin-eosin), and 200 cells were counted in distinct histological slide fields under a light microscope (Axioskop 40, Carl Zeiss, Oberkochen, Germany) at × 400 magnification. Cells were counted on duplicate slides. White spermatozoa (unstained) were considered to have an intact membrane and those with pink or red heads were considered to have damaged membranes. Membrane integrity was calculated as the percentage of unstained cells [24].

An aliquot of sperm was diluted (1: 1000) in citrate formaldehyde solution (2.9% sodium citrate, 4% commercial solution of formaldehyde 35%, and distilled water) for posterior evaluation of sperm concentration and sperm morphology. Briefly, sperm concentration was determined through the observation of a 10 µL aliquot of the diluted sperm in a Neubauer-type hemacytometer chamber (Boeco, Hamburg, Germany) under a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×400 magnification, following the methodology described by Sanches et al. [40].

For morphologic analysis of sperm, the diluted samples were stained with Rose Bengal (3: 20; stain: sperm) and, cells were analyzed on duplicate slides [28]. Each sample was observed under a light microscope (Olympus® CX22LED, Tokyo, Japan) at ×1000 magnification and the morphology of two hundred sperm cells was evaluated. Primary damage in sperm (head degeneration, midpiece degeneration, tail stump, fractured tail, strongly coiled tail, macrocephaly, and microcephaly) and secondary damage in sperm (free normal head, simple bent tail, proximal, and distal droplet) were considered (Fig. S1) [29]. Data were recorded as the percentage of abnormal sperm cells.

Sperm cryopreservation

For the cryopreservation process, six sperm pools were performed. For the formation of each pool, sperm from seven animals were used, totaling 42 males (52.2 ± 7.3 g of body weight). Six freezing media comprising combinations of three extenders and two permeable cryoprotectant agents (CPAs) were tested. The choice of extenders and cryoprotectants was performed according to the methodologies described by Orfão et al [34] and Viveiros et al [46]. The commercial extenders tested were: BTS™ 5.5% (353 mOsm/Kg), MIII™ 5.5% (321 mOsm/Kg) or Androstar Plus™ 5.5% (354 mOsm/Kg) (Minitube™, Tiefenbach/Landshut, Germany) (Table 1), osmolality was tested using a vapor pressure osmometer (Wescor Vapro 5520, Logan, USA). As cryoprotectants, dimethyl sulfoxide (DMSO) $(CH_3)_2SO$ (Sigma-Aldrich, Missouri, USA) or methyl glycol (Methyl) $[CH_3O(CH_2)_2OH]$ (Vetec Química Fina Ltda., Duque de Caxias, Brazil) were used at 10% concentration.

Each sperm pool was diluted in the freezing media at a final ratio of 1:50 [10 μL sperm (2% V/V) + 440 μL extender (88% V/V) + 50 μL cryoprotectant (10% V/V)] [22]. Samples were loaded into 0.25 mL straws (total of 108 straws; 6 pools × 3 replicate straws × 6 treatments) and placed on racks. The racks were frozen 6 cm from the surface of the liquid nitrogen for 12 min, as described for the Atheriniformes order [22] and then transferred to a cryogenic tank (18HC, Taylor-Wharton, Baytown, USA) at –196°C for storage.

Post-thaw sperm analysis

Straws (n = 2 straws per pool) were individually thawed in a water bath (FE-370, Felisa, Guadalajara, Mexico) at 35°C for 10 s, as described for the Atheriniformes order [22]. Motility rate, motility quality score and duration of sperm motility after activation in glucose solution (150 mM), and membrane integrity, and sperm morphologic analysis were performed as described previously.

Fertilization Assays

For the fertilization assays, oocytes of *C. estor* (n = 3 females) were collected by manual abdominal pressure. After collection, the oocytes were divided into aliquots (average 100 oocytes) and deposited into 50 ml disposable cups. One straw from each treatment (n= 42 straws; 6 pools × 6 treatments) was thawed (35°C for 10 s) and added to the cups (one straw per cup). Fresh sperm from two males were collected and used as control (10 µL of sperm). The fertilization process described was carried out at 20°C. To activate fertilization, 5 mL of a glucose solution at 150 mM [17,47] was added to the cups, circular motions were performed for 120 s. The eggs were randomly transferred to experimental incubators (PVC pipe, 50 mm in diameter, 15 cm in height, with a 0.5 mm mesh bottom). The incubators were arranged in a tank with constant water recirculation at approximately 19-21°C.

