UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

MARITZA PÉREZ ATEHORTÚA

PROTOCOLO PARA REMOÇÃO DO CRIOPROTETOR DO SÊMEN CRIOPRESERVADO DE *Rhamdia quelen*

Porto Alegre
2021

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PROTOCOLO PARA REMOÇÃO DO CRIOPROTETOR DO SÊMEN CRIOPRESERVADO DE *Rhamdia quelen*

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Dissertação apresentada como requisito para obtenção do Grau de Mestre em Zootecnia, na Faculdade de Agronomia, da Universidade Federal do Rio Grande

Orientador: Prof. Dr. Danilo Pedro Streit Jr.

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“A vida é como andar de bicicleta. Para ter equilíbrio você tem que se manter em movimento”

Albert Einstein

A família é lugar onde começa a vida e o amor nunca acaba”

PROTOCOLO PARA REMOÇÃO DO CRIOPROTETOR DO SÊMEN CRIOPRESERVADO DE *Rhamdia quelen*[1]

Autora: Maritza Pérez Atehortúa
Orientador: Prof. Dr, Danilo Pedro Streit Jr.

Resumo: A criopreservação oferece vários benefícios, entre estes o armazenamento de espermatozoides a longo prazo. No entanto, a falta de padronização ou a redução da qualidade espermática após o congelamento-descongelamento são desafios que ainda precisam ser resolvidos. O uso de tecnologias de reprodução assistida, como a centrifugação, tem sido relatada como uma ferramenta que permite separar a melhor porção de espermatozoides do sêmen criopreservado. O objetivo da pesquisa foi desenvolver um método de centrifugação para sêmen criopreservado de *Rhamdia quelen* e sua influência sobre a funcionalidade e capacidade de fecundação dos espermatozoides separados por gradiente de densidades AllGrad® 90%. Para isto foram realizados dois estudos. O primeiro estudo teve como objetivo a padronização de um método de centrifugação por gradiente de densidades para a separação do sêmen criopreservado de *R. quelen* a través do gradiente AllGrad® 90%. Seguidamente o método foi validado por meio de análises de qualidade (concentração, motilidade e morfologia espermática). Ao final deste estudo, a centrifugação de 1000 x g por 10 min, permitiu obter em média 8,19 x 10^6 espermatozoides normais/mL (22,25 ± 4,64%). Este valor representa um aumento de 9,25% dos espermatozoides normais em comparação ao sêmen criopreservado não centrifugado (P = 0,0013). Esse resultado deu lugar a um segundo estudo, cujo objetivo foi validar a eficiência da técnica de centrifugação por gradiente de densidades sobre a funcionalidade espermática e capacidade de fertilização. Foram comparados três tratamentos: i) controle (sêmen descongelado não centrifugado), ii) a centrifugação diferencial ou peletização e, iii) centrifugação por gradiente de densidades com AllGrad® 90%. A centrifugação diferencial resultou em melhores taxas de motilidade (42,58 ± 6,05%). Tanto a centrifugação diferencial quanto por gradiente de densidades resultou em melhores índices de atividade mitocondrial (1,58 ± 0,79 e 2,02 ± 1,88 AU/100 milhões sptz, respectivamente), taxa de fertilização (76,68 ± 15,22% e 69,85 ± 17,85%, respectivamente) e de eclosão (54,62 ± 17,39% e 50,87 ± 20,59%, respectivamente). Portanto, os métodos de centrifugação aplicados pela primeira vez no sêmen criopreservado de *R. quelen*, mostraram ser eficientes para obter uma melhor fração de espermatozoides.

Palavras chaves: catfish, qualidade espermática em peixes, morfologia espermática, centrifugação de sêmen de peixes, teleósteos

[1] Dissertação de Mestrado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil (135 p.). Abril, 2021.

PROTOCOL TO REMOVE THE CRIOPROTECTOR FROM THE CRYOPRESERVED MILT FROM *Rhamdia quelen*[1]

Author: Maritza Pérez Atehortúa
Advisor: Prof. Dr. Danilo Pedro Streit Jr.

Abstract: Cryopreservation offers several benefits, including long-term storage of sperm. However, the lack of standardization or the reduction of sperm quality after freezing-thawing are challenges that still need to be solved. The use of assisted reproduction technologies, such as centrifugation, has been reported as a tool that allows the best portion of sperm to be separated from cryopreserved milt. The objective of the research was to develop a centrifugation method for cryopreserved milt from *Rhamdia quelen* and its influence on the functionality and fertilization capacity of spermatozoa separated by 90% AllGrad® density gradient. For this, two studies were carried out. The first study aimed to standardize a density gradient centrifugation method for the separation of *R. quelen* cryopreserved milt through the AllGrad® 90% gradient. Then the method was validated through quality analysis (concentration, motility and sperm morphology). At the end of this study, the centrifugation of 1000 x g for 10 min, allowed an average of 8.19 x 106 normal sperm/mL to be obtained (22.25 ± 4.64%). This value represents a 9.25% increase in normal sperm compared to non-centrifuged cryopreserved milt (P = 0.0013). This result gave rise to a second study, whose aim was to validate the efficiency of the density gradient centrifugation technique on sperm functionality and fertilization capacity. Three treatments were compared: i) control (non-centrifuged thawed semen), ii) differential centrifugation or pelletization, and iii) density gradient centrifugation with AllGrad® 90%. Differential centrifugation resulted in better motility rates (42.58 ± 6.05%). Both differential centrifugation and density gradient resulted in better rates of mitochondrial activity (1.58 ± 0.79 and 2.02 ± 1.88 AU/100 million sptz, respectively), fertilization rate (76.68 ± 15.22% and 69.85 ± 17.85%, respectively) and hatching (54.62 ± 17.39% and 50.87 ± 20.59%, respectively). Therefore, the centrifugation methods applied for the first time in the cryopreserved milt of *R. quelen*, proved to be efficient to obtain a better fraction of sperm.

Keywords: catfish, fish sperm quality, fish sperm morphology, fish milt centrifugation, teleost

[1] Master dissertation in Animal Science – Animal Production, Faculty of Agronomy, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (135 p.). April 2021.

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CAPÍTULO II

LISTA DE ABREVIATURAS E SÍMBOLOS

$CaCl_2$ – Cloreto de cálcio
CASA - Análises espermática assistida por computador
cm - centímetro(s)
CPE – Extrato pituitário de carpa
DNA - Ácido desoxirribonucleico
h – Hora(s)
IP – Iodeto de propídio
KCl - Cloreto de potássio
Kg - Quilograma(s)
L – Litro(s)
Me_2SO - Dimetilsulfóxido
mg – Miligrama(s)
min – Minuto(s)
mL – Mililitro(s)
mM - miliMolar
mOsm - miliOsmolar
MTT - 3- (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NaCl – Cloreto de sódio
$NaHCO_3$ – Bicarbonato de sódio
pH – Potencial Hidrogeniônico
PVP - Polivinilpirrolideno
RCF - força centrífuga relativa ou força g (X g)
RNA - Ácido ribonucleico
RPM - Revoluções por minuto
s – Segundos
UA – Unidades de absorbância
v - Volume
µL - Microlitros
ºC – Graus centígrados

CAPÍTULO I

1. INTRODUÇÃO GERAL

A criopreservação permite o armazenamento por tempo indefinido de material biológico (Sheikhi et al., 2011), fornecendo inúmeras maneiras de gerar, manter e distribuir recursos genéticos (Tiersch & Green, 2011). Permite o gerenciamento ideal dos riscos comerciais (mudanças ou demandas econômicas e de mercado em características desejáveis) e biológicos (perda de animais por doenças) associados ao melhoramento seletivo (Demoy-Schneider et al., 2020). Mundialmente, a produção de peixes tem crescido a uma taxa, cerca de 10% anual, na última década (Betsy & Kumar, 2020). No Brasil, o crescimento em 2020, quando comparado a 2019, foi de 5,93%, atingindo 802.930 toneladas, no qual 31,1% dessa produção é representada pela região Sul do país (Associação Brasileira da Piscicultura, 2021). Nessa região, o *Rhamdia quelen*, é o peixe nativo de água doce mais promissor para a piscicultura (Amaral Júnior, 2013). Aparentemente são bem adaptados a diferentes ambientes, com bom desempenho produtivo e domínio do processo reprodutivo em cativeiro (Bombardelli et al., 2006). Adicionalmente, esta espécie tem sido aceita no mercado consumidor por apresentar uma carne saborosa sem espinhas intramusculares (Amaral Júnior, 2013). Além dos atributos zootécnicos, essa espécie também é usada como modelo animal (Brasil, 2019), por exemplo, para o desenvolvimento de novos fármacos (Rodrigues et al., 2019). Portanto, a busca e implementação de técnicas, como a criopreservação de sêmen são cada vez mais emergentes e necessárias na comunidade científica (Marques & Godinho, 2004).

A criopreservação de células reprodutivas em peixes resulta em soluções para a manutenção de espécies usadas como modelo animal (Tiersch & Green, 2011; Howe et al., 2013), em programas de preservação de espécies ameaçadas (Tiersch, 2008), ou para a melhora ou criação de novas indústrias (Tiersch & Green, 2011). Entre os benefícios na criopreservação de material reprodutivos de peixes, Suquet et al. (2000) relacionaram a sincronização para a disponibilidade dos gametas, para o uso do volume total de sêmen disponível, para a diminuição de manutenção do material reprodutor e para o transporte de gametas (quando gametas masculinos e femininos são coletados em locais diferentes, podendo ser de ambientes naturais). Assim, esses autores

destacaram a importância da técnica para evitar o envelhecimento dos espermatozoides, utilização para programas experimentais e, por fim conservação da variabilidade genética em populações domésticas.

Os processos envolvidos na criopreservação (exemplo: congelamento/descongelamento) tem potenciais efeitos deletérios sobre a função dos espermatozoides, podendo prejudicar os compartimentos subcelulares e as suas moléculas (Cabrita et al., 2014). As injúrias causadas na criopreservação estão inversamente relacionadas com a qualidade dos espermatozoides (Li et al., 2006), gerando efeitos negativos na morfologia (Streit Junior et al., 2009), funcionalidade das mitocôndrias (Hagedorn et al., 2012), integridade do DNA (Zilli et al., 2003), perfil proteico (Horokhovatskyi et al., 2018), e consequentemente na motilidade (Gallego & Asturiano, 2019). Portanto, o desenvolvimento de técnicas que permitam manter a qualidade dos espermatozoides durante a criopreservação tem sido necessário, tais como o uso de proteínas, glicoproteínas anticongelantes, (Xin et al., 2019), ou então antioxidantes (Costa & Streit Junior, 2019). Outra ferramenta auxiliar, que vem sendo usada, é a centrifugação por gradiente de densidades. Segundo Machado et al. (2009), essa técnica tem mostrado resultados eficientes na procura de características específicas em sêmen (sexagem de espermatozoide, separação de espermatogônias, limpeza de patógenos, plasma seminal/crioprotetores, qualidade espermática, entre outros), em diferentes espécies (humanos, ovinos, bovinos, equinos, cães) e seu uso em peixes também foi reportado (Rivers et al., 2020).

Em peixes, a centrifugação por gradientes de densidades tem sido documentada para a separação das diferentes populações de células reprodutivas de machos em espécies como por exemplo, Sterlet (*Acipenser ruthenus*) (Dzyuba et al., 2014), tenca (*Tinca tinca*) (Linhartová et al., 2014), pejerry (*Odontesthes bonariensis*) (Majhi et al., 2014), catfish (*Ictalurus furcatus*) (Shang et al., 2018), piracanjuba (*Brycon orbignyanus*) (Silva et al., 2019) e carpa comum (*Cyprinus carpio*) (Franěk et al., 2019). Em adição, outros estudos (Li et al., 2010; Horokhovatskyi et al., 2018) mostraram que a técnica de centrifugação por gradientes de densidades promete ser eficiente na seleção dos espermatozoides, com aumento na capacidade de sobrevivência nos processos de criopreservação. Li et al. (2010), usaram sêmen criopreservado de *C. carpio* e avaliaram

a motilidade, integridade de membrana e velocidade espermática, obtendo uma diferença do sêmen separado em relação ao tratamento controle. Para a espécie *A. ruthenus,* também foram comparados os parâmetros de qualidade, tais como a porcentagem de motilidade e de células vivas, encontrando uma melhora de 62,4% e 26,5%, respectivamente, nos espermatozoides que foram separados (Horokhovatskyi et al., 2018).

Em nosso estudo, a técnica de centrifugação por gradiente de densidades foi aplicada pela primeira vez para a espécie *R. quelen*, por conta disso, foi necessária a padronização do método. Por tanto, o objetivo inicial do trabalho foi identificar a melhor força e tempo de centrifugação para a separação do sêmen criopreservado, considerando como parâmetros de avaliação a formação e a concentração espermática no pellet. Seguidamente, os grupos considerados como sendo os melhores, foram escolhidos e comparados em termos de motilidade, integridade de membrana, morfologia e concentração dos espermatozoides. Por fim um protocolo foi escolhido. Desta vez os parâmetros de motilidade com o software CASA, a concentração espermática, a integridade de membrana com SYBR-14/IP por citometría de fluxo, a atividade mitocondrial com o teste de MTT, e, a taxa de sobrevivência embrionária e eclosão, foram avaliados e comparados. Finalmente, um protocolo foi determinado com a finalidade de ser usado em rotinas de criopreservação do sêmen da espécie em estudo. Espera-se também que esse protocolo sirva de base e possa ser adaptado e aplicado para outras espécies de peixes teleósteos.

2. REVISÃO BIBLIOGRÁFICA

2.1. Criopreservação

A criobiologia (do grego *kryos* = frio, *bios* = vida e *logos* = ciência) é uma área da biologia que têm como objetivo principal estudar os efeitos das baixas temperaturas em células, tecidos e organismos vivos. A interação da criobiologia com outros campos da ciência (fisiologia celular, patologia, bioquímica, biofísica e físico-química, termodinâmica e matemática) (Pretunkina, 2007; Benson et al., 2012) dão origem à criopreservação, tecnologia que permite manter células, tecidos ou embriões a baixas temperaturas, tendo como premissa a preservação da composição e da viabilidade das células por tempo indefinido (Silva & Guerra, 2011).

Existem dois métodos de criopreservação, a vitrificação (Yamaki et al., 2002) e o congelamento lento (Shaw et al., 2000; Sanches, 2009). No congelamento lento a desidratação da célula e a redução da temperatura se dá de forma gradual, visando reduzir o estresse térmico na fase de transição das soluções do estado líquido para o estado sólido (Sanches, 2009), evitando ou reduzindo a formação de cristais de gelo (Shaw et al., 2000). Com o congelamento lento, o material biológico geralmente é congelado sob o controle de, por exemplo, um freezer programável. Neste caso, a desidratação acontece quando a temperatura se encontra entre -30 °C a -80 °C, e posteriormente as células são estocadas à temperatura de -196 °C (Paynter, 2000).

A variação da qualidade dos espermatozoides quando submetidos ao processo de criopreservação é influenciada por muitos fatores, por exemplo, a técnica de congelamento (Guimarães et al., 2018) ou mesmo pela quantidade ou tipo de crioprotetor empregado (Silva & Guerra, 2011). Os crioprotetores são solutos que promovem a desidratação celular (perda de até 95% de água) durante a criopreservação, estabilizando as membranas e as proteínas (Karlsson & Toner, 1996; Müller et al., 2019).

Existem dois tipos de soluções crioprotetoras: permeáveis (capacidade de penetrar a célula) e impermeáveis (não realizam penetração celular). Os crioprotetores permeáveis são solventes orgânicos de baixo peso molecular entre os que podem se mencionar o etilenoglicol ($C_2H_6O_2$), o dimetilsulfóxido (Me_2SO), o metanol (MeOH), o metilglicol ($C_2H_4(OH)_2$) (Castro et al., 2011; Silva, 2014). Os crioprotetores impermeáveis recobrem

a superfície celular e estabilizam a membrana minimizando os possíveis danos celulares causados pelo processo de congelamento. Podem ser açúcares (sacarose ou glicose) e proteínas ou aminoácidos (BSA - albumina de soro bovino, glicina, gema de ovo e leite em pó. Tanto a gema de ovo como o leite em pó não são só compostos de proteínas, por exemplo, o leite em pó tem a lactose que é um açúcar) (Silva & Guerra, 2011). Contudo, a eficiência destes crioprotetores podem variar em função da estrutura (célula ou tecido) a ser criopreservada, do tipo, da concentração e do tempo de exposição, ao agente crioprotetor, utilizado antes do congelamento.

Por outro lado, algumas características tóxicas têm sido atribuídas as soluções crioprotetoras, principalmente a temperatura ambiente (Müller et al., 2019). No momento do descongelamento a água é reequilibrada nas células, e é neste momento em que o maior número de lesões podem acontecer (Pretunkina, 2007). Portanto, considera-se que a concentração destas soluções nas amostras de sêmen é um dos fatores que mais interferem na qualidade dos espermatozoides após descongelamento e, portanto, nas taxas de fertilização (Pretunkina, 2007).

2.2. Centrifugação

Segundo Griffith (2010), a centrifugação é um processo utilizado para separação de diferentes partículas biológicas (células, organelas subcelulares ou macromoléculas) através da força centrífuga. Este mesmo autor indica que na pesquisa biológica existem dois métodos de separação: a centrifugação diferencial ou peletização e a centrifugação por gradiente de densidades. O primeiro método permite separar partículas de diferentes coeficientes de sedimentação, na qual as partículas de maior tamanho ou peso vão primeiro para o fundo do tubo. Para isso, um tubo é inicialmente preenchido com uma mistura uniforme de solução de amostra. Por meio de cada centrifugação são obtidas duas frações: um pellet (material sedimentado) e um sobrenadante. Quanto à separação por gradiente de densidades, é um método que pode ser mais complicado do que a centrifugação diferencial, mas com vantagens compensatórias. Segundo Morrel (2006), esse método possibilita, por exemplo, a separação de vários ou todos os componentes em uma suspensão de células (exemplo: células germinativas, células somáticas, detritos

celulares, espermatozoides), ou isola uma partícula de um material químico, físico ou biológico, a fim de quantificar os efeitos destes sobre a partícula estudada, exemplo, efeito do plasma seminal sobre a motilidade espermática. Partindo deste contexto é possível afirmar que a separação por gradiente de densidades é um método eficaz para a separação de células com base ao tamanho. Na prática, o gradiente age como uma "peneira molecular", capturando as células de maior tamanho nas camadas superiores, permitindo a passagem das células de menor tamanho (Rivers et al., 2020).