The fertilization rate was determined 16 h after fertilization when the blastopore closure can be observed [30] by analyzing all eggs from each incubator using a binocular stereomicroscope (L-Z2000, Leica Microsystems, Wetzlar, Germany) at ×10 magnification. The result was given by the formula: fertilization rate (%) = (number of fertilized eggs/ total number of eggs) × 100 [32].

Statistical analysis

A completely randomized design with six replicates (pools from seven fish) and six treatments arranged in a 3 × 2 factorial structure (extenders: BTS, MIII and Androstar Plus × cryoprotectants: dimethyl sulfoxide DMSO- and methyl glycol METHYL) was applied. Data were expressed as mean ± standard deviation (SD). Data were tested for normal distribution using Shapiro–Wilk test and for significant differences using ANOVA, followed by Student–Newman–Keuls test, when applicable. For motility quality score, a categorical parameter, data were expressed as median ± interquartile range (IQR) and statistical analysis was performed through the Friedman test. Statistical analyses were conducted using the R

software version 3.3.2 (R Development Core Team, 2016) and the level of significance for all statistical tests was set to 5% ($P < 0.05$).

Results

Fresh sperm analysis

The observed values for body weight and fresh sperm characteristics evaluated in this study are presented in Table 2. Regarding sperm morphology, primary damages are mainly represented by fractured tail (4.0 %), strongly coiled tail (2.4 %), tail stump (1.7 %), and head degeneration (1.6 %). While secondary damages are mainly represented by the simple bent tail (32.2 %) and free normal head (3.1 %).

No significant differences were observed in the sperm motility rate after activation in tank water (80%) and glucose solution (87.5%). However, a higher motility quality score (5.0) and duration of sperm motility (285 s) were observed in the samples activated in glucose solution if compared to the samples activated in tank water (3.75 of motility quality score; 31 s).

Post-thaw sperm motility

No significant interactions among the extenders and cryoprotectants were observed for post-thaw sperm motility. However, a higher significant motility rate was observed for the samples cryopreserved in MIII+Methyl (40%) and Androstar+Methyl (48%), compared with other treatments (20-30%) (Fig. 1). Samples cryopreserved in MIII+Methyl and Androstar+Methyl showed higher values for motility quality score than samples containing BTS+DMSO and Androstar+DMSO (Table 3). There were no significant differences among treatments for motility duration and membrane integrity ($P > 0.05$). The same was observed for post-thaw sperm morphology, for primary damages (23-32%), secondary damages (38-48%), and cell normality (28-33%), where no statistical differences were observed among treatments ($P > 0.05$). Assessing the morphological damages individually, the main primary

damages observed were tail stump (7.9%), head degeneration (7.5%), and fractured tail (7.2%). While the main secondary damages observed were simple bent tail (31.4%) and free normal head (8.6%).

Fertilization Assays

For the fertilization rate, a significant interaction was observed between the extenders and cryoprotectants ($P < 0.05$). All samples with the cryoprotectant Methyl presented fertilizing capacity, independently of the extenders used. While for DMSO, fertilizing capacity was observed only in samples cryopreserved with the extender Androstar (Table 4). Higher fertilization rate was observed when the Androstar extender was used together with the cryoprotectant Methyl, compared with DMSO. The fresh sperm control yielded a fertilization rate of 36%, with a minimum of 3% and a maximum of 72%.

Discussion

In males, the quantity and quality of sperm may determine fertilization capacity and reproductive success in both natural and artificial fertilization [20]. Males of *Chirostoma estor* have low sperm volume but high sperm concentration. The observed sperm volume is in accordance with that reported for the species [3]. However, the sperm concentration was higher than that reported in the same species and for *C. jordani* and *C. humboldtianum* [3,5,6]. The difference in sperm concentration may be related to the male body weight, since for *C. estor*, a positive correlation between sperm concentration and body weight has been reported [3].