Nesse contexto das técnicas de separação para espermatozoides, Henkel & Schill (2003) relatam que a técnica de separação espermática ideal deve (i) ser rápida, fácil e econômica, (ii) isolar o máximo possível de espermatozoides móveis, (iii) não causar danos aos espermatozoides ou alterações não fisiológicas das células espermáticas separadas, (iv) eliminar espermatozoides mortos e outras células, incluindo leucócitos e bactérias, (v) eliminar substâncias tóxicas ou bioativas, e (vi) permitir o processamento de volumes maiores do sêmen liberado. Contudo, o sucesso da separação é determino pela conformação de bandas dentro do gradiente, que corresponde as populações celulares (Rivers et al., 2020).

Para a realização da separação por centrifugação com gradientes de densidades várias soluções podem ser usadas. Uma solução normalmente usada, é a sílica revestida com polivinilpirrolidona (PVP – comercialmente conhecido como Percoll), cujo uso foi reportado na década de 1980s para a separação de células e organelas celulares (Kjellén & Pertoft, 1978). Ao longo dos tempos outras soluções compostas por partículas de sílica revestidas com silano têm sido desenvolvidas, tais como: AllGrad®, PureSperm®, ISolate®, SpermGrade™, Sydney IVF Spermient™ (Meryman, 1971; Muhammad & Fakhrildin, 2019), em especial para uso na reprodução assistida em humanos. Outras soluções espécie-específicos que podem ser encontradas, para o uso com animais, são o EquiPure™, Androcoll® para equinos (Alvarenga et al., 2017) e CapriPure® para caprinos (Batista et al., 2011).

As soluções de sílica revestida com silano apresentam benefícios tais como, baixos níveis de endotoxinas e, capacidade de permanecerem estáveis por longos períodos em soluções salinas, o que permite o uso de formulações comerciais prontas para uso (Morrell & Rodriguez-Martinez, 2009). Provavelmente estas características levaram este

tipo de soluções a serem usadas como substitutas das soluções de sílica revestida com PVP (Henkel & Schill, 2003; Allahbadia et al., 2017), na reprodução assistida em humanos (Mortimer, 2000; Muhammad & Fakhrildin, 2019), ou em algumas formulações espécie-específicas para animais (Morrell, 2006).

Em particular, soluções como o AllGrad® apresentam uma média de pH e osmolaridade de 7,4 e 310 mOsm, respectivamente, características de importante cuidado quando se trabalha com sêmen de peixes (Morisawa & Suzuki, 1980; Mochida et al., 1999; Cosson, 2004; Alavi & Cosson, 2005; Cherr et al., 2008). Os espermatozoides de peixes de água doce têm como característica permanecerem imóveis e são ativados ao entrar em contato com a água (baixa osmolaridade), e por isso, durante a centrifugação deve ser garantida a não ativação espermática. Embora a centrifugação por gradiente de densidades já tenha sido usada em peixes, como por exemplo em *C. carpio* (Li et al., 2010) ou em *A. ruthenus* (Horokhovatskyi et al., 2018), o uso de AllGrad® ainda não foi reportado para sêmen criopreservado de peixes. Somado a isso, é importante considerar que, amostras de sêmen submetidas a protocolos de separação podem apresentar diferentes forças e tempo de centrifugação e, consequentemente, influenciar nos parâmetros de qualidade, efeitos que ainda não foram comparados (Marzano et al., 2020).

2.3. Qualidade espermática

A avaliação da qualidade dos gametas é um componente essencial para a criopreservação bem-sucedida e o desenvolvimento de bancos de germoplasma (Tiersch & Green, 2011). Para garantir a qualidade seria necessária realizar avaliações em todas as etapas envolvidas nesse processo. Por exemplo, avaliação da motilidade dos espermatozoides no momento da coleta, após os procedimentos de suspensão em diluente, armazenamento refrigerado ou envio das amostras diluídas, equilíbrio no crioprotetor e descongelamento (Martínez-Páramo et al., 2017). As avaliações de qualidade podem ser agrupadas em avaliações da membrana, da mitocôndria, do DNA ou do RNA (Cabrita et al., 2014). Métodos típicos de avaliação de qualidade para peixes incluem estimativas de motilidade subjetiva ou objetiva com análises espermática assistida por computador como CASA (Gallego et al., 2018), avaliação de várias

propriedades celulares por citometria de fluxo (Daly & Tiersch, 2011), estresse oxidativo (Costa & Streit Junior, 2019) e, morfologia (Streit Junior et al., 2004a). Informações que estão relacionadas com o sucesso da fertilização na aquicultura.

2.3.1. Concentração espermática

A concentração espermática é um parâmetro quantitativo que auxilia a fertilização artificial e permite controlar e otimizar a taxa de fertilização e por sua vez, promove o uso racional dos reprodutores em um sistema de cultivo (Felizardo et al., 2010; Sanches et al., 2011). Existem vários métodos que permitem mensurar a concentração de espermatozoides no sêmen (Ciereszko et al., 2020). Entre as técnicas usadas estão a contagem no microscópio ou a determinação dos valores de espermatócrito (Sanches et al., 2011). Ciereszko et al. (2020) também citaram os métodos de quantificação por espectrofotometria e por citometria de fluxo. A quantificação espermática do sêmen de peixes no microscópio, com o uso da câmara de Neubauer, tem sido a metodologia regularmente referenciada, como por exemplo, em *Brycon opalinus* (Narahara et al., 2002), *Prochilodus lineatus* (Streit Junior et al., 2004b), *Brycon opalinus* (Viveiros et al., 2012) e *R. quelen* (Costa et al., 2019). Cabe ressaltar que esta avaliação é considerada importante nas rotinas de reprodução artificial por permitir, por exemplo, o uso do volume de sêmen apropriado para uma determinada massa de oócitos, influenciando positivamente as taxas de fertilização (Felizardo et al., 2010).

2.3.2. Motilidade espermática

A motilidade espermática é um dos parâmetros mais usados no estudo da biologia dos espermatozoides, sendo considerado um pré-requisito fundamental para determinar a qualidade e a capacidade de fertilização do sêmen (Kime et al., 2001; Alavi & Cosson, 2005). A maioria das espécies de peixes, em contraste com os mamíferos, realizam a fertilização externa, sendo que os espermatozoides são imóveis até entrar em contato com a água (Mochida et al., 1999; Cherr et al., 2008). A motilidade pode ser iniciada por alteração da osmolaridade, da concentração de íons (Morisawa & Suzuki, 1980; Mochida et al., 1999; Cosson, 2004; Cherr et al., 2008), pelo pH ou temperatura da água (Alavi &

Cosson, 2005). Na maioria das espécies de água doce, os espermatozoides em média se movem por menos de 2 min, sendo, no geral, considerados como altamente ativos por menos de 30 s (Alavi & Cosson, 2005).

Segundo Rurangwa et al. (2004), existem três métodos diferentes que permitem a avaliação da motilidade: o método subjetivo, o método semiquantitativo e o método quantitativo. O método subjetivo tem sido a técnica mais usada ao longo da história. Esta técnica permite determinar, com observação direta no microscópio óptico, a porcentagem de espermatozoides móveis e a duração da motilidade dos espermatozoides (Fauvel et al., 2010). Portanto, é uma técnica que vai depender da experiência do observador, e de outros aspectos, como densidade do esperma ou velocidade dos espermatozoides, que podem causar superestimações ou subestimações dos resultados (Gallego et al., 2018).

O método semiquantitativo foi uma técnica proposta para superar a subjetividade e permitir maior confiabilidade da análise. Estas medidas são realizadas por meio de gravações de vídeos, permitindo obter resultados de taxa e tempo de motilidade dos espermatozoides, além de medidas de velocidade (Rurangwa et al., 2004).

Já a técnica quantitativa ou análise de esperma assistida por computador (CASA), é um método mais sensível, que permite avaliar os vários parâmetros de motilidade dos espermatozoides, de forma objetiva, por meio de um computador equipado com um software de análise de imagem (Rurangwa et al., 2004; Gallego et al., 2018). Os resultados gerados após avaliações com o software CASA são os resultados médios de motilidade espermática (MOT), velocidade espermática curvilínear (VCL), velocidade espermática média de deslocamento (VMD), velocidade espermática em linha reta (VLR), retilinearidade, balanço, progressão, frequência de batimentos e o número de espermatozoides considerados na análise (Neumann, 2013).

2.3.3. Morfologia

Os espermatozoides estão compostos por três compartimentos diferentes, cabeça, peça intermediária e cauda (Ginzburg, 1972; Nunes et al., 2015). A maioria dos espermatozoides dos peixes são carentes de acrossoma (vesícula com enzimas capaz de digerir a parede do oócito), mas a fertilização é feita por meio de um orifício presente no córion dos oócitos conhecido como micrópila (Ginzburg, 1972; Nagahama, 1983;

Cosson et al., 1999). Para garantir o bom desempenho dos espermatozoides é necessário que suas estruturas (cabeça, peça intermediária e cauda) se encontrem íntegras (Kavamoto et al., 1999). Sendo assim, é fundamental a avaliação morfológica dos espermatozoides tanto de amostras de sêmen fresco quanto criopreservado, uma vez que permite a caracterização dos mesmos, ajudando explicar a redução da motilidade dos espermatozoides e, consequentemente, perda da capacidade fertilizante (Streit Junior et al., 2008).

Muito embora, em bovinos e equinos há recomendações para a não utilização de sêmen com índices de espermatozoides com anormalidade acima de 30%, em bovinos e equinos, e acima de 20% para ovinos e suínos (CBRA, 1998). Para peixes essas referências ainda não foram estabelecidas. Todavia, algumas propostas de classificação morfológica já foram realizadas como no caso da espécie *Prochilodus lineatus* (Miliorini et al., 2011). Outros estudos recentes de Costa et al. (2019) e Costa et al. (2020) e a meta-análise de Lassen et al. (2021), também estabeleceram uma proposta de avaliação de patologias morfológicas de espermatozoides para sêmen criopreservado para espécies de peixes neotropicais.

A análise morfológica dos espermatozoides é realizada com auxílio de corantes específicos (Rosa Bengala, Vermelho Congo, entre outros) e a diluição do sêmen em soluções como formol salina tamponado (Streit Junior et al., 2004a). Esta análise é realizada por meio da observação dos espermatozoides em microscopia de luz, e a classificação das anormalidades morfológicas é obtido de forma subjetiva como sugerido por Herman et al. (1994). Estes autores caracterizaram as alterações morfológicas dos espermatozoides em patologias primárias (flagelo quebrado, enrolado, curto, degenerado, macrocefalia, microcefalia) e patologias secundárias (flagelo dobrado, cabeça isolada, gotas citoplasmáticas proximal e distal) (Herman, 1994). Nesse contexto, as patologias primárias estão relacionadas com a espermatogênese e as patologias secundárias com fatores ambientais ou com procedimentos de manipulação dos reprodutores ou do sêmen. Cabe ressaltar que, para peixes, a origem destas anormalidades morfológicas vem sendo discutidas e propostas novas abordagem de acordo com Costa et al. (2019, 2020).

2.3.4. Integridade de membrana

A membrana plasmática controla os intercâmbios de água e íons entre o meio interno e externo da célula (Fauvel et al., 2010). A avaliação das membranas espermáticas pode ajudar a indicar o sucesso da criopreservação, por serem extremamente suscetíveis às lesões criogênicas (Li et al., 2006), e sua integridade é amplamente verificada pelo uso de corantes fluorescentes ou não fluorescentes específicos (Boryshpolets et al., 2020). Do ponto de vista prático, a integridade da membrana tem sido estudada pela avaliação da capacidade de penetração diferencial de corantes como eosina-nigrosina ou eosina sozinha, por simples observação ao microscópio (Blom, 1950; Fauvel et al., 2010).

Entre os corantes fluorescentes usados para determinar a permeabilidade da membrana estão o SYBR-14, o iodeto de propídio (IP), a rodamina 123 (Rh 123), entre outras (Li; et al., 2006). Esta avaliação pode ser realizada em microscópio de fluorescência ou por citômetro de fluxo (Daly & Tiersch, 2011; Boryshpolets et al., 2020). Desta forma, o ensaio de fluorescência normalmente compreende duas substâncias de fluorescência, PI e SYBR-14. Neste caso, os espermatozoides que não tenham a membrana íntegra, apresentam coloração vermelha pela ação do IP, enquanto o SYBR-14, permeável à membrana, apresenta fluorescência verde, após ligação com os ácidos nucléicos (Boryshpolets et al., 2020). Ressaltando que o SYBR-14 é deslocado pelo IP, quando o espermatozoide apresenta a membrana danificada (Silva & Gadella, 2006). O uso de SYBR-14/IP age diretamente no DNA, o que garante que a fluorescência identificada pertence a uma célula e não a outras partículas, vantagem do uso destes corantes. A importância de destacar esse ponto é que a presença de partículas não espermáticas, usadas para melhorar as soluções de criopreservação (gema de ovo, gorduras do leite em pó), ao não apresentarem DNA não terão a expressão de nenhuma destas fluorescências, predizendo unicamente sobre a viabilidade dos espermatozoides (Silva & Gadella, 2006).

2.3.5. Função mitocondrial por ensaio de metil-tiazolil-tetrazólio (MTT)

A avaliação da funcionalidade mitocondrial é outro parâmetro importante na determinação da viabilidade dos espermatozoides (Tiersch & Green, 2011). O MTT (3-

(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) é um teste colorimétrico, considerado simples, rápido e confiável, tendo como objetivo avaliar a funcionalidade mitocondrial ou estimar a porcentagem de espermatozoides viáveis (Aziz, 2006). Assim, os espermatozoides com metabolismo ativo (células viáveis) têm a capacidade de converter o MTT em um produto, chamado de cristais de formazan, de cor púrpura, o que não ocorre em células mortas (Nasr-Esfahani et al., 2002; Riss et al., 2004). Acredita-se que o sistema de succinato desidrogenase (ativo em células vivas) é o encarregado de converte o MTT em cristal de formazan (insolúvel em água) (Nasr-Esfahani et al., 2002). Essa característica de insolubilidade determina a necessidade de uso de solventes orgânicos, como o dimetilsulfóxido (Me_2SO), para que o formazan possa ser dissolvido (Riss et al., 2004). A quantidade de formazan, é mensurada registrando as mudanças na absorbância a 570 nm, a partir do uso de espectrofotômetro de leitura de placa, podendo ser utilizado ou não um comprimento de onda de referência de 630 nm (Riss et al., 2004).

2.3.6. Fecundação e taxas de eclosão

A capacidade de fecundação e de contribuir no desenvolvimento embrionário inicial bem-sucedido, são medidas que devem ser usadas como determinantes diretos da qualidade de uma amostra de sêmen (Rurangwa et al., 2004; Cabrita et al., 2014). A medida de fecundação é considerada uma das melhores variáveis para definir a qualidade do sêmen (Bobe & Labbé, 2010; Fauvel et al., 2010). A taxa de sobrevivência embrionária é uma variável que permite estimar o número de oócitos fecundados, ou seja, a porcentagem de embriões que atingem a fase de fechamento do blastóporo (Felizardo, 2008).

Contudo, a sobrevivência embrionária é um índice que não deve ser avaliada de forma individualizada e sim considerando outros aspectos próprios da espécie (Rurangwa et al., 2004), além de outros parâmetros de qualidade dos espermatozoides (Bombardelli et al., 2006) ou então a qualidade dos oócitos (Felizardo et al., 2010).

Bombardelli et al. (2004) reportaram que uma proporção adequada de espermatozoide:oócito, em fecundação artificial em peixes, pode melhorar o número de embriões e possibilitar a economia de gametas, uma vez que uma amostra de sêmen poderia ser utilizada para fecundar os oócitos de várias fêmeas. Assim, conhecer a

proporção correta de espermatozoide:oócito é importante para programas de criopreservação de gametas de espécies com fins de conservação da biodiversidade ou para uso em programas de melhoramento genético em fazendas de cultivo (Bombardelli et al., 2006).

2.4. Jundiá (*Rhamdia quelen*, Quoy & Gaimard, 1824)

A espécie *R. quelen*, é um teleósteo de água doce (Perdices et al., 2002), pertencente à família Heptapteridae (Silfvergrip, 1996), com distribuição neotropical (Silfvergrip, 1996), podendo ser encontrado no centro da Argentina ao Sul de México e região Sul do Brasil (Valladão et al., 2016). Dentre as características zootécnicas importantes, este peixe pode atingir 600–800 g de peso corporal em oito meses a uma densidade de 2 a 4 peixes/m^2 (Barcellos et al., 2004). Adicionalmente, tem uma boa aceitação no mercado consumidor por apresentar uma carne saborosa e sem espinhos intramusculares (Amaral Júnior, 2013). Apesar destas características produtivas positivas para a piscicultura, esta espécie prefere temperaturas da água mais amenas, porque, embora consigam sobreviver em uma ampla faixa de temperaturas, apresentam melhor crescimento a temperaturas cerca dos 24 °C (Valladão et al., 2016).

Esta espécie de couro tem a coloração variando de marrom-avermelhado claro a cinza ardósia (Silfvergrip, 1996). Prefere locais calmos e profundos dos rios e próximos às margens e a vegetação (Baldisserotto & Radünz Neto, 2004). Segundo Valladão et al. (2016), esta espécie tem hábito alimentar onívoro, com tendências piscívoras. No entanto aceita uma dieta artificial com ingredientes de origem vegetal, desde os primeiros dias de vida.

Reprodutivamente, o *R. quelen* atinge maturidade sexual no primeiro ano de vida, com dois picos reprodutivos durante o ano (primavera e verão, com múltiplas desovas) no ambiente natural (Baldisserotto & Radünz Neto, 2004). A maturação das gônadas começa à temperatura da água de 17 °C, mas, é prejudicada por fatores como a densidade de estocagem, o manejo e o tipo de dieta (Narahara et al., 1988). O início de maturação das gônadas e o potencial reprodutivo dos machos de dessa espécie se dão com 13,4 cm e 16,5 cm de comprimento corporal, respectivamente, no caso das fêmeas

ocorre com comprimentos respectivos de 16,5 cm e 17,5 cm (Narahara et al., 1988; Baldisserotto & Radünz Neto, 2004). Os machos tendem a maturar precocemente em relação as fêmeas, e por consequência a produção de sêmen ocorre quando as fêmeas ainda não estão aptas para reprodução (Baldisserotto & Radünz Neto, 2004).

3. HIPÓTESES E OBJETIVOS

3.1. Hipóteses

- O uso da técnica de centrifugação do sêmen criopreservadas de *R. quelen* por gradientes de densidade com AllGrad® 90%, vai auxiliar na obtenção da melhor fração de espermatozoides.
- A separação por gradiente de densidade com AllGrad® 90%, resultará em uma maior população de espermatozoides viáveis, avaliada a partir de motilidade espermática, integridade de membrana e do potencial de membrana.
- A população de espermatozoides escolhida a partir da centrifugação por gradiente de densidade com AllGrad® 90% vão permitir uma maior taxa de sobrevivência embrionária e de eclosão, em comparação ao sêmen não centrifugado.

3.2. Objetivos

3.2.1. Objetivo Geral

Desenvolver o método de centrifugação para sêmen criopreservado de *R. quelen* e sua influência sobre a funcionalidade e capacidade de fecundação dos espermatozoides separados por gradiente de densidades AllGrad® 90%.

3.2.2. Objetivos específicos

- Padronizar um método de centrifugação por gradiente de densidades para sêmen criopreservado de *R. quelen* a partir de AllGrad® 90%.
- Quantificar a concentração de células espermáticas da fração de amostra separada com centrifugação por gradiente de densidades com AllGrad® 90%.
- Determinar a integridade de membrana, atividade mitocondrial e morfologia dos espermatozoides separados por gradiente de densidades com AllGrad® 90%.
- Avaliar a capacidade de fecundação e as taxas de eclosão do sêmen criopreservado de *R. quelen,* depois de passar por um protocolo de centrifugação por gradiente de densidades com AllGrad® 90%.

CAPITULO II[1]

Sperm selection of cryopreserved milt of *Rhamdia quelen* by density gradient centrifugation with AllGrad® 90%

[1] Artigo elaborado conforme as normas do periódico *MethodosX* (https://www.journals.elsevier.com/methodsx)

Sperm selection of cryopreserved milt of *Rhamdia quelen* by density gradient centrifugation with AllGrad® 90%

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Abstract

Density gradient centrifugation is a technique used to wash or separate samples of cryopreserved milt, mainly in humans and bovines allowing, for example, reducing the concentration of cryoprotectants or choosing the best portion of sperm. We propose a method that allows reducing the presence of cryoprotectant in cryopreserved milt of the *Rhamdia quelen* species and subsequently improve quality of the spermatozoa. The method was based on density gradient centrifugation with AllGrad® 90%, comparing characteristics such as centrifugation times and forces. The method showed that density gradient centrifugation represented a better percentage of motility compared to the milt samples non-separated. In addition, head damage and the presence of gout damage were significantly higher in non-separated milt. An average of 8.19 x 10^6 normal spermatozoa (22.25 ± 4.64%) were obtained with the rate of 1000 X g for 10 min, 4 °C, 9.25% more than non-centrifuged milt ($p = 0.0013$). This indicates that the method allows to reduce the morphological damage caused by the cryoprotectant in the thawed sperm samples, achieving thus a larger portion of normal sperm.

- Density gradient centrifugation with AllGrad® 90% is proposed as a tool of easy adaptation and application for the separation of cryopreserved sperm of *Rhamdia quelen*.
- The density gradient centrifugation method with AllGrad® 90% at a centrifugation time and force of 1000 X g for 10 min allows obtaining a larger portion of normal sperm.

Keywords: catfish, fish sperm quality, fish sperm morphology, fish sperm centrifugation, teleost

Graphical abstract

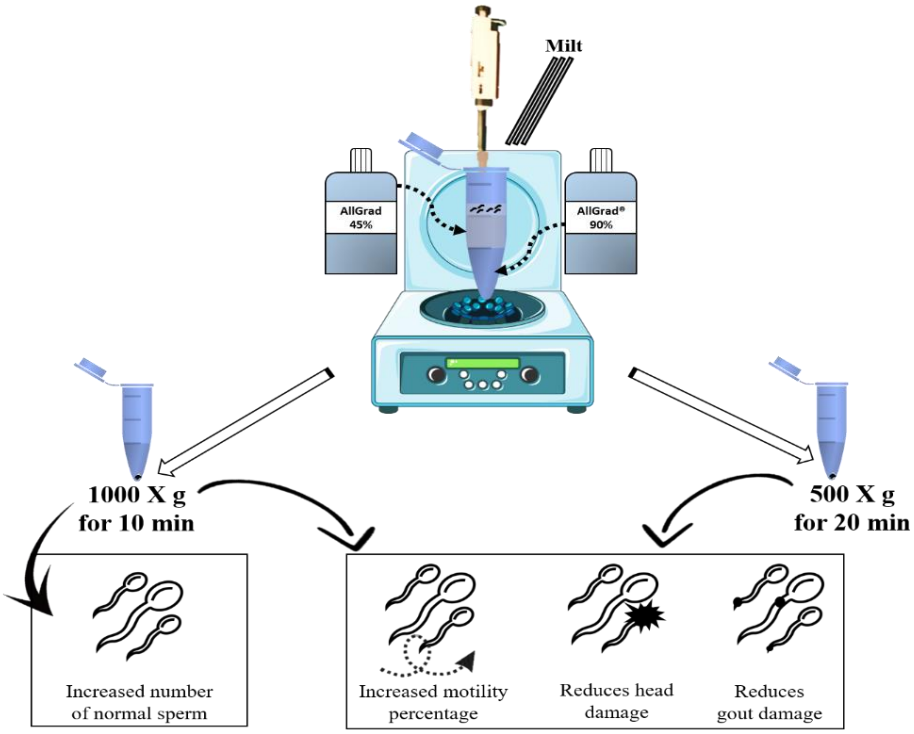

Figure 1. Abstract graphic of the standardization of the density gradient centrifugation method.

1. Method details

1.1. Background

Cryopreservation allows the maintenance of viable biological material for indefinite time under temperatures like -80 °C or -196 °C [3,26]. The cryopreservation of fish sperm is a useful tool for both the preservation of species and storage of valuable genotypes for animal research and production [40,41]. To reach success in the cryopreservation techniques it is necessary to use substances named cryoprotectants.

The cryoprotectants are substances that promote cellular dehydration (deplete up to 95% of the water in the cell) during the cryopreservation process, stabilizing membranes and proteins [16,25]. The dehydration avoid or reduce the formation of ice crystals [33], and depending on the molecular weight of the cryoprotective solution used, it may or may not penetrate the cell membrane [6,7]. At ideal concentration or exposure

time, cryoprotectant solutions are less toxic when at low temperature than at room temperature [16]. In thawing, the sample goes from -196°C to 30°C in a few minutes, that is the moment where the greatest number of injuries can happen [27]. Damage occurs, probably due to the cytotoxic activity of the cryoprotectant, mainly at higher temperatures. Therefore, the use of techniques that allow reducing the concentration of cryoprotectants after thawing of the milt samples would be a possible solution to reduce these cytotoxic effects, which can promote injuries on cryopreserved spermatozoa diminishing the sample quality.

The density gradient centrifugation technique has been used for auxiliary tool, in assisted reproduction in mammals, such as humans [14], bovines [12], and lastly in fish [29]. In fish, the use of this technique has a relatively new approach, having already been used in some species such as *Cyprinus carpio* [19], *Acipenser ruthenus* [15], *Salmo salar* [5], allowing a proportion of sperm with greater motility, speed, membrane integrity and fertilization capacity. For the shaping of gradients, silica-based solutions are used, which can be coated with polyvinylpyrrolidone (PVP), or with silane [2]. However, the use of silane-coated silica separation gradients has begun to replace PVP-coated silica [2,14] for use in humans cells [24], and some species-specific formulations for animals, for example, BoviPure™ for bull, EquiPure™ for stallion and PorciPure™ for sperm boar [22]. The silane-coated silica present low levels of endotoxicity and the ability to remain stable for long periods in saline solutions, allowing the use of ready-to-use commercial formulations [23]. However, quality parameters, such as sperm viability and morphology, can be affected by centrifugation force and time that have not yet been compared [21]. Therefore, this study aims to describe a method that allows identifying the best centrifugation time and force, for the separation of the best portion of sperm from cryopreserved milt, based on the AllGrad® 90% gradient. The *Rhamdia quelen* species was used as an animal model, being the first time, it is used for this purpose. The adaptations were made from other studies performed to separate male reproductive cells from fish (Supplementary Table 1).

1.2. Required reagents and equipment

- Fish cryopreserved milt. To the experiment, of the catfish (*R. quelen*).

- AllGrad® 90% (Catalogued Numbers: AG90-050, AG90-100, LifeGlobal Group, Europe).
- Carp pituitary extract (CPE)
- Distilled water.
- Cryopreservation solution (50 g/L fructose [Sigma- Aldrich®], 50 g/L powdered milk [Molico®-Nestlé] and 100 mL/L methanol [Sigma- Aldrich®])
- Saline-buffered formaldehyde (10%).
- Ginsburg extender solution (123.2 mM NaCl, 3.75 mM KCl, 3.0 mM CaCl2, 2.65 mM $NaHCO_3$ -300mOsm, pH 7.5).
- Eosin dye solution (3%).
- Nigrosin dye solution (5%).
- Rose Bengal dye solution.
- Neubauer chamber.
- Straws (0.25 mL - Minitube®).
- Micropipettes P10 µL, P100 µL, P200 µL and P1000 µL.
- Eppendorf microcentrifuge tubes of 1.5 mL and 2.0 mL.
- Falcon tube 15 mL.
- Refrigerated centrifuge. For standardizing this method, the Eppendorf Centrifuge 5403 was used.
- Microscope slides.
- Light microscope.

1.3. Animals

All animals were handled in accordance with the regulations of the Ethics Committee in the Use of Animals (CEUA) of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, with approval protocol No. 38722. The animals, from the Nossa Senhora Aparecida Fish Farm (Rodovia RS 155, S/N Km 6, Ijuí - RS, 98700-000), were acclimated for one month in 500 L plastic tanks, in a recirculation system at AQUAM Laboratory from the same University. A commercial ration was provided, until apparent satiety, twice a day.

1.4. Milt samples

The samples were obtained from seven adult males of *R. quelen*. The males were randomly divided into two groups and the milt was collected at different times. A pool was forming from each group. The mean ± SD weights of the animals in each group were 702 ± 171 g (group [i], n= 3) and 351 ± 110 g (group [ii], n= 4).

1.5. Milt collection and cryopreservation

Each male was induced intramuscularly with a single dose of CPE 3 mg/kg body weight [1]. Milt collection was carried out after completing 240 degree-hours (10 h; water at 24 ºC) [4,39]. For this, the urogenital papilla was dried, followed by a gentle abdominal massage (cephalo-caudal direction). The first portion of the milt was discarded to avoid possible contamination with urine, feces, or blood [41]. The collection was performed in a 15 mL Falcon conical tube. The milt samples were kept at 4 ºC until the total of males was collected and previous evaluations were carried out after freezing.

Before forming the milt pool, the samples were evaluated under an optical microscope to certify the absence of sperm motility and the ability to start motility when exposed to distilled water. The motility evaluation was adapted from Fauvel et al., (2010). For sperm activation, an aliquot of 1 µL of milt was diluted at 5 mL of distilled water and the chronometer was immediately started. The motility rate (0 – 100%) was evaluated between 10 to 15 s after activation [11]. The assessment of motility and sperm concentration in each pool was performed with a microscope (Nikon Eclipse E200, Tokyo, Japan), 40 X objective.

The sperm concentration was analyzed with a Neubauer chamber, with previous fixation of the sperm in saline-buffered formaldehyde (10%), in a ratio (v/v) 1: 999 µL [4,31,34]. The rate and mean motility time of the fresh milt pool in group (i) was 80 ± 5% and 40 ± 3 s. Regarding group (ii), the results were 68.33 ± 7.64% and 48.33 ± 9.07 s, respectively. The concentration for each group (number of sperm [cells/mL]) was $4.20x10^{11} \pm 6.41x10^{10}$ and $1.81x10^{11} \pm 8.41x10^{9}$, group (i) and (ii), respectively.

The cryopreservation of the pools was performed by diluting the milt in the cryopreservation solution (1:3 ratio), in distilled water. The dilution was placed on 0.25 mL straws (Minitube®) [8,35]. The straws were conditioned in a canister and kept in liquid

nitrogen steam for 18 h (dry-shipper CP300, -170ºC), and finally stored in liquid nitrogen (-196 ºC), for a minimum period of seven days, until thawing (25 ºC for 10 s) [35].

Before thawing, a random distribution of each group was carried out for each of the phases involved in standardization. For this reason, the pool corresponding to group (ii) was used in the first phase and group (i) was used in the second phase. Both experimental phases are detailed in section 1.6.

1.6. Standardization of the density gradient centrifugation procedure

The standardization process of the centrifugation methodology by density gradients with AllGrad® 90% was divided into two validation experiments (Fig. 2). (i) Standardization of centrifugation forces and times, as well as the volume of the layers formed by the gradients, and (ii) method validation through sperm quality measurement after centrifugation at different speeds and times (that were selected in the first experiment).

In short, each validation experiment consisted of two procedures: (i) Cryopreservation of milt (section 1.5 of this document), (i) milt thawing and centrifugation using density gradient AllGrad® 90%, and (iii) evaluation of sperm quality parameters. For the first experiment, only the sperm concentration and pellet formation were evaluated, and in the second experiment, in addition to sperm concentration, sperm motility and morphology were measurement.

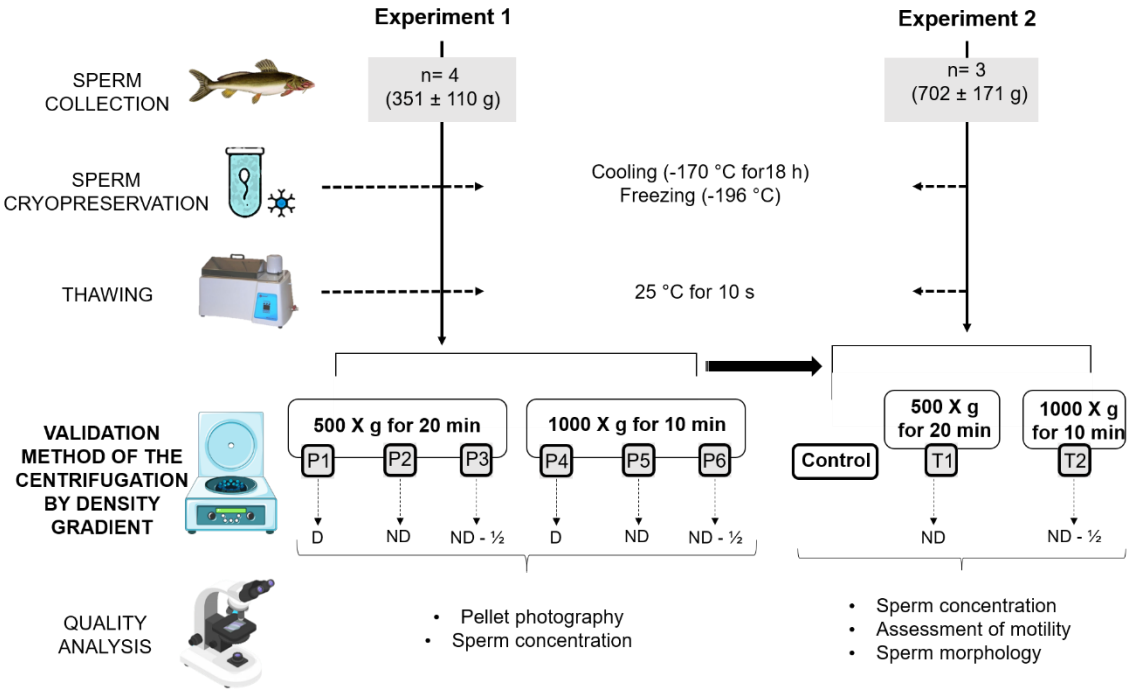

Figure 2. Validation method of the density gradient centrifugation with AllGrad® 90% for criopreservation sperm of catfish (*R. quelen*). **D:** dilution sperm. **ND:** no dilution sperm. **ND-½:** no dilution sperm with half the volume of the AllGrad layers. **Control:** milt samples that after thawing were not centrifuged treatment applied in experiment two to compare the quality of the sperm with the treatments where the milt was centrifuged (T1 and T2).

1.6.1. Standardization of centrifugation forces and times

The purpose was to determine the treatments (**Table 1**) that allow a well-formed pellet and to determine if there is any statistical difference between them when comparing the sperm concentration of each pellet. The treatments were determined and adapted as described below. In short, the centrifugations were performed at 4 °C [9, 10, 15, 18, 20, 32], and density gradients of 90% and 45% were used [19].

At the end of the AllGrad gradient column a layer of thawed milt (100 µL) was gently placed. The milt dilution, considered in some treatments (P2 and P5), is based on the fourth principle of centrifugation [28]. This indicates that the sedimentation rate increases when the viscosity of a solution decreases. This process was thought-out to obtain a larger pellet or with a difference in sperm concentration in relation to the other treatments. The step-by-step method is described in sections 1.6.1.1 and 1.6.1.2 and is schematized in Fig. 3.

Table 1. Tratments of the standardization of centrifugation forces and times by density gradients with AllGrad® 90% of cryopreserved fish sperm of the catfish *R. quelen*.

Treatment	Centrifugal Forces (X g)	Time centrifugation (min)	AllGrad layer 90% (µL)	AllGrad layer 45% (µL)	Milt layer (µL)	Milt dilution
P1	500	20	200	200	100	ND
P2	500	20	200	200	100	D
P3	500	20	100	100	100	ND
P4	1000	10	200	200	100	ND
P5	1000	10	200	200	100	D
P6	1000	10	100	100	100	ND

Dilution (D) or no dilution (ND) of the sperm in the Ginsburg solution.

1.6.1.1. Thawing and dilution of milt samples

1. Prior to conformation of density gradients and centrifugation, thawing of milt samples was performed. The straws were taken from liquid nitrogen (-196 °C) and placed in a water bath at 25 °C for 10 min.
2. Samples that did not need to be diluted were gently placed on the top of the gradient immediately after thawing. In the case of diluted samples, in a 2 mL Eppendorf, three parts of thawed milt were diluted in four parts of Ginsburg solution, using a 200 µL pipette, and an aliquot was placed at the top of the gradient. In both cases, a milt volume of 100 µL was used.

1.6.1.2. Preparation of density gradients

3. The AllGrad 45% solution was built from the commercial AllGrad® 90% stock solution. For this purpose, the AllGrad® 90% was diluted in Ginsburg extender solution, in a ratio of 1:1 (v:v).
4. From AllGrad solutions (45% and 90%), the gradient columns were formed in a 1.5 mL Eppendorf tube. The layers of AllGrad 90% and 45% were placed, respectively, and at the top of the gradient layers, the thawed milt sample was gently applied. To place all layers within the gradient, a 200 µL micropipette and tip was used.

However, for the last two layers (AllGrad 45% and milt sample), a 10 µL tip was adapted to the 200 µL tip, allowing it to be poured more smoothly to avoid mixing.