The reproductive season for *C. estor* occurs during most of the year, but there is greater reproductive activity between January and June [3,30]. The constant production of sperm suggests that, for the species under study, the spermiation process occurs continuously, when kept in ideal conditions for reproduction. And the relationship between sperm volume and sperm concentration, there is great variation among species. In Percidae

species, low sperm volume and high sperm concentrations are observed, while for Acipenseridae species high sperm volumes and low sperm concentrations are observed [20]. Thus, the parameters of sperm volume and concentration are possibly related to the physiology and reproductive strategy of the species. However, factors such as hormonal induction, the number of sperm collections from the same individual during the reproductive period, and time of collection during the reproductive period are also related to sperm volume and concentration parameters [20].

Sperm of *C. estor* presented high membrane integrity, however, the average observed in the present study is lower than observed in the literature for the genus [3,5]. In regard to sperm morphology, there is no information available in consulted literature for the species under study. Sperm malformations damages are associated with functional deficiencies and cause reduced motility and fertilization capacity, becoming an important analysis in this and future studies regarding sperm quality [38]. The specific assessment of primary and secondary damages may provide accurate and reliable information on the sperm fertilization capacity, however, there is no indication in consulted literature about

level of damages of these variables in fish [37].

Among the main factors to be analyzed for assessing sperm quality, motility rate is one of the most important as sperm of most external fertilization fish species is immotile in the testis and seminal plasma and only activated when in contact with the aquatic environment or other activating agents [11]. Thus, the osmolality, ionic composition, pH, and temperature of activating agents influence the activation of sperm motility [12,13]. In the present study, a high motility rate was observed in tank water and glucose solution activating agents tested for fresh sperm, however, the glucose activating solution provides higher motility quality score and motility duration compared to the tank water. Similar results were observed in fresh sperm of *Brycon orbignyanus* and *Prochilodus vimboides*

[17]. These results may be related to the fact that the tank water has a lower osmolality, generating an extreme osmotic condition, which causes damage to the spermatozoa induced by a large water influx [2,7]. In view of the results presented above, the estimation of fresh sperm quality is a basic tool for the scientist working with endangered species in which high quality sperm is necessary to perform successful cryopreservation for cryobank [20].

This is the first study to demonstrated the effects of the combination of three commercial extenders and two permeable cryoprotectants on sperm quality after thawing *C. estor* sperm. The extenders BTS, MIII, and Androstar Plus have similar formulas and the same chemical contents in different concentrations (Table 1), except for the absence of KCl in MIII and the presence of cysteine in Androstar Plus. The extenders tested in the present study were developed to cool storage swine sperm, but BTS has been used successfully in cryopreservation protocols in characiforms *Brycon orbignyanus* [17,23], *Brycon nattereri* [45], *Brycon insignis* [47], *Prochilodus lineatus* [15], *Colossoma macropomum* [43], cyprinodontiformes *Austrolebias minuano* [16] and siluriformes *Steindachneridion scriptum* [35]. The success of using commercial extenders may be related to its major chemical component being glucose, which acts not only as an extender but also as an external cryoprotectant [19]. The use of commercial extenders also increases the standardization of the cryopreservation process on farms, since the acquisition of the complete extender is easier to access and inexpensive than several chemical reagents, for the extender manufacturer.

In relation to the cryoprotectants tested, it is observed that regardless of the extender tested, the cryoprotectant Methyl provided a higher motility (38%) rate and a motility quality score (3.4) when compared to DMSO (24% of motility rate; 2.5 of motility quality score). Despite the fact that DMSO cryoprotectant is widely used in the cryopreservation of fish sperm [4], in the present study there was a higher sperm toxicity of DMSO compared to

Methyl. Methyl glycol, known as 2-methoxyethanol or ethylene glycol monomethyl ether, is derived from methanol (CH_3OH) and ethene oxide (CH_2OCH_2) [23]. In *S. scriptum* post-thawed sperm, concentrations of 5-15% Methyl (42.9-47.3%) promote a higher motility rate than concentrations of 5-15% DMSO (22.2-31.1%) [35]. Methyl is also capable of promoting higher post-thaw velocities of sperm compared to DMSO in *Prochilodus brevis* [36]. Similarly, higher toxicity of DMSO in relation to Methyl has been reported in species of characiforms [46]. In contrast to the studies reported above, *C. jordani* sperm can be cryopreserved with DMSO at 10%, with motility rates of 48.8% [6], these values are higher than those found in the present study in *C. estor*, which presented a maximum motility rate of 30% when cryopreserved in DMSO.