5. After shaping the gradients, the samples were centrifuged, at 4 °C, in a refrigerated centrifuge (Eppendorf Centrifugue 5403), following the centrifugation forces and times described for each treatment. The force (X g) was calculated from the following equation:

$$\boldsymbol{RCF\ (X\ g)} = 1{,}12\ x\ r(mm)\ x\ \left(\frac{RPM}{1000}\right)^2$$

Where, **RCF:** relative centrifugal force or g force (X g), **1.1**2: constant value, **r:** radius in millimeters, **RPM:** revolutions per minute and **1000:** constant value.

6. Immediately after centrifugation, each conformed pellet was photographed which allowed the analysis of pellet formation and subsequent classification (**Supplementary Table** 2). This classification was performed using a score that was determined, subjectively, for the purposes of this standardization (**Supplementary Table** 3).
7. The supernatant from each centrifuged sample was discarded using a 1000 µL pipette with a 10 µL tip adapter, to keep the pellet intact.
8. Then, each pellet was resuspended in 200 µL of Ginsburg extender solution.
9. Resuspension was again centrifuged at 500 X g for 5 min [32].
10. The supernatant was removed again following the same procedure explained in number seven, and then resuspended in 150 µL of Ginsburg solution. This final sample was used to perform the different quality analyzes required. The samples were kept at 4 °C until the completion of the centrifugation of all samples, and quality analysis.

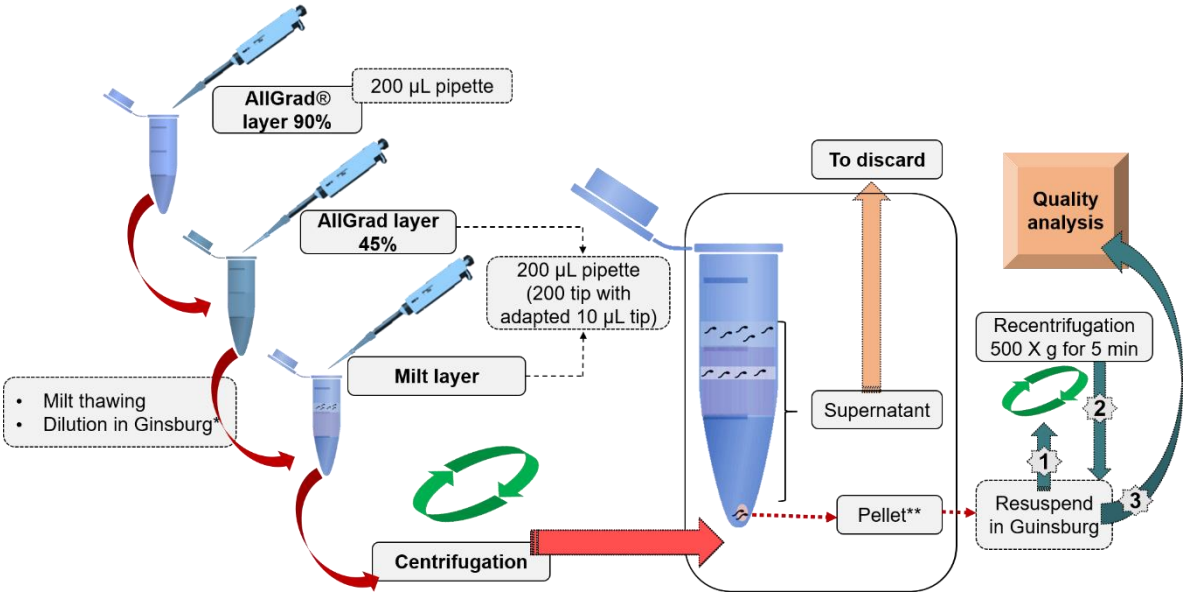

Figure 3. Scheme of the density gradient centrifugation protocol from AllGrad® 90% with cryopreserved milt of the catfish *R. quelen*. *Milt dilution (three parts of thawed milt were diluted in four parts of Ginsburg solution, in a 2 mL Eppendorf, using a 200 µL pipette) was only for cases where the procedure was necessary. **For all treatments, the pellet was resuspended in the Ginsburg extender solution, centrifuged again at 500 X g for 5 min. The final pellet was again resuspended in the Ginsburg solution and used to carry out the different quality analyzes.

2. Method validation

2.1. Results of standardization of centrifugation forces and times

Statistically only P1 and P6 (T1 and T2, respectively, for the second method validation experiment) were different from P4 (lower sperm concentration, Fig. 4), they were chosen because they presented a better pellet conformation score (3 – Fig. 5).

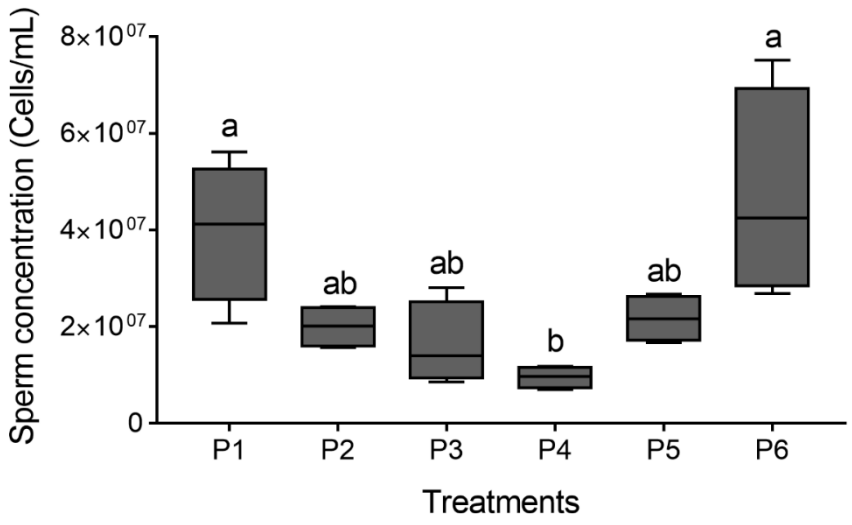

Figure 4. Sperm concentration after application of treatments (P1-P3: 500 X g for 20 min and P4-P6: 1000 X g for 10 min. Details of treatments in Table 1) in catfish (*R. quelen*) cryopreserved milt. Kruskal-Wallis analysis followed by Dunn's test (p = 0.0051). Different letters indicate difference between treatments.

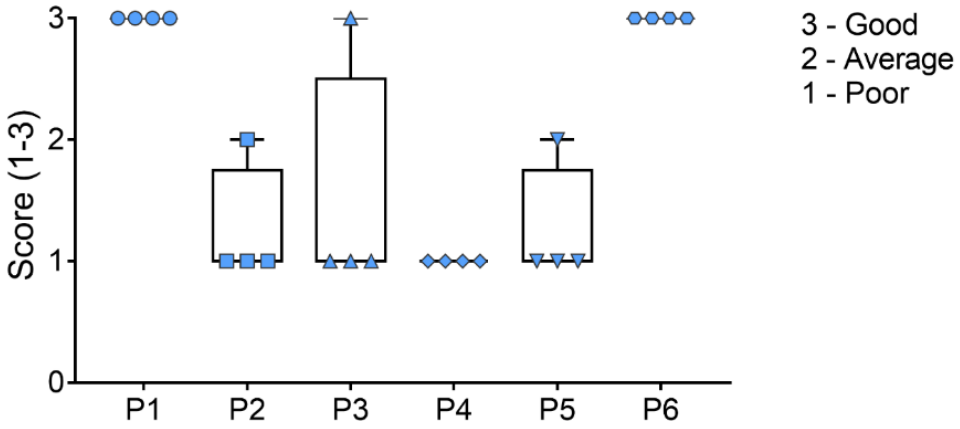

Figure 5. Score for pellet formation for each treatment applied in the standardization of the centrifugation method by density gradient of catfish (*R. quelen*) milt with AllGrad® 90%. P1-P3: 500 X g for 20 min and P4-P6: 1000 X g for 10 min. Details of treatments in Table 1. The blue symbols represent the score of the treatments in each repetition, with four repetitions performed.

2.2. Method validation through sperm quality measurement

The treatments for the second experimental phase of the method validation were defined in **Table** 2. A control treatment was considered (milt samples that after thawing

were not centrifuged) to be able to make the comparisons. For all treatments, in addition to sperm concentration, the following quality assessments were also performed: motility and sperm morphology. For theses analysis, adaptations were made for the species under study, from test/error, until reaching the most appropriate proportions for each quality assessment (**Supplementary Table 4)** due to the different forces and centrifugation times applied. On the other hand, it is important to clarify that due to the difference that exists between the species of fish and even within the same species, it will most likely always be necessary to make these adjustments.

Table 2. Method validation treatments of the centrifugation by density gradients with AllGrad® 90% of cryopreserved fish sperm of the catfish *R. quelen.*

Treatment	Centrifugal Forces (X g)	Time centrifugation (min)	AllGrad layer 90% (µL)	AllGrad layer 45% (µL)	Milt layer (µL)
Control	Milt samples thawed and not centrifuged				
T1	500	20	200	200	100
T2	1000	10	100	100	100

2.2.1. Sperm concentration

Sperm concentration was performed with the help of the Neubauer chamber, as previously described. In this case, it is evident the decrease in the sperm concentration of the treatments that were centrifuged in comparison to the control treatment (8.30×10^9 cells/mL).

2.2.2. Sperm motility

Motility was assessed using the semi-quantitative method with adaptations [30]. Immediately after thawing, videos of each treatment were taken using the camera of a cell phone (Iphone 8, apple) fitted in one of the microscope eyepieces and with a 40 X objective. The aliquots to be evaluated were placed in the Neubauer chamber. In total 12 videos were made per treatment. The videos were evaluated on the computer screen, by a technician with intermediate experience [13]. All videos were randomly numbered before being sent to the evaluator. The quantified variables were motility rate (0% - 100%) and motility time (s).

2.3. Sperm morphology

The evaluation of sperm morphology was performed using milt previously fixed in buffered formaldehyde solution (10%). An aliquot of 100 µL of fixed milt (same sample used in the sperm concentration) was mixed with 10 µL of the Rose Bengal dye [36]. The adapted methodology includes conformation of the slides per drained drop, placing three drops of 10 µL, each, on a histological slide. The slides were read with the 100 X objective, using immersion oil. The morphology evaluated were [37]: Normal spermatozoa, short tail, distally curled tail, strongly curled tail, broken tail, folded tail, degenerate head, macrocephaly, microcephaly, loose head, proximal gout, distal gout. In total, six repetitions were performed per treatment, counting 200 sperm per slide, in duplicate.

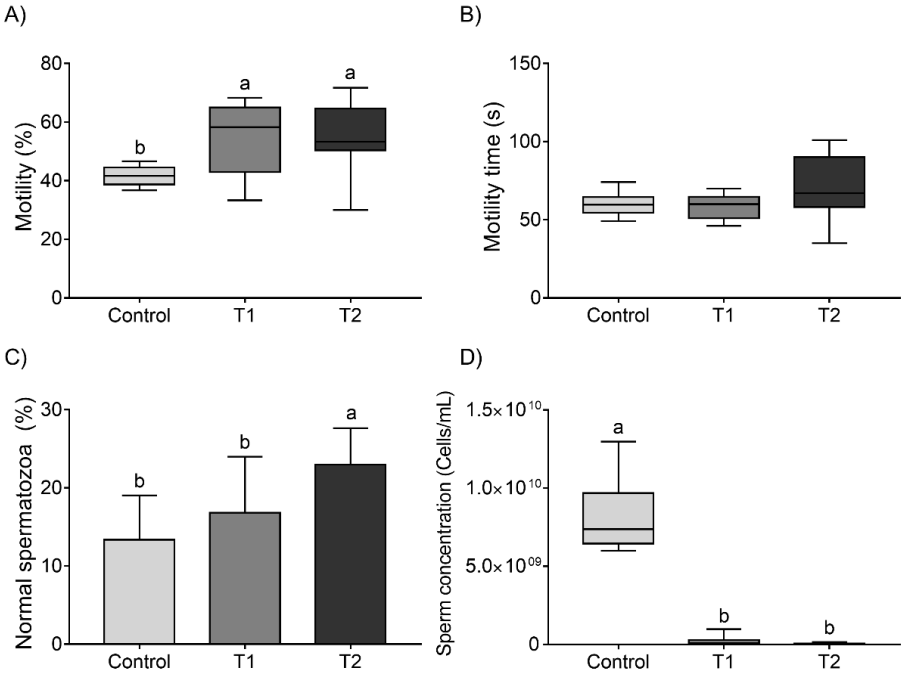

Figure 6. Sperm quality of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and centrifuged milt at 500 X g for 20 min) and T2 (thawed and centrifuged milt at 1000 X g for 10 min) treatments were applied. More details of treatments in Table 2. A) Motility ($p = 0.0020$); B) Motility time ($p = 0.1554$); C) Spermatozoa with normal morphology ($p = 0.0013$); D) Sperm concentration ($p < 0.0001$). Kruskal-Wallis analysis followed by Dunn's test (Figures A, B and D). Analysis of variance followed by Tukey's test (Figure C). Different letters indicate difference between treatments.

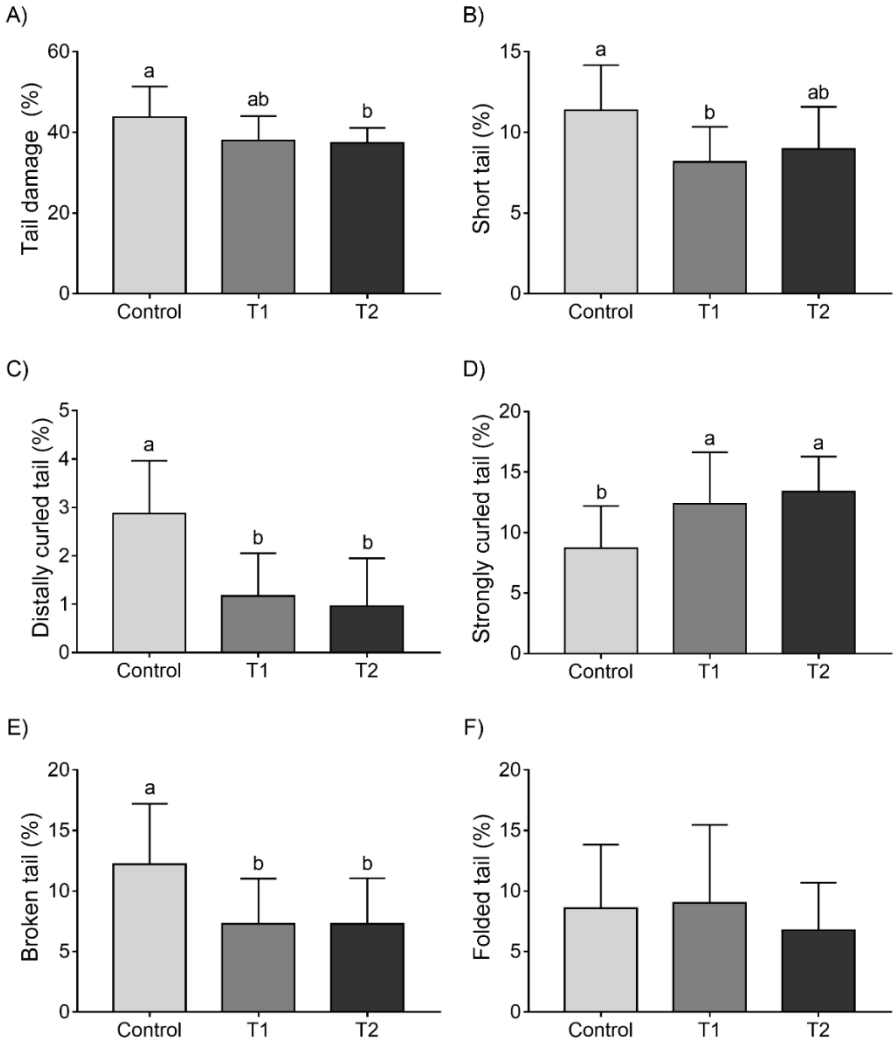

Figure 7. Tail morphological abnormalities of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and centrifuged milt at 500 X g for 20 min) and T2 (thawed and centrifuged milt at 1000 X g for 10 min) treatments were applied. More details of treatments in Table 2. A) Total tail pathologies ($p = 0.0253$); B) Short tail ($p = 0.0110$); C) Tail curled distally ($p < 0.0001$); D) Tail strongly curled ($p = 0.0080$); E) Broken tail ($p = 0.0091$); F) Tail folded ($p = 0.6061$). Analysis of variance followed by Tukey's test. Different letters indicate difference between treatments.

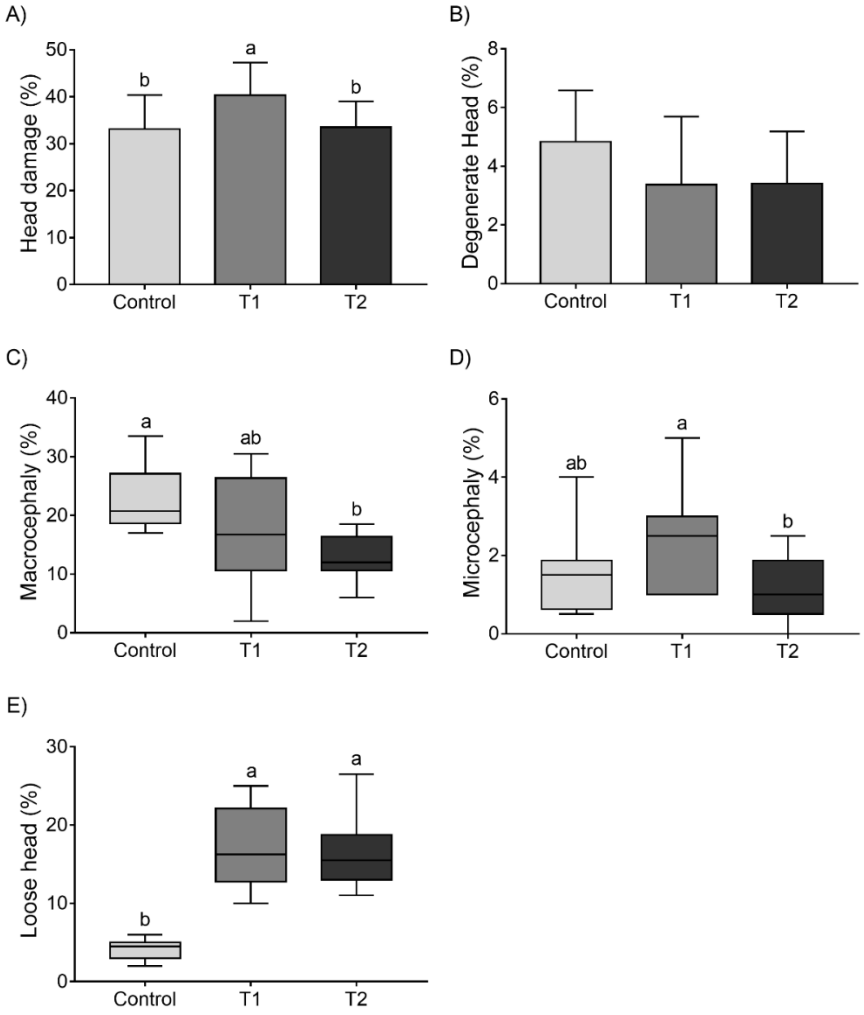

Figure 8. Head morphological abnormalities of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and centrifuged milt at 500 X g for 20 min) and T2 (thawed and centrifuged milt at 1000 X g for 10 min) treatments were applied. More details of treatments in Table 2. A) Total head diseases ($p = 0.0191$); B) Degenerate head ($p = 0.1329$); C) Macrocephaly ($p = 0.0011$); D) Microcephaly ($p = 0.0426$); E) Head loose ($p < 0.0001$). Analysis of variance followed by Tukey's test (Figures A and B). Kruskal-Wallis analysis followed by Dunn's test (Figures C, D and E). Different letters indicate difference between treatments.

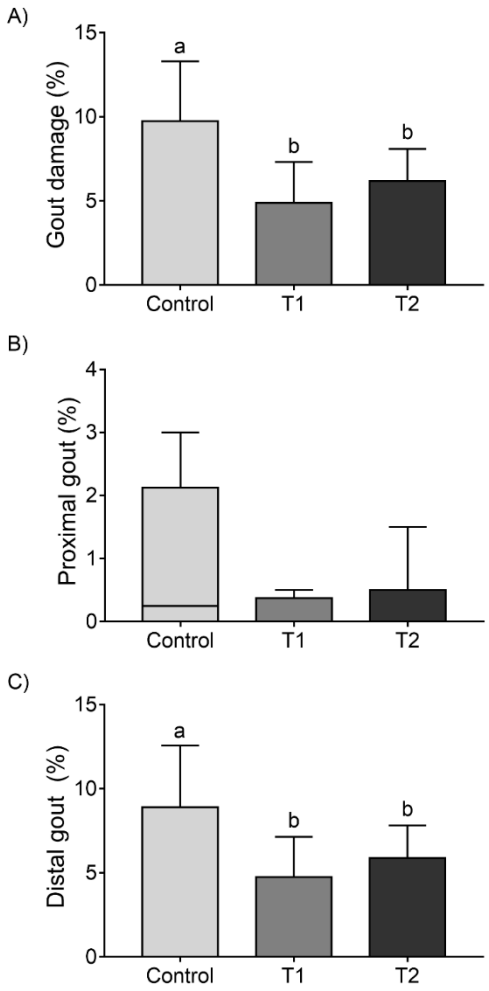

Figure 9. Gout morphological abnormalities of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and centrifuged milt at 500 X g for 20 min) and T2 (thawed and centrifuged milt at 1000 X g for 10 min) treatments were applied. More details of treatments in Table 2. A) Total cytoplasmic gout pathologies (p = 0.0003); B) Proximal drop (p = 0.2397); C) Distal drop (p = 0.0023). Analysis of variance followed by Tukey's test (Figures A and C). Kruskal-Wallis analysis followed by Dunn's test (Figure B). Different letters indicate difference between treatments.

The centrifuged milt represented a high percentage of motility compared to the control treatment (Fig. 6A). Density gradient centrifugation with AllGrad revealed the notable reduction in sperm concentration after application of the method (Fig. 6D). The presence of normal cells was better when the thawed milt was centrifuged at a speed of 1000 X g for 10 min, 4 °C (Fig. 6C), 22.25 ± 4.64%, which is equivalent to 8.19 x 10^6 normal spermatozoa, this is 9.25% more than the milt that was not centrifuged. The presence of tail morphological abnormalities was lower in the centrifugation of 1000 X g

for 10 min compared to the control treatment (Fig. 7A). In addition, head damage (Fig. 8A) and the presence of gout damage (Fig. 9A) were also significantly lower in separated milt whit AllGrad. The importance of this result is that the good performance of the spermatozoa is in the integrity of its structures (head, intermediate piece and tail) [39]. The presence of morphological damage will lead to the reduction of parameters of quality such as motility or capacity sperm fertilizer [40].

3. Conclusion

The centrifugation method with AllGrad density gradients (90% - 45%, volume of each layer: 100 uL), at a speed of 1000 X g for 10 min, 4 °C, and with recentrifugation of 500 X g for 5 min, 4 °C, it proved to be a promising technique for obtaining a portion of better-quality sperm from cryopreserved milt of the *R. quelen* species. This method allowed better sperm characteristics, specifically, a better percentage of normal spermatozoa, and an important characteristic in fertilization processes. This method can be easily adapted and applied to work with cryopreserved milt of the same species and even for other species of teleost fish, allowing to selection and separate out a larger number of normal sperm.

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Supplementary information

Supplementary table 1. Studies where the centrifugation technique was used, with or without density gradients, in fish, to separate reproductive cells or seminal plasma.

Authors	Species	Cell type	Density gradient	Protocol				Observation*
				Gradient concentration	Container	Centrifugation (Force/Time)	°C	
Majhi et al., (2009) [12]	*Odontesthes bonariensis*	Germ cell	Percoll	20%, 25% e 12%	-	200 X g for 20 min	20	-
Yoshikawa et al., (2009) [16]	*Carassius auratus*	Dissociation of testicular tissue cells	Percoll	65%, 40%, 36%, 30%	-	800 X g for 30 min	-	-
Lacerda et al., (2006) [7]	*Oreochromis niloticus*	Germ cells	Percoll	-	-	800 X g for 30 min	25	
Lacerda et al., (2010) [8]	*Oreochromis niloticus*	Germ cells	Percoll	-	-	200 X g for 10 min		Pellet resuspended in Dulbecco Modified Eagle medium/Ham F-12 (DMEM/F12)
Li et al., (2010) [10]	*Cyprinus carpio*	Spermatozoas	Percoll	90% e 45%	Eppendorf tube (1.5 mL)	300 X g for 10 min	-	-
Lahnsteiner & Mansour, (2010) [9]	*Lota lota, Perca fluviatili, Alburnus alburnos & Salmo trutta*	Separate seminal fluid and spermatozoa	-	-		300 X g for 10 min e 1000 X g for 10 min	4	Spermatozoa were diluted in sperm motility-inhibiting saline solution (SMIS), centrifuged a second time to remove remnants of seminal fluid
Bellaiche et al., (2014) [1]	*Oncorhynchus mykiss*	Germ cells	Percoll	90%	-	500 X g for 40 min and 500 X g for 5 min	-	The cell pellets were resuspended in L-15 medium with 1% bovine serum albumin

								(BSA). Recentrifugação at 5 min at 50 X g to remove cell clusters, erythrocytes, and most spermatozoa, if present
Dzyuba et al., (2014) [3,4]	*Acipenser ruthenus*	Sperm centrifuga-tion for obter seminal fluid	-	-	-	300 X g for 10 min	4	Supernatants obtained was recentrifuged at 5000 X g for 15 min to obtain seminal fluid
Linhartová et al., (2014) [11]	*Tinca tinca*	Germ cells	Percoll	5% e 30%	-	500 X g for 30 min	4	Pellets with testicular cells were transferred into 1.5 mL Eppendorf tubes, diluted with PBS to 0.5 mL, and kept on ice at 4 °C for analysis
Majhi et al., (2014) [13]	*Odontesthes bonariensis*	Germ cells	Percoll	20%, 25% e 12%	-	200 X g for 20 min	20	-
Pšenička et al., (2015) [14]	*Acipenser ruthenus*	Germ cells	Percoll	50%, 40%, 30%, 20%, 10% e 5%	-	800 X g for 30 min	20	Each cell fraction was removed from the gradient and transferred to a tube, diluted in PBS 1:10, and centrifuged again at 800 X g for 30 min. The pellets were resuspended in PBS
Horokhovatskyi et al., (2018) [6]	*Acipenser ruthenus*	Spermatozoas	Percoll	90% e 45%	15 mL plastic tube	2000 X g for 20 min	4	The resulting pellets were washed at a 1:10 ratio with artificial seminal fluid and centrifuged at 3000 X g for 10 min, 4 ˚C, and then resuspended

Shang et al., (2018) [15]	*Ictalurus furcatus*	Germ cells	Percoll	35%, 45% e 70%	15 mL centrifuge tube	800 X g for 40 min	4	After gradient centrifugation, cells from the same band from the same replicate of fish were pooled together into a 50 mL centrifuge tube and washed with two volumes of the cell suspension with HBSS. Centrifugation was carried out at 500 X g for 5 min. The cell pellets were resuspended in 2 mL HBSS
Franěk et al., (2019) [5]	*Cyprinus carpio*	Germ cells	-	-	-	200 X g for 10 min	-	The supernatant was removed and the pellet was resuspended by a gentle pipetting with addition of an appropriate volume of L-15 medium
De Siqueira-Silva et al., (2018) [2]	*Brycon orbignyanus*	Germ cells	-	40%, 30%, 25% e 10%	15 mL conic tubes (Falcon)	800 X g for 30 min	25	-

* The observation column corresponds to the cases where the pellet resuspension process or recentrifugation was specified. (-) Unspecified information.

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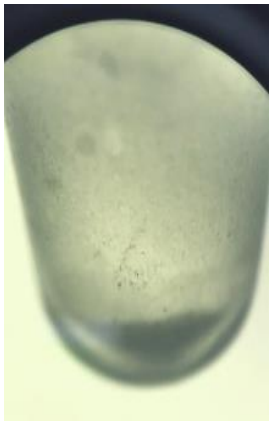
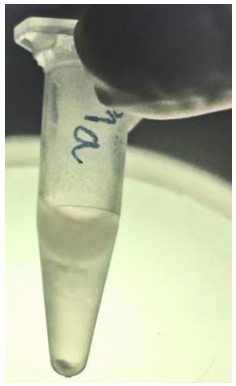
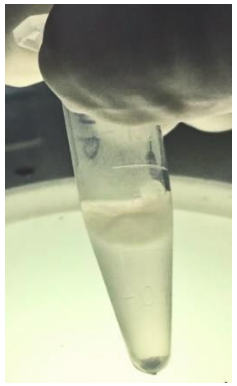
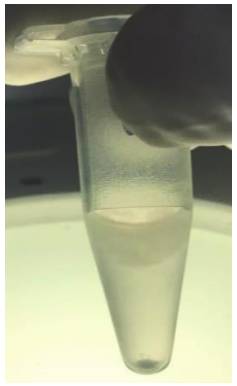
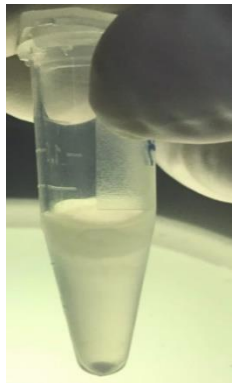
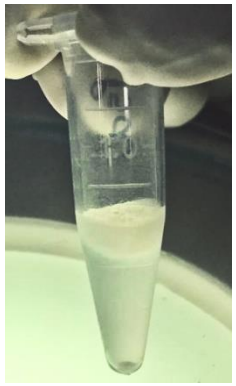
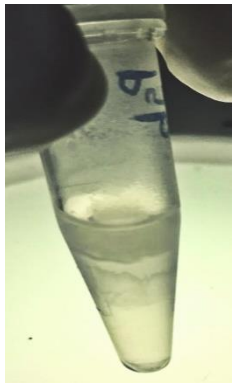
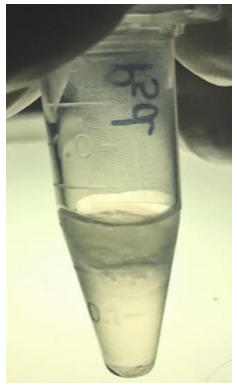
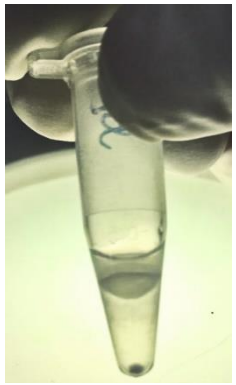
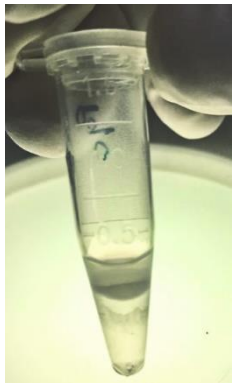
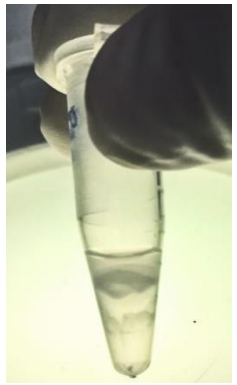
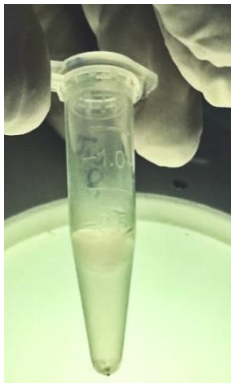
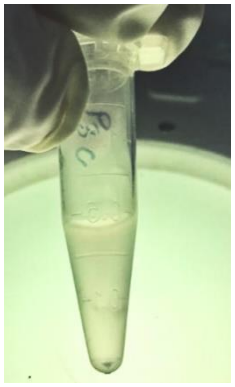
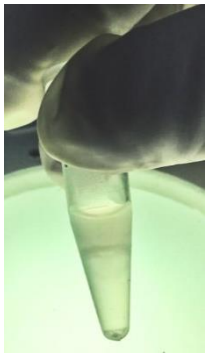
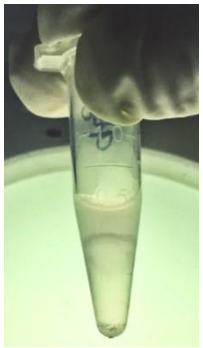
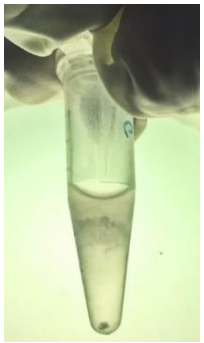
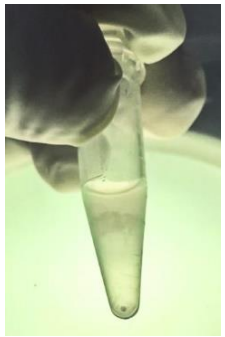
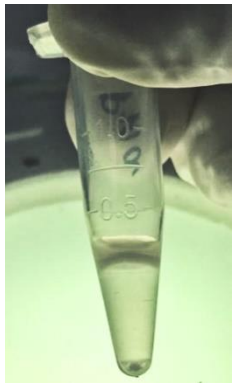
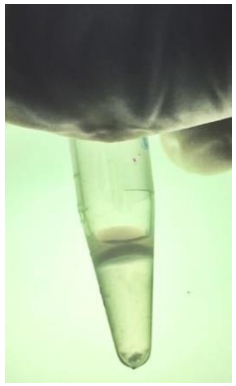
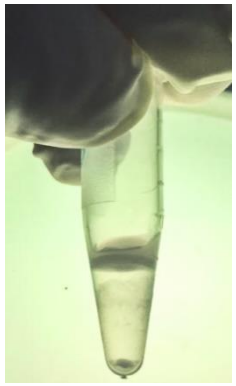
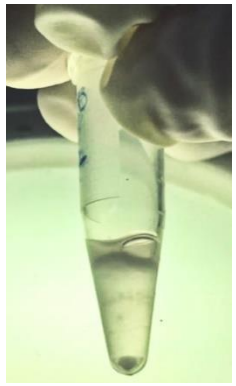
Supplementary table 2. Capture images after the application of the different treatments, applied to adapt the centrifugation method with density gradients with AllGrad® 90% with cryopreserved semen from Catfish (*Rhamdia quelen*)

Treatments	Treatment repetitions				Pellet	Score
P1	P1a	P1b	P1c	P1d		3
						
P2	P2a	P2b	P2c	P2d		1
						
P3	P3a	P3b	P3c	P3d		1

						
P4	**P4a**	**P4b**	**P4c**	**P4d**		1
						
P5	**P5a**	**P5b**	**P5c**	**P5d**		1

						
P6	**P6a** 	**P6b** 	**P6c** 	**P6d** 		3

Lowercase letters (a, b, c, d) indicate the repetitions made for each of the treatments applied.

Supplementary table 3. Score defined to categorize the pellet formation in each of the repetitions within the treatments.

Score	Definition	Observation
3	Good	Good pellet conformation and no difficulty in visualization for each repetition.
2	Average	Average conformation of the pellet, smaller than the previous size and with easy visualization of the pellet in at least three of the repetitions.
1	Poor	Poor conformation of the pellet, very small and more difficult to visualize the pellet in two or more repetitions.

Supplementary table 4. Proportions for each quality assessment

Treatment	Milt concentration (v:v - µL)	Motility (v:v - µL)	Eosin-Negrosin (v:v - µL)	Morphologies (v:v - µL)
Control	1:999	1:1000 (a)	5:30	100:10
P-T	50:150	1:1 (b)	30:20	100:10

Control: milt samples thawed without being subjected to density gradient centrifugation. P-T: Treatments applied in the two stages of standardization of the centrifugation method with density gradient, AllGrad® 90%, starting from cryopreserved milt *R. quelen*. In the v:v (µL) ratio, the first volume is indicated for milt samples. (a) The milt was initially diluted in a Falcon tube 15 mL in distilled water and a 1 µL aliquot was placed in the Neubauer chamber, and the motility parameters were quantified. (b) 1 µL of milt was placed directly in the Neubauer chamber, followed by 1 µL of distilled water, to activate motility and make a video for later quantification of motility parameters. The motility time was counted from the milt activation, starting the chronometer until the total motility stop.