Extenders and cryoprotectants evaluated in *B. orbignyanus* frozen sperm, (BTS+DMSO and MIII+DMSO) resulted in nonexistent null motility rates after thawing and were only observed in the cryosolutions of BTS+Methyl (70%) and MIII+Methyl (10%) [23]. In the present study, although the cryopreserved solutions in DMSO allowed motility rates, independently of the extender, fertilization did not occur. Evaluating the cryoprotectant DMSO, only the samples cryopreserved with Androstar showed fertilization rates (14-20%). The damage that occurs during the cryopreservation process induces the increase in the generation of reactive oxygen species (ROS), resulting in lipid peroxidation in the plasma membrane and mitochondria, as well as DNA and RNA fragmentation [9]. Antioxidants such as glutathione which act in reducing cellular levels of ROS and regenerating other antioxidant molecules [14] may attenuate the deleterious effects of the cryopreservation process. It is thus possible that cysteine, a precursor to glutathione and part of the Androstar composition may have indirectly reduced the toxicity of DMSO and attenuated the deleterious effects of the cryopreservation process in *C. estor*. This has been previously reported in *Cyprinus carpio* in which the addition of cysteine to extenders

significantly increased sperm motility and duration, and fertilization in addition to decreased DNA damage [33] whilst other antioxidants have also been tested with species-specific results [14].

In the present study, fresh sperm of *C. estor* had an adequate quality to be cryopreserved. For sperm activation, the use of the activating agent glucose at 150 mM is recommended, as it allows for highest quality of motility and motility duration. About the cryopreservation process, the best results obtained in this study indicate that the extenders developed for swine sperm cool storage (MIII™ and Androstar Plus™) can be used for the cryopreservation of sperm together with the methyl glycol (Methyl) cryoprotectant. However, the use of the dimethyl sulfoxide (DMSO) cryoprotectant at the concentration of 10 % is not recommended for this species. As this is the first cryopreservation work performed on sperm from *Chirostoma estor*, it is suggested that further studies be developed to determine the ideal cryopreservation protocol. Surveys should include the process of dilution rate, freezing, thawing, and sperm viability over the years during cryopreservation.

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Declaration of interest

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Table 1

Chemical composition of extenders BTS™, MIII™, and Androstar Plus™.

	BTS™	**MIII™**	**Androstar Plus™**
Glucose monohydrate	76.06	89.20	79.57
Sodium citrate	15.89	3.05	12.38
EDTA	2.65	3.05	3.05
Sodium bicarbonate	3.31	4.20	2.66
Gentamicin sulphate	0.50	0.50	0.50
Potassium chloride	1.60	-	1.60
Cysteine	-	-	0.21

Note: g reagent / 100g extender. Minitube™, Tiefenbach/Landshut, Germany

Table 2

Body weight and fresh sperm characteristics of *Chirostoma estor* (n= 22).

Parameters	**Mean ± SD**	**Range**
Body weight (g)	51.8 ± 8.7	38.0-69.0
Sperm volume (µL)	23.4 ± 8.3	10.0-40.0
Concentration (spermatozoa×10^9/mL)	29.2 ± 10.5	11.3-56.5
Membrane Integrity (%)	82.4 ± 5.7	69.0-93.0
Normal sperm cells (%)	53.0 ± 10.3	36.4-80.3
Primary damages[1] (%)	10.8 ± 4.7	3.8-19.4
Secondary damages[2] (%)	36.2 ± 8.8	14.7-53.4
Motility rate (%)	85.0 ± 5.2	80.0-90.0
Motility quality[3] (score)	4.6 ± 0.4	3.0-5.0

Data were expressed as mean ± standard deviation (SD). [1]Primary damages: head degeneration; midpiece degeneration; tail stump; fractured tail; strongly coiled tail; macrocephaly and microcephaly. [2]Secondary damages: Free normal head; simple bent tails; proximal and distal droplets. [3]Score: rectilinear and very fast progressive movement (score 5); rapid rectilinear progressive movement (score 4); intermediate movement (score 3); slow movement (score 2); oscillatory movement (score 1); and absence of movement (score 0).

Table 3

Post-thaw motility quality score, duration of motility, and membrane integrity of *Chirostoma estor* sperm cryopreserved in six media.

Treatment	Motility quality (score*)[1]	Duration of motility (%)[1]	Membrane Integrity (%)
Fresh sperm control	5.0 ± 0.0	285 ± 65	82.4 ± 5.7
BTS+DMSO	2.5 ± 1.7^{b}	116 ± 104	54± 9
BTS+Methyl	3.0 ± 2.0^{ab}	153 ± 93	60 ± 9
MIII+DMSO	3.5 ± 1.7^{ab}	183 ± 86	60 ± 8
MIII+Methyl	4.0 ± 1.0^{a}	213 ± 105	60 ± 11
Androstar+DMSO	2.0 ± 1.5^{b}	114 ± 84	54 ± 6
Androstar+Methyl	4.0 ± 1.0^{a}	222± 109	54 ± 3

[1]Activation in glucose solution (150 mM); *Score: rectilinear and very fast progressive movement (score 5); rapid rectilinear progressive movement (score 4); intermediate movement (score 3); slow movement (score 2); oscillatory movement (score 1); and absence of movement (score 0); Dimethyl sulfoxide (DMSO) and Methyl glycol (Methyl); Data were expressed as median ± interquartile range (IQR) for motility quality score ($P < 0.05$, Friedman test); Data were expressed as mean ± standard deviation (SD) for the duration of motility and membrane integrity ($P > 0.05$, Scott-Knott test); n = 6 sperm pools (7 males/pool) ^{a,b}Means followed by different superscript in the same column differ.

Table 4

Fertilization rate (%) of *Chirostoma estor* sperm cryopreserved in six media, after activation in glucose solution at 150 mM.

	Extender		
Cryoprotectant	BTS™	MIII™	Androstar Plus™
DMSO	0 ± 0^{bB}	0 ± 0 ^{bB}	14 ± 2.8^{aB}
Methyl	19.5 ± 2.1^{aA}	12.0 ± 2.8^{bA}	20.0 ± 3.5^{aA}
	Fresh sperm 36 ± 26		

Dimethylsufoxide (DMSO) and Methylglycol (Methyl); Data were expressed as mean ± standard deviation (SD); n = 6 sperm pools (7 males/pool). $^{a,b;\ A,B}$ Means followed by different superscripts (lowercase for lines and uppercase for columns) are different ($P < 0.05$; Scott-Knott).

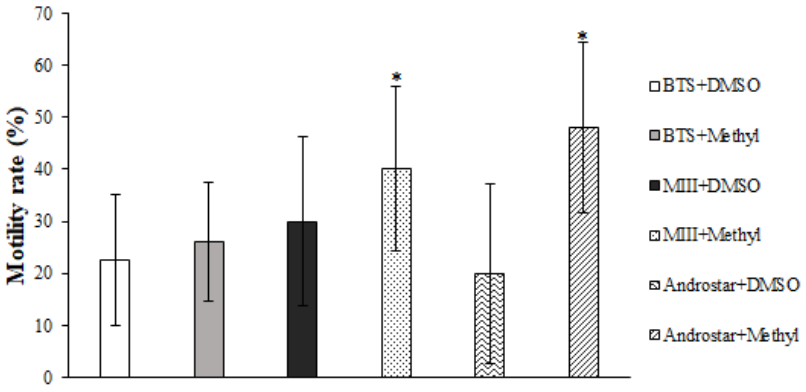

Fig. 1. Post-thaw motility rate of *Chirostoma estor* sperm cryopreserved in six media and analyzed subjectively after activation in glucose solution (150 mM). Dimethyl sulfoxide (DMSO) and Methyl glycol (Methyl); Bars indicate mean ± SD; n = 6 sperm pools (7 males/pool). *Means followed by an asterisk were significantly higher ($P < 0.05$, Scott-Knott).