CAPITULO III[1]

AllGrad® 90% separation of cryopreserved South American catfish (*Rhamdia quelen*) spermatozoa, effect on sperm function and fertilizing capacity

[1] Artigo elaborado conforme as normas do periódico *PLoS One* (https://journals.plos.org/plosone/)

AllGrad® 90% separation of cryopreserved South American catfish (*Rhamdia quelen*) spermatozoa, effect on sperm function and fertilizing capacity

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Abstract

Cryopreservation provides several benefits, such as long-term storage of sperm cells. Nevertheless, the lack of standardization or the low quality of sperm after freeze-thaw, are challenges that still must be faced. The use of technologies in assisted reproduction, such as the use of centrifugation, has been reported as a tool that allows separating the best portion of sperm from cryopreserved semen. The aim of this work was to validate and evaluate the efficiency of the density gradient centrifugation technique, on sperm functionality and fertilization capacity. Three treatments were compared: control, differential centrifugation or pelletization (T1), and density gradient centrifugation (T2). Motility percentage was better for T2 (mean ± standard deviation [± SD], 44 ± 6.05%) than for the control treatment (32.21 ± 6.033%). Membrane integrity was not different between treatments. The presence of normal sperm was higher in T2 (13.55 ± 5.82%) when compared to the control (10.72 ± 3.53%) and T1 (3.97 ± 1.95%). In the MTT test (AU/100 million sptz), the centrifuged treatments resulted in values of 1.58 ± 0.79 (T1) and 2.02 ± 1.88 (T2), better than the treatment control (0.20 ± 0.08). Likewise, the fertilization and hatching rates, respectly, were better for the T1 (76.68 ± 15.22% and 54.62 ± 17.39%) and T2 (69.85 ± 17.85% and 50.87 ± 20.59%) treatments, than the control treatment (40.03 ± 11.67% and 24.14 ± 13.97%). These methodologies were proposed for the first time for the *Rhamdia quelen* species, making it possible to show the efficiency of the centrifugation technique to obtain the best portion of sperm from cryopreserved milt.

Keywords: fertilization, hatch, fish sperm quality, fish sperm morphology, fish milt centrifugation, teleost

Introduction

The world production of fish was estimated at 179 million tonnes in 2018 with a per capita consumption of 20.5 kg, and growth of 13% is expected for the year 2030. The Americas are in third place, representing a 14% of the world production [1]. Brazilian fish farming reached 802,930 tons in 2020, which represents an increase of 5.93% in relation to 2019, being 31.1% represented by the South region [2]. In this Region, the neotropical species, *Rhamdia quelen*, has acquired great economic importance due to its resistance to handling, accelerated growth, even at low temperatures, good feeding efficiency and, above all, for presenting a tasty meat, without intramuscular spines and with acceptance consumer market [3]. In addition, it is a species that responds positively to hormonal induction [4], which allows its production in captivity [5]. Even so, according to CONCEA (2019), it is a species that also has importance as an experimental model [6], for example, for the development of new pharmacological approaches [7]. Thus, the use of techniques of artificial propagation or biotechnologies of reproduction, such as cryopreservation, is fundamental when it is desired to use programs of reproduction to contribute to the production and survival of offspring in fish [8,9].

Cryopreservation of fish gametes is considered tool for artificial reproduction and genetic improvement of aquaculture species. This biotechnology involve solutions both for the maintenance of fish species used as an animal model [10], and for preservation programs for the endangered species [11], to implement at the industry level [12]. Cryopreservation provides several benefits, such as synchronizing the availability of male and female gametes, long-term storage of cells without aging, convenient transport of genetic material, and reducing the number of broodstock required in hatchery facilities and living gene banks [13]. Nevertheless, in the cryopreservation of fish milt, there are still challenges that must be faced, such as the lack of standardization of the existing cryopreservation protocols [8,14], or the low quality of sperm after freeze-thaw [15]. The sperm quality is considered not only as the capacity of this cell to reach an oocyte and fertilize it, but also to contribute to the successful initial embryonic development [16].

Assisted reproductive technologies have been a great ally in obtaining quality sperm in different species [17]. Among these, the selection techniques, for example, swin-up or centrifugation by density gradient [18,19], can be mentioned. Centrifugation by density gradient has shown efficient results in the search for specific characteristics in semen (sperm sexing, sperm separation, pathogen cleaning, seminal/cryoprotectant plasma, sperm quality, among others), in different species (humans, sheep, cattle, horses, dogs) [20]. In humans, for example, this technique has allowed sperm to be separated according to their density or motility, in order to overcome infertility [21].

Density gradient centrifugation has been documented for the separation of different populations of male reproductive cells for various fish species, for example, sterlet (*Acipenser ruthenus* [22]), tench (*Tinca tinca* [23]), Patagonian pejerry (*Odontesthes hatcheri* [24]), blue catfish (*Ictalurus furcatus* [25]), piracanjuba (*Brycon orbignyanus* [26]), common carp (*Cyprinus carpio* [27]). In addition, other studies have shown that density gradient centrifugation technique promises to be an efficient technique for selecting the sperm that best survive the cryopreservation processes. An example of this is a work done with *C. carpio* [28]. These authors reported as being the first time that the technique was used to separate sperm from fish. They evaluated membrane integrity, motility, and sperm speed, showing significant improvements in the separated milt. Even so, for *A. ruthenus* species, quality parameters were also compared, such as the percentage of motility and living cells, finding an improvement of 62.4% and 26.5%, respectively, in the separate sperm regarding control [29]. The aim of this work was to validate and evaluate the efficiency of the density gradient centrifugation technique, using the AllGrad® 90% gradient, on sperm functionality and fertilization capacity.

Materials and methods

The Ethics Committee of the Paulista State University "Júlio de Mesquita Filho" (UNESP) approved all protocols reported in this study with the Project Number: 38722.

Experimental framework and animals

Eleven males (146.2 ± 141.7 g) and one female (872 g) *Rhamdia quelen* were used. All originating from the São Paulo State University Júlio de Mesquita Filho (UNESP),

Campus de Registro, Agrochá Unit: BR-116, Km 449 - Registro/SP - CEP 11.900-000, place where the experiment was carried out. The fish remained stocked in 10000 L geomembrane tanks, in a recirculation system, until the moment of milt collection and fertilization. They were fed three times a day, until apparent satiety and feeding was restricted 24 hours before collection of gametes to avoid contamination by feces. Female able to reproduce was selected; the female exhibited a rounded abdomen, reddish protruding urogenital papilla and uniform oocyte size [30]. The males selected released milt when subjected to mild celomatic pressure [4].

Collection and evaluation of fresh milt

To collect the milt, the males were transferred to the reproduction laboratory and weighed individually, identified, and allocated in plastic tanks of 500 L, in a recirculation system equipped with aeration and with a temperature of 24 °C. They were hormonally induced with carp pituitary extract (CPE), to promote spermiation, using a single intramuscular application in the dorsal region of 3 mg CPE/1 mL 0.9% NaCl solution/kg body weight per specimen [30,31]. Milt collection was performed after the period corresponding to 240 accumulated thermal units (ATU; 10 h; water at 24 °C), counted from hormone application [31,32].

The collection of gametes was individually performed for each breeder, in graduated 15 mL Falcon tubes by abdominal massage in the cephalocaudal direction. The handling of the specimens was carried out with wet cloth. The urogenital area was wiped dry to prevent contamination with water or mucus and the first samples of milt released were discarded to avoid possible contamination with urine, mucus, feces, or water. On average, 3.00 ± 3.09 mL was collected from each breeder. Immediately after milt collection, the sperm motile was validated with the Computer Assisted Sperm Analysis (CASA) system, to detect the presence or absence of motile sperm in fresh milt. The samples showing motile sperm were discarded.

After collection and validation of the presence of motility, sperm samples were mixed to provide a milt *pool* and to minimize the differences of sperm quality. Immediately after forming *pool*, was analyzed sperm kinetics parameters by CASA system. In sequence, one microliter sample was removed from milt *pool* and fixed in 999 µL of saline-

buffered formaldehyde for measurement of the sperm concentration ($1.21x10^{10}$) and the rate of sperm morphological (64.75 ± 0.35 % normal sperm). The kinetic parameters obtained for the fresh milt *pool* were: Motility time: 40.50 ± 1.71 s, motility percentage (MOT): 76.63 ± 10.08%, curvilinear velocity (VCL): 117.02 ± 9.27 µm/s, average path velocity (VAP): 92.25 ± 1.00 µm/s, straight-line velocity (VSL): 84.64 10.18 µm/s, straightness index (STR): 91.74 ± 1.82%, wobble (WOB): 78.59 ± 2.74%, progression (PROG) 3621 ± 431.99 µm, and beats per seconds (BCF): 44.81 ± 1.91 beats/s. In total, 48 ± 13 sperm were counted. Measurements were performed 5 s after milt activation with distilled water, in a ratio of 1:600 (v:v).

Sperm cryopreservation

The pool milt was cryopreserved following the methodology proposed by Adames et al., (2015) [31]. In short, sperm was diluted at a ratio of 1:3 with the cryomedium containing 5% fructose (Sigma- Aldrich®), 5% powdered milk (Molico®-Nestlé) and 10% methanol (Sigma- Aldrich®). Sperm was loaded into 0.25 mL straws (Minitube®) which were subsequently conditioned in a canister and kept in liquid nitrogen steam for 18 h (*dry-shipper* CP300, -170 ºC). After that time has elapsed, the straws were plunged into liquid nitrogen and stored in canister. Twenty-four hours after the cryopreservation, the *R. quelen* sperm samples were thawed in a 25 °C water bath for 10 s and sequentially, the samples were divided into three experimental groups or treatments. In all cases, concentration, sperm functionality (CASA motility, membrane integrity and mitochondrial activity) was evaluated, as well as the capacity for fertilization.

Experimental design

Three treatments were compared: control, differential centrifugation, or palletization (T1), and density gradient centrifugation (T2). Thus, for the control treatment, sperm quality was evaluated immediately after thawing. In the case of the second treatment, the sperm quality was measured after centrifugation of the thawed semen with previous dilution in the Ginsburg extender solution (NaCl 123.2 mM, KCl 3.75 mM, $CaCl_2$ 3.0 mM, $NaHCO_3$ 2.65 mM [300mOsm, pH 7.5]). For the third treatment, centrifugation by density gradients was applied. In the end, sperm concentration and functionality were

compared, as well as the fertilization capacity of the sperm from the three experimental groups (Fig 1).

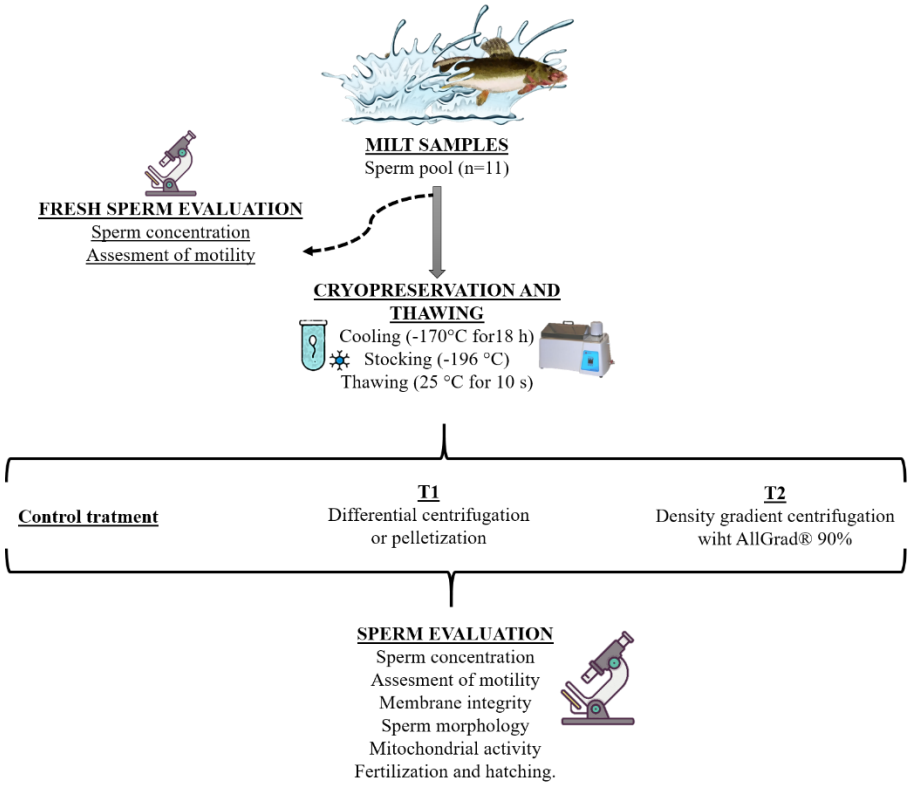

Fig 1. Experimental design developed to evaluate the use of AllGrad 90% for *R. quelen* milt

Centrifugation and conformation of density gradients

For the second treatment, differential centrifugation or pelleting [33] was applied, therefore, 100 µL of thawed milt was mixed with 200 µL of the Ginsburg solution and was then centrifuged. For the third treatment, a density gradient was formed before centrifugation. A density gradient (90% and 45%) was formed using the AllGrad® 90% gradient (Catalog numbers: AG90-050, AG90-100, LifeGlobal Group, Europe) and subsequently centrifuged. This methodology was defined from trails previously performed in the laboratory (Method in process of publication). In summary, to prepare a 45% AllGrad solution, the AllGrad® 90% was mixed at a 1:1 ratio with Ginsburg solution. One hundred microliters of 90% AllGrad solution was placed into a 1.5 mL Eppendorf tube and 100 µL of 45% AllGrad was smoothly layered over this. At the end of the AllGrad gradient column a layer of 100 µl of thawed milt was gently placed. To place all layers within the gradient, a 200 µL micropipette and tip was used. However, for the last two layers (45% AllGrad

solution and milt sample), a 10 µL tip was adapted to the 200 µL tip, allowing it to be poured more smoothly to avoid mixing.

Both treatment two and three, separation was performed by centrifugation at 1000 X g for 10 min at 4 °C, in a refrigerated centrifuge (SP-701/6000 Serial No.: 08/13-0001). After centrifugation, the supernatant above the sperm fraction was carefully removed and the pellet material were resuspended in 200 µL of Ginsburg solution. A second centrifugation was performed (500 X g for 5 min at 4 °C), the supernatant was again discarded, and the pellet was resuspended in 150 µL of extender solution. This sperm suspension was used to carry out the analysis of concentration, functionality, and fertilization capacity.

Spermatic concentration

The sperm concentration was analyzed with the help of a Neubauer chamber, with previous fixation of the sperm in saline-buffered formaldehyde [30,32,34]. The dilution rate for control treatment was performed in the same proportion as for fresh milt (1: 999 uL). For treatments two and three, the proportions 50:450 uL and 50:150 uL were used, respectively. An aliquot of 10 µL was placed on each side of the chamber. For each treatment, eight counts were made. The reading was performed with the help of a microscope (Nikon Eclipse E200, Tokyo, Japan) and a 40 X objective lens.

Assessment of sperm function

Sperm motility

Eight duplicate recordings were carried out for each of the treatments, to evaluate the movement characteristics of the spermatozoa of the evaluated sperm samples. Sperm motility was initiated with the use of distilled water, and the proportions (v:v) varied according to the treatment analyzed. Therefore, for control tratment a ratio of 1:100 µL was used. For the treatments subjected to centrifugation, the ratios of 1:50 µL and 1:10 µL were used, for treatments one and two, respectively.

According to the methodology adapted from Sanches et al. (2010 & 2013) [35,36], 10 µL of the mixture was placed in a mirrored Neubauer chamber. A phase contrast trinocular microscope Solaris Bel, planochromatic objectives and LED illumination (Italian

model, 20 X objective) were used. Videos were recorded from 5 to 50 s post-activation using a digital video camera (BALSER, acA640-120uc, Germany) coupled to the microscope. All samples were analysed 5 s after activation by assessment at a rate of 100 frames per second.

Sperm kinetics parameters were analysed with the CASA-ImageJ system (U.S. National Institutes of Health, Bethesda, Maryland, USA). Briefly, the capture of the videos was made in the .avi format, edited in the software VIRTUALDUB- 1.10.4 (http://www.virtualdub.org/), and exported as a sequence of images, corresponding to 1 s of video, in .jpg format. Then the image sequence files were imported into ImageJ 1.51j8/JAVA 1.8.0_112 (http://imagej.nih.gov/ij) free software and analyses using the CASA plug-in (open-source software available at http://wilson-leedy.com/CASA/ instructions.html).

The sperm parameters calculated by the open-source CASA were the same as those evaluated for fresh milt. There was use of the following input variable values in the CASA plugin: a= 1, b= 60, c= 50, d= 10, e= 3, f= 10, g= 20, h= 5, i= 1, j= 15, k= 10, l= 20, m= 80, n= 80, o= 50, p= 60, q= 100, r= 561.7978, s=0, t=0).

Membrane integrity

The evaluation of the membrane integrity or sperm viability of the samples after thawing were performed using an Accuri C6 flow cytometer (BD Biosciences) equipped with a 488 nm solid state laser and a 640 nm diode laser. Before measuring the membrane integrity, the control treatment was subjected to a centrifugation of 1000 X g for 10 min, the supernatant was discarded, and the pellet was used for measure plasma membrane integrity. Plasma membrane integrity was assessed by double-staining with SYBR-14 (for live cell nucleic acid staining - Thermo Fisher)/ propidium iodide (PI, for dead cell nucleic acid staining that penetrates through damaged plasma membrane - Thermo Fisher), adapted from the method proposed by [37], with modifications. In brief, for each treatment, aliquots of 50 µL of milt were mixed with 0.25 µL SYBR-14 (0.02 mM) and incubated in the dark at room temperature for 4 min. After, 1.0 µL PI (1.19 mM) was added to the samples, which were incubated for 1 min, also in the dark and at room temperature. After

final incubation, the samples were diluted (1:10) in Ginsburg solution and analyzed on flow cytometry.

Fluorescence signals of SYBR-14, gathered via 533/30 nm band-pass filter, and PI, gathered via 670 nm long-pass filter, were plotted on logarithmic scales. The sperm population was gated referring to the expected forward- and side-scatter signals. Ten thousand events per sample were acquired.

Sperm morphology

One hundred microliters of the milt previously fixed in saline-buffered formaldehyde was mixed with 10 µL of Rose Bengal dye [38]. The adapted methodology includes conformation of the slides per drained drop, placing three drops of 10 µL, each, on a histological slide. The slides were performed per drained drop, with a volume of 10 µL per drop. The morphology evaluated were [39]: Normal spermatozoa, short tail, distally curled tail, strongly curled tail, broken tail, folded tail, degenerate head, macrocephaly, microcephaly, loose head, proximal gout, distal gout (Fig 2). The slides were read with the 100 X objective, using immersion oil. In total, two hundred sperm were counted per slide. Eight duplicate counts were performed for each treatment.

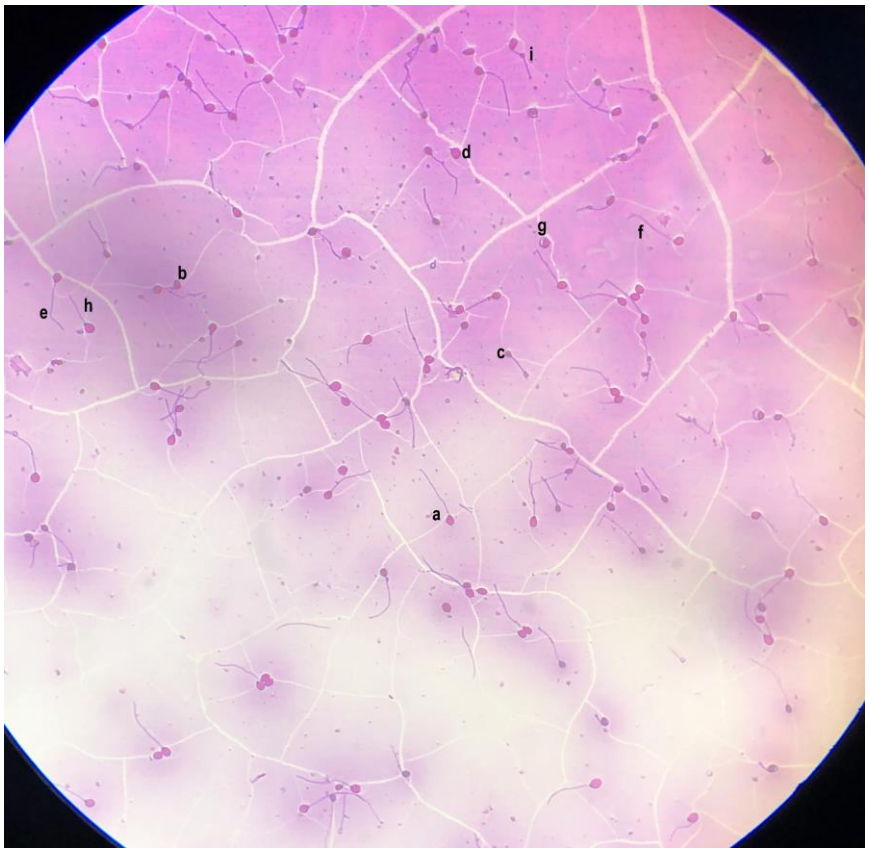

Fig 2. Some morphological abnormalities of catfish (*R. quelen*) sperm. a) Normal sperm, b) Loose head, c) Microcephaly, d) Macrocephaly, e) Broken tail, f) Folded tail, g) Strongly curled tail, h) Short tail and i) Proximal gout.

Mitochondrial activity

Mitochondrial activity was assessed using the MTT (3- (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) test. This technique is based on the principle of MTT reduction to formazan crystals by the mitochondrial succinyl dehydrogenase enzyme, which is active only in living cells [40]. MTT reduction assay was adapted of Aziz, (2006) [41], with modifications. In short, after thawing of the control treatment, two hundred microliters of milt sample were centrifuged (1000 X g for 10 min). For treatments two and three, after the second centrifugation, and for the control treatment, the pellet was resuspended in 400 µL of MTT (Invitrogen®, Thermo Fisher Scientific) stock solution (5 mg of MTT/mL of PBS) and were placed into a 1.5 mL Eppendorf tube. The suspension was incubated in an oven for 2 h at 28 °C. After incubation, all tubes were centrifuged (1000 X g for 5 min). The supernatant was discarded, and the sedimentation was strongly resuspended in 400 µL de dimethyl sulfoxide (Me_2SO) with the help of a pipette. Finally,

eight wells, in triplicate, of the 96-well microplate were used, for each treatment. The absorbance analysis was performed on a Spectrophotometer (SpectraMax® M2 250 Microplate Readers), at the wavelengths of 570 and 630 nm. The absorbance level was calculated by the difference between both wavelengths, and the final value was adjusted for 100 million sperm.

Fertility assessment

For fertilization, oocytes from one female were used in equal quantities (85 ± 7 oocytes) and they were mixed with a volume of milt indicated for each treatment (control: 3.50 µL, second treatment: 6.26 µL, and third treatment: 92.01 µL). The oocytes were obtained with the application of two hormonal doses of CPE. The first dose was 0.5 mg CPE/1 mL of NaCl solution 0.9%/kg body weight and eight hours later, the second dose (2.5 mg CPE/1 mL). After 240 ATU, counted from the application of the second hormonal dose, the female was extruded, following the care already described for the males.

The inseminating dose [30] was calculated from the sperm concentration and the percentage of motile sperm, allowing a total of 70,000 sperm per oocyte [42]. The quantity of oocytes to be fertilized was measured with the help of an insulin syringe (1 mL). Then the tip of the syringe was cut off, and 0.1 mL of oocytes were measured. Each portion of oocytes were individually placed in plastic cups (10 mL) and the volume of milt was added. The activation of the milt and hydration of the oocytes was carried out with 5 mL of distilled water. A gentle mixing, for one minute, was done. The incubation was carried out in fine mesh plastic strainers, kept in 500 L plastic tanks, with constant control of the water temperature (24 °C) and oxygen feeding.

The embryonic survival rate was performed between eight (initiates blastopore formation begins) to ten hours (blastopore closing forming germination ring) after fertilization of the oocyte. Thirty-eight hours later fertilization the hatching rate was measured from the total embryos that survived. Was considered the number of normal (larvae without any abnormality in the spine, yolk sac, or head) or deformed larvae [43]. These analyzes were carried out in a stereoscopic microscope (10 X objective). In total, eight repetitions were performed per treatment in duplicate.

Statistical methodology

The data obtained in treatments applied to cryopreserved milt were subjected to normality tests (Shapiro-Wilk, Kolmogorov-Smirnov and/or D'Agostino-Pearson) and Bartlett's homogeneity. When necessary, data were transformed (LOG) and outliers were excluded when present. The data showing normal distribution were analyzed using one-way analysis of variance (One-Way ANOVA), and when a significant difference was observed, the treatments were compared using the Tukey test. The results analyzed by means of parametric analyzes are presented in bar graphs using the mean and standard deviation (SD). Nonparametric data were analyzed using Kruskal-Wallis analysis, and when differences were observed, treatments were compared using the Dunn test. The data analyzed by means of non-parametric analysis are presented in Box and Whiskers graphs (maximum and minimum). Statistical analysis was performed using Statistical Analysis System - (SAS) v.9.0 software and GraphPad Prism 7.0.

Results

The decrease in sperm concentration was evident in T2 ($2.79x10^8 \pm 1.71x10^8$ cells/mL) compared to the control treatment ($5.01x10^9 \pm 1.03x10^9$ cells/mL) ($P < 0.0001$, Fig 3)

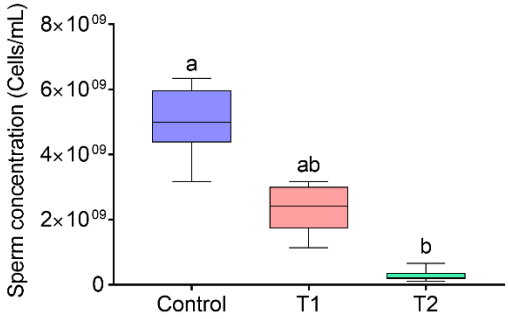

Fig 3. Sperm concentration of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. ($P < 0.0001$). Kruskal-Wallis analysis followed by Dunn's test. Different letters indicate difference between treatments.

The results showed that the differential centrifugation (T1), presented a better percentage of motility compared to the control treatment (42.58 ± 6.05% and 32.44 ±

6.033%, respectively, P = 0.0427, Fig 4A). The motility time (Fig 4B) was shorter for the centrifugal treatment by density gradient T2 (32.38 ± 0.92 s) than for treatment T1 (36.69 ± 3.62 s), P = 0.0072. The other sperm kinetics parameters measured did not show differences between the treatments (P = 0.4114, Fig. 5).

Membrane integrity was not different between treatments (Fig 4C). The percentages of integral membrane for each treatment were: control= 72.96 ± 7.30%, T1= 79.56 ± 6.35% and T2= 76.27 ± 4.16%. The presence of normal sperm (Fig 4D) was higher in T2 when compared to the control and T1 treatments. The values obtained were 10.72 ± 3.53%, 3.97 ± 1.95% and 13.55 ± 5.82%, for the control groups, T1 and T2, respectively.

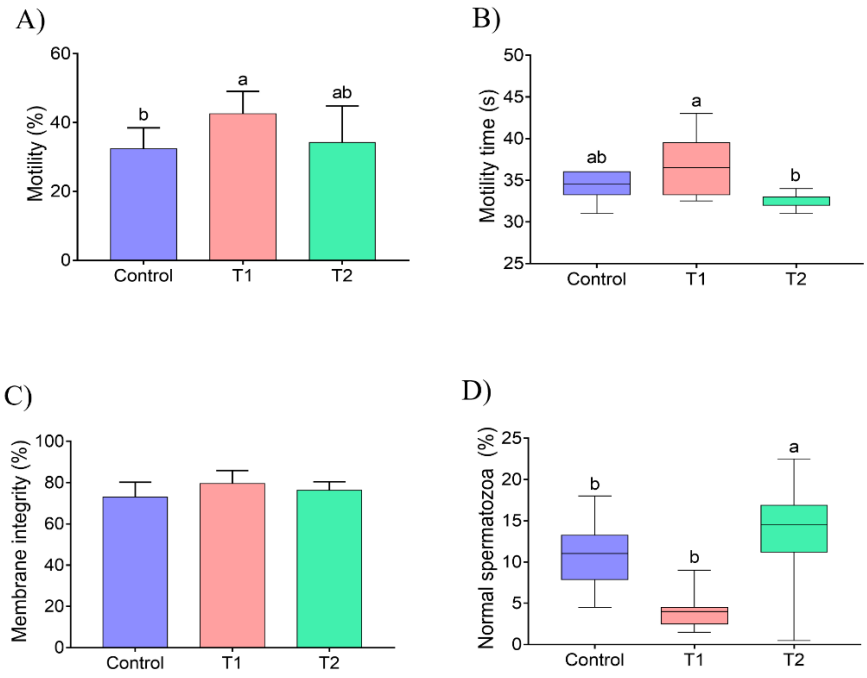

Fig 4. Sperm quality of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. A) Motility (P = 0.0427); B) Motility time (P = 0.0072); C) Membrane integrity (P = 0.1190); D) Normal sperm (P < 0.0001). Analysis of variance followed by Tukey test (Figure A and C). Kruskal-Wallis analysis followed by Dunn's test (Figures B and D). Different letters indicate difference between treatments.

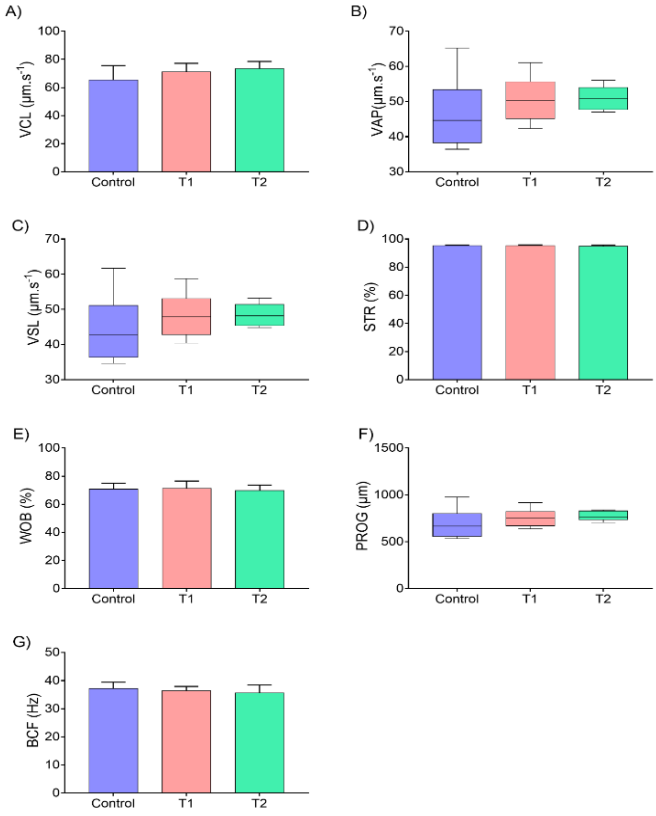

Fig 5. Sperm kinetic parameters of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. A) VCL (P = 0.1084); B) VAP (P = 0.3594); C) VSL (P = 0.3764); D) STR (P = 0.4114); E) WOB (P = 0.7816); F) PROG (P = 0.3009); G) BCF (P = 0.9506). Analysis of variance followed by Tukey's test (Figures A, D, E and G). Kruskal-Wallis analysis followed by Dunn's test (Figures B, C and F). Different letters indicate differences between treatments.

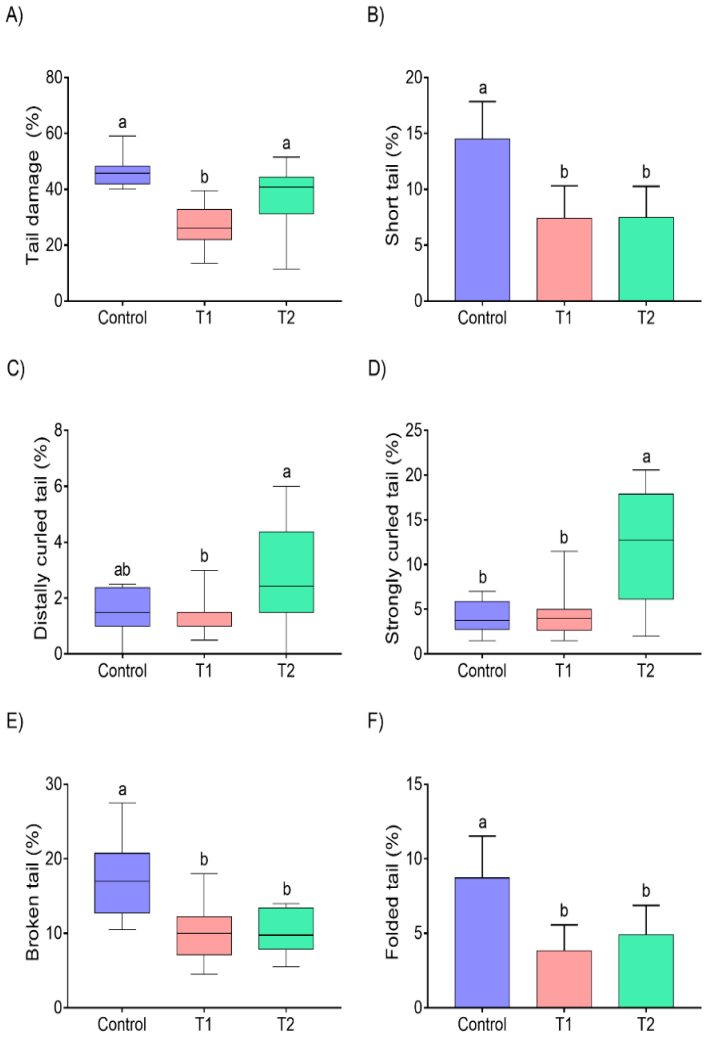

Fig 6. Tail morphological damage of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. A) Total tail moforlogic damage ($P < 0.0001$); B) Short tail ($P < 0.0001$); C) Tail curled distally ($P = 0.0459$); D) Tail strongly curled ($P < 0.0001$); E) Broken tail ($P < 0.0001$); F) Tail folded ($P < 0.0001$). Analysis of variance followed by Tukey's test (Figures B and F). Kruskal-Wallis analysis followed by Dunn's test (Figures A, C, D and E). Different letters indicate difference between treatments.

Regarding tail damage, T1 represented the lowest rate (Fig 6A, $P < 0.0001$). However, this treatment was the one that represented a greater portion of spermatozoa with a damaged head (Fig 7A, $P < 0.0001$). Finally, there was no difference ($P = 0.7473$) between the treatments for sperm damage due to the presence of gout (Fig 8).

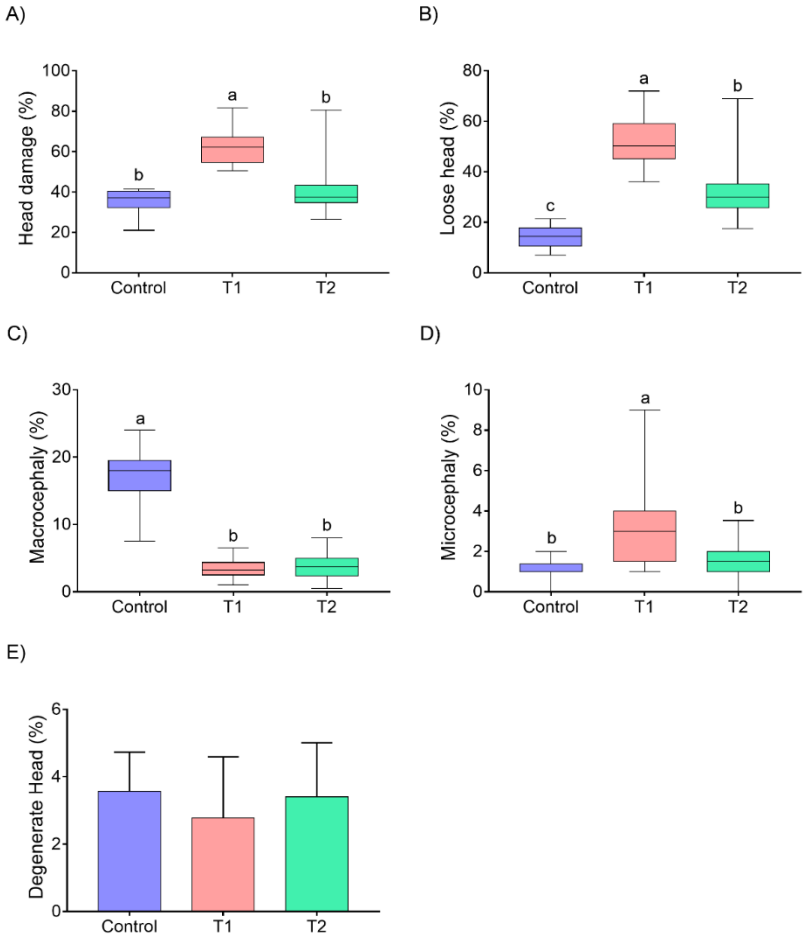

Fig 7. Head damage of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. A) Total head morphological damage ($P < 0.0001$); B) Degenerate head ($P = 0.3311$); C) Macrocephaly ($P < 0.0001$); D) Microcephaly ($P = 0.0002$); E) Head loose ($P < 0.0001$). Analysis of variance followed by Tukey's test (Figure E). Kruskal-Wallis analysis followed by Dunn's test (Figures A, B, C and D). Different letters indicate difference between treatments.