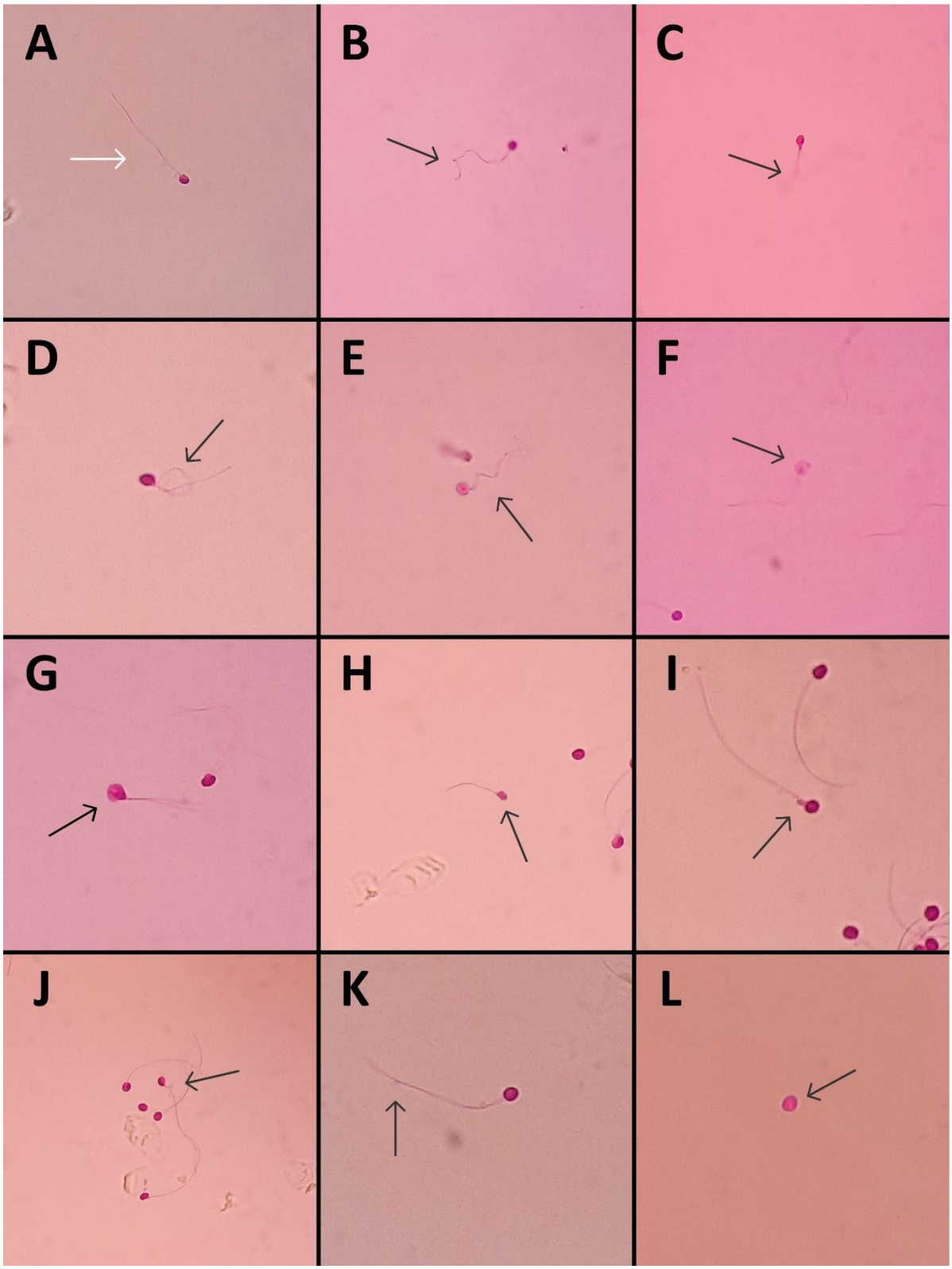

Fig. S1. Optical microscopy photographs of the sperm morphology of *Chirostoma estor*. The white arrow indicates normal sperm (A). Black arrow indicates morphological damages: fractured tail (B), tail stump (C), strongly coiled tail (D), simple bent tail (E), head degeneration (F), macrocephaly (G), microcephaly (H), midpiece degeneration (I), proximal droplet (J), distal droplet (K), and free normal head (L). ×1000 magnification.