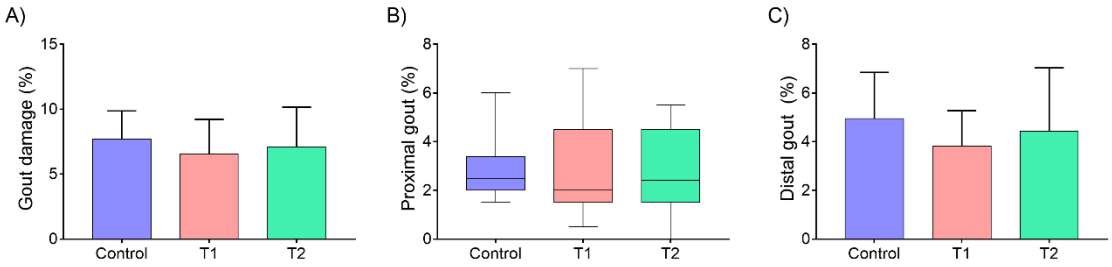

Fig 8. Gout damage of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied A) Total gout damage (P = 0.7473); B) Proximal drop (P = 0.6574); C) Distal drop (P = 0.3053). Analysis of variance followed by Tukey's test (Figures A and C). Kruskal-Wallis analysis followed by Dunn's test (Figure B).

The T1 and T2 treatments were different from the control treatment in the MTT test (Fig 9, P = 0.0007). The control group showed MTT values of 0.20 ± 0.08 AU/100 million sptz, meanwhile the centrifuged treatments resulted in values of 1.58 ± 0.79 AU/100 million sptz (T1) and 2.02 ± 1.88 AU/100 million sptz (T2).

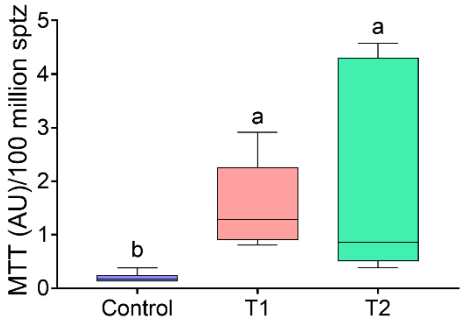

Fig 9. Sperm quality. MTT of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. (P = 0.0007). Kruskal-Wallis analysis followed by Dunn's test. Different letters indicate difference between treatments.

Regarding the embryo survival rate (Fig 10A) and hatching (Fig 10B), when the centrifugation was applied in the milt, the results were superior to the control (P < 0.0001). The Embryonic survival for the control treatment was 40.03 ± 11.67%, 76.68 ± 15.22% (T1), and 69.85 ± 17.85% (T2). On the other hand, the hatching rate for the three

treatments was 24.14 ± 13.97%, 54.62 ± 17.39% and 50.87 ± 20.59%, respectively. Regarding the presence of normal larvae (Fig 10C), no difference was obtained between treatments (P = 0.4336).

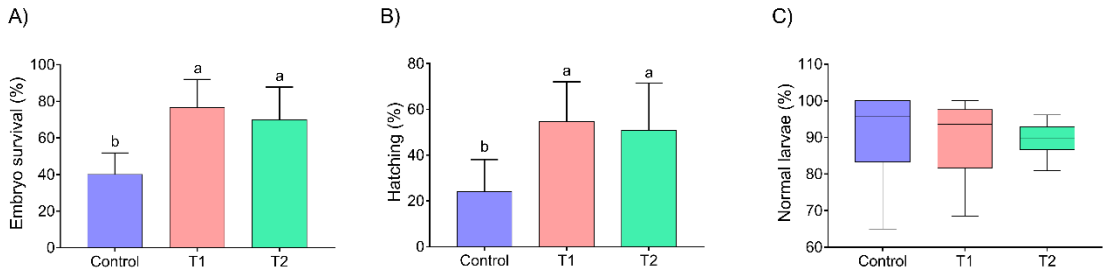

Fig 10. Embryo survival and hatch rate of catfish (*R. quelen*) cryopreserved milt after control (thawed and non-centrifuged milt), T1 (thawed and differential centrifugation milt at 1000 X g for 10 min) and T2 (thawed and density gradient centrifugation milt at 1000 X g for 10 min) treatments were applied. A) Embryonic survival rate (P < 0.0001); B) Hatch rate (P < 0.0001); C) Normal larvae (P = 0.4336). Analysis of variance followed by Tukey's test (Figures A and B). Kruskal-Wallis analysis followed by Dunn's test (Figure C). Different letters indicate difference between treatments.

Discussion

In the present study, the AllGrad 90% centrifugation gradient was used for the first time with cryopreserved milt of the *R. quelen* species. Likewise, it was the first time differential, and density gradient centrifugation techniques were compared in fish.

The results obtained in this study, for the sperm concentration, were expected considering the effects of centrifugation, since this process allows the sedimentation of the densest cells more quickly. In the case of density gradient centrifugation, the gradient column acts as a sieve, therefore, the larger or less dense particles will become trapped along the gradient, finally allowing sedimentation, in this case, of the densest cells, and with sufficient size to cross these gradients (45% and 90%).

The loss of sperm motility, and consequently of the fertilization capacity after cryopreservation process is compromised owing to the increased number of abnormalities that occur, in the head as well as in the tail [44]. Our results showed that the milt samples that were subjected to differential centrifugation presented 10.14% more motile sperm than the control treatment. Therefore, the results obtained for T1 are consistent with the

presence of sperm with a loose head, since 42.48% of the sperm were motile. Li et al. (2010) showed that samples of cryopreserved milt of the species *C. carpio* separated by density gradient presented high motility compared to non-separated sperm [28]. Additionally, Horokhovatskyi et al. (2018) reported a high percentage of sperm motility after separating with density gradients when using semen of the *A. ruthenus* species [29]. Another study carried out with the species *S. salar*, showed that when using a density gradient of 45%/90%, the percentage of mobile cells increased by 5.8% compared to the control treatment [45]. Although our results when the density gradient centrifugation was applied did not show any difference with the other treatments, it allowed us to know the benefits of differential centrifugation on cryopreserved milt samples, considering that, on the one hand, it was better than the non-separated milt with respect to the numbers of mobile cells and on the other hand, it would be more economical if it was thought of a use for productive purposes.

A greater selection of viable sperm in a milt sample is an important factor for the success of assisted reproductive techniques. Herein lies the importance of selection techniques, which allow the use of viable sperm with a relatively higher fertilization capacity [29]. Our results did not show differences between the percentage of viable cells or cells with an intact membrane for the different treatments. In the *A. ruthenus* species, the percentage of viable sperm was 26.5% higher when comparing separated cryopreserved milt samples with samples that were not separated [29]. These results lead us to consider two possibilities. The first of these refers to the specific characteristics of fish sperm, such as osmolality or ionic composition in the surrounding medium, which vary between species or even within the same species [11,29], or by the presence of sperm structures such as the acrosome, present in the spermatozoa of the *A. ruthenus* species and absent in the *R. quelen* species [46]. The second possibility is related to the differential centrifugation, performed in the present study, to the control treatment at the time of applying the membrane integrity analysis protocol. This process could positively influence the presence of viable sperm in this treatment, leading it to not be different from the other treatments. This hypothesis can be confirmed with the definition given by Griffith, 2010. He indicates that differential centrifugation allows the separation of particles with

different sedimentation coefficients, therefore the cells of greater size or weight will precipitate first [33], thus, integral spermatozoa will precipitate more quickly.

The results of staining with Bengal Rose dye for morphological evaluations in the present study, allowed to show that the density gradient centrifugation method allowed to obtain a larger portion of normal sperm. Dead and abnormal spermatozoa as well as sperm debris may be an obstacle to successful fertilization in *in vitro* conditions [47]. Unlike fish, in mammals the assessment of sperm morphological changes is considered an important parameter [38]. According to CBRA (1998), for a good evaluation of the semen, some species must not exceed 20% (swine and sheep) or 30% (equine and bovine) of sperm pathologies in order not to harm the fertility index [48]. in fish, some classifications of morphological abnormalities have already been proposed [38,49]. Based on the classification carried out by Miliorini et al. 2011 we planted a classification of the morphological abnormalities of *R. quelen* spermatozoa as mild and severe (Fig 11).

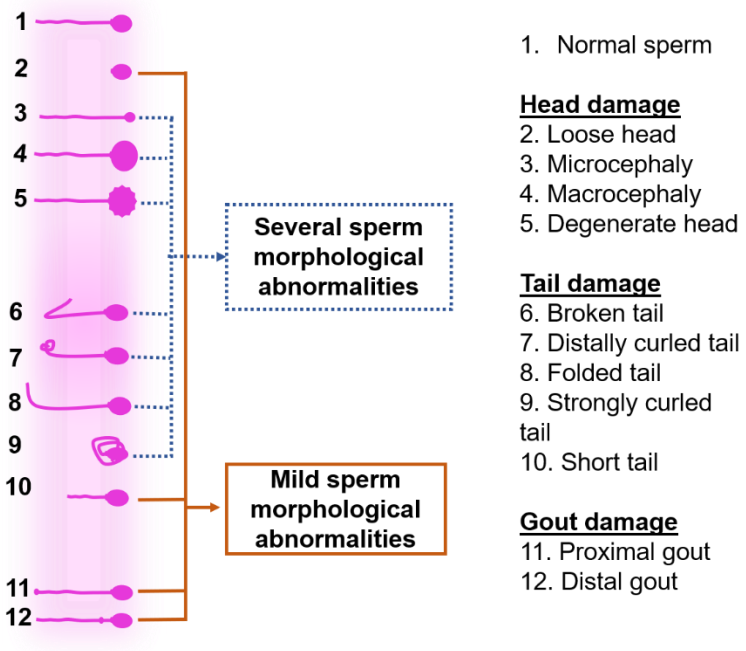

Fig 11. Classification of the morphological pathologies of the fish spermatozoa. Adapted from Miliorini et al. (2011), proposal for curimba (*Prochilodus lineatus*) sperm damages after cryopreservation.

In this sense, the differential and density gradient centrifugation treatments were the ones that represented the largest amount of mild morphological abnormalities (71.12% and 52.67%, respectively), being represented mainly by loose heads (Fig 7B, 52.34% and

33.22%, respectively). These results may be related to the centrifugation process when considering what Herman (1994) said. This author reports that the presence of pathologies such as loose head are due to the manipulation of milt [50]. In another work carried out for *R. quelen*, it showed that cryopreserved milt presented a greater number of morphological abnormalities, mainly macrocephaly [44]. For the present study where the control treatment is represented by a greater number of severe pathological morphologies (44.07%), mainly for broken tail (6,75%, Fig 6E) and macrocephaly (16.91%, Fig 7D). These severe morphological abnormalities decreased by 19.16% and 10.29% for the differential and density gradient centrifugation treatments, respectively. Although the comparison of the pathological morphologies of fresh milt with cryopreserved milt was not carried out in our work, it is important to highlight the results obtained by Da Costa et al. (2019), since it allows us to see the benefits of applying centrifugation methods, where pathologies such as macrocephaly were reduced, a pathology that showed a negative correlation (-0.88) with sperm motility [44]. Although studies have already been carried out evaluating the morphologies in fish, including for the species under study [44], however, there are no indices that indicate the sperm quality of fish from normal pathologies, such as in mammals, or whether mild pathologies could also be considered within the normal range, since the *in vitro* fertilization in fish, in practice, is performed by a gentle mixture of the gametes after being hydrated (oocytes) and activated (sperm). This movement leads us to hypothesize that; for example, sperm with a loose but viable head coincide with the micropyle of the oocyte, fertilizing it, making it necessary to carry out future studies to verify this theory.

The functioning of sperm cells and their metabolic activity is dependent on the mitochondria and its components, such as proteins. This organelle is the main source of energy and cellular homeostasis.[16] and are very vulnerable to low temperature stress [51], so validating its functionality after cryopreservation is very important. The mitochondrial dehydrogenase enzyme, present in the mitochondrial membrane, it is in charge of reducing MTT to thiazolyl blue tetrazolium bromide, however, enzyme only acts when the cell is viable [52,53]. In our study, it was found that the centrifuged treatments had greater mitochondrial functionality than the control. A study carried out with cryopreserved milt of the species Atlantic salmon (*Salmo salar*) showed that samples that

were separated by a density gradient a percentage higher membrane potential than the control treatment [45]. Although the membrane potential was not assessed in our study, this variable is considered to be a measure that indicates mitochondrial functionality [54]. However, future MTT tests for milt thawed after centrifugation could be recommended in order to obtain other explanations considering the possibility of latent injuries [55].

The success of artificial reproduction, based on cryopreservation programs, depends on the maximum use of available gametes, which means fertilizing the largest number of oocytes with the least amount of sperm [30]. Additionally, the use of selection techniques is of great importance since it allows concentrating spermatozoa that maintain the fertilization capacity [29]. For the present study, the sperm of the milt samples that were centrifuged showed better embryonic survival and hatching rates. Cabrita et al. (2014) indicated that the best determinant of sperm quality is not only the ability of sperm to fertilize an oocyte, but also to contribute to the initial embryonic development. A review carried out for aquatic species reported that high fertilization rates in artificial reproduction should be greater than 70% to 80%, depending on the species [56]. Considering these values, we could assume that from the differential centrifugation it was possible to obtain high fertilization rates positively influencing the embryonic survival, which was 36.36% higher when compared to the control treatment. Finally, although no differences were found in the percentage of normal larvae, a future study that includes monitoring their growth would be very interesting, considering that the growth of the offspring may be affected by the use of cryopreserved sperm in induced reproduction routines [57]. The previous placement, part of the fact that the present study allowed to identify that the negative effects generated by cryopreservation can be reduced when using the sperm separation method.

In conclusion, the development of this work made it possible to identify that the centrifugation methods benefit the quality of thawed milt samples of *R. quelen*, resulting in better rates of embryonic survival and hatching. In another sense, the differential centrifugation allowed to obtain a greater quantity of motile spermatozoa. Considering this point and possibly economic aspects, of sperm separation by differential centrifugation could be considered a promising technique in induced reproduction programs, as an

auxiliary tool for the milt cryopreservation of this species. Furthermore, the use of this technique can probably be adapted to other species of teleost fish.

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CAPÍTULO IV

4. CONSIDERAÇÕES FINAIS

- As metodologias propostas pela primeira vez para a espécie *R. quelen,* permitiram demonstrar que as técnicas de centrifugação diferencial e por gradiente de densidades são eficientes para obter uma porção de espermatozoides de maior qualidade a partir do sêmen criopreservado desta espécie, uma vez que resultaram em melhores taxas de sobrevivência embrionária e taxa de eclosão.
- A centrifugação diferencial é uma técnica promissora nos programas de reprodução *in vitro* a partir do sêmen criopreservado da espécie *R. quelen* pois vez que permitiu maior porcentagem de células espermáticas móveis com referência ao sêmen não centrifugado, conforme resultados obtidos no segundo estudo.
- Em atendimento a premissa de que a técnica de centrifugação deve ser rápida, fácil e econômica, destaca-se a separação dos espermatozoides por centrifugação diferencial. Esse método é promissor em programas de reprodução induzida, como ferramenta auxiliar para a criopreservação do sêmen de *R. quelen*. Por fim, o uso desta técnica poderá ser adaptado a outras espécies de peixes teleósteos.

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APÊNDICES

Apêndice I – Normas do periódico MethodsX

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All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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All Western blots should be presented with molecular weights noted, replicated, quantified and with statistical analysis. Cropping of the image is acceptable but must be clearly indicated. Merging images together to give the appearance of one image is not acceptable. The method of normalization to total protein, or where appropriate, a loading control (e.g. cell signaling studies) should be explicitly stated in the text. Images may be subjected to analysis for manipulation prior to publication and authors may be requested to provide copies of the original data.

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Apêndice II – Normas do periódico MethodsX

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Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., “$a^2 + b^2 = c^2$”), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

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Ending section	*The following elements are required, in order:* • Acknowledgments • References • Supporting information captions (if applicable)
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Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

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- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

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The introduction should:

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- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
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These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

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Source	Format
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Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity. arXiv:1403.3301v1 [Preprint]. 2014 [cited 2014 March 17]. Available from: https://128.84.21.199/abs/1403.3301v1 Kording KP, Mensh B. Ten simple rules for structuring papers. BioRxiv [Preprint]. 2016 bioRxiv 088278 [posted 2016 Nov 28; revised 2016 Dec 14; revised 2016 Dec 15; cited 2017 Feb 9]: [12 p.]. Available from: https://www.biorxiv.org/content/10.1101/088278v5 doi: 10.1101/088278
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html

Source	Format
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Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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Example caption

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- *PLOS ONE* guidelines, for clinical trials requirements
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In the methods, include a section on statistical analysis that reports a detailed description of the statistical methods. In this section:

- List the name and version of any software package used, alongside any relevant references
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Statistical reporting guidelines:

- Identify research design and independent variables as being between- or within-subjects
- For pre-processed data:
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- Define the threshold for significance (alpha)
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- For analyses of variance (ANOVAs), detail any post hoc tests that were performed
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- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).

- For step-wise multiple regression analyses:
    - Report the alpha level used
    - Discuss whether the variables were assessed for collinearity and interaction
    - Describe the variable selection process by which the final model was developed (e.g., forward-stepwise; best subset). See SAMPL guidelines.

- For Bayesian analysis explain the choice of prior trial probabilities and how they were selected. Markov chain Monte Carlo settings should be reported.

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This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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VITA

Maritza Pérez Atehortúa nasceu em 26 de maio de 1988 em Amalfi, Antioquia, Colômbia. Filha de Lucely Amparo Atehortúa Hernández e Humberto de Jesús Pérez Rúa.

Concluiu o estudo fundamental em 1999 na Instituição Educativa María Auxiliadora e o ensino médio em 2005 na Instituição Educativa Eduardo Fernández Botero, ambos em Amalfi, Antioquia, Colômbia. Em 2012 iniciou a graduação em Zootecnia pela Universidad de Antioquia em Medellín, Colômbia. No último ano da graduação realizou o Estágio Curricular na Universidade Federal do Rio Grande do Sul, apoiando atividades de pesquisa na área de nutrição em ruminantes, focada a gado de corte. A graduação foi concluída em 2018.

No ano de 2019 ingressou no mestrado do Programa de Pós-graduação em Zootecnia na área de concentração de Produção Animal, pela Universidade Federal do Rio Grande do Sul, sendo bolsista CAPES. Durante o tempo do mestrado realizou e apoiou diferentes atividades no Grupo de Pesquisa AQUAM (Produção e Conservação da Biodiversidade das Espécies Aquáticas). O trabalho realizado durante este período se focou no desenvolvimento de métodos que permitissem melhorar a qualidade durante o processo de criopreservação de gametas de peixes. Aplicou a metodologia de centrifugação como técnica de reprodução assistida, além de validação da qualidade e aplicação de protocolos de reprodução e a verificação da sobrevivência embrionária e quantificação das taxas de eclosão.

Recentemente (abril, 2021) iniciou os estudos Doutorais em Ciências Agropecuárias pela Universidad Católica de Temuco, Chile, na área de concentração de Produção Animal.