

**Sperm quality and cryopreservation in
teleost: effect of seminal plasma
component and climate change**



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Sperm quality and cryopreservation in teleost: effect of seminal plasma component and climate change

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DEDICATION

To my children Madlyn and David for their strength that gives me every day.

To my parents and my family for all the support in my personal and professional life.

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RESUMO

A seleção de gametas de alta qualidade é um requisito essencial a ser levado em consideração nos programas de reprodução assistida. O desenvolvimento de ferramentas biotecnológicas como a criopreservação de gametas desempenha um papel importante na produção aquícola e na formação de bancos de germoplasma, que posteriormente contribuirão para o melhoramento genético das populações de peixes, principalmente aqueles em perigo e que poderão ser mais afetados pela mudanças climáticas futuras. Esta tese está sendo implementada no âmbito de um acordo de supervisão conjunta entre a Unesp e a UPV. Na primeira fase desta tese realizada na Unesp, trabalhamos com uma espécie neotropical de grande importância econômica para a região Suramericana. A Segunda fase realizada na UPV trabalhou com a Enguia Europeia (*Anguilla angilla*), espécie classificada na Lista Vermelha da União Internacional para a Conservação da Natureza (IUCN) como espécie “criticamente ameaçada”. Na primeira fase, buscamos caracterizar a composição bioquímica do plasma e as características seminais da espécie. Avaliamos as possíveis relações entre esses parâmetros. Na composição do plasma seminal, observamos principalmente íons sódio (Na^+) e dentro dos componentes orgânicos, destacaram-se proteínas totais e glicose. A análise de componentes principais (PCA) demonstrou que a motilidade teve forte correlação positiva com o tempo de motilidade, concentração espermática e proteína total. Essas análises serviram de base para a criação de uma solução diluente utilizada posteriormente na substância crioprotetora. Em seguida, foi determinada a influência do plasma seminal como constituinte da solução crioprotetora na criopreservação do sêmen

de *P. reticulatum*. Foram utilizados três tratamentos: glicose 5% + metanol 10% (T1), plasma seminal natural 30% (T2) e plasma seminal artificial 30% foram adicionados neste crioprotetor com base nos resultados dos componentes bioquímicos do plasma determinados. espécie no experimento anterior (T3). Parâmetros de motilidade espermática, capacidade de fertilização do sêmen criopreservado, bem como a fragmentação do DNA foram avaliados. O tratamento T1 resultou nos melhores valores de motilidade seguido do T2, sendo que a capacidade de fertilização desses dois tratamentos foi semelhante ao controle, porém, o tratamento T2 apresentou menos danos ao DNA. A PCA demonstrou que T1 teve uma melhor associação com fertilidade e motilidade total e progressiva. Avaliamos ainda, as estruturas das subpopulações espermáticas em cada um dos tratamentos utilizados. Por meio da análise multivariada em duas etapas, determinados três subpopulações espermáticas no sêmen criopreservado da espécie, SP1 (linear rápido), SP2 (não linear rápido) e SP3 (linear lento). O T1 apresentou maior percentual de SP1, sendo confirmado pela eficácia em proteger as células deste tratamento no processo de criopreservação da espécie. Na segunda fase foi realizada na UPV, objetivamos determinar o efeito da temperatura e do pH da água do mar na motilidade espermática da Enguia europeia. O baixo pH da água do mar (6.5-7.4) diminuiu a motilidade dos espermatozoides da enguia em comparação com o controle (pH= 8.2). Quando estudamos o efeito combinado do pH do plasma seminal artificial e do pH do ASW (7.8 e 8.2), não encontramos diferenças na motilidade e cinética espermática em relação ao pH do plasma seminal artificial, mas sim no pH da água do mar. Maiores valores de motilidade total (MOT), FA e ME foram encontrados em pH 8.2 do que em pH 7.8. Em contraste, a temperatura da

água do mar não afetou os parâmetros de motilidade espermática ou longevidade espermática. Para estudar o efeito da interação entre a temperatura da água do mar e o pH na motilidade espermática, foram testadas duas temperaturas: 4 e 24 °C, e dois pHs: 7.8 e 8.2. Houve diferenças significativas entre temperatura e pH em vários parâmetros cinéticos, como MP, VCL, VSL, VAP, ME e SL, onde os menores valores para MP, VCL, VSL e VAP foram observados em amostras ativadas em baixa temperatura e pH baixo (4 °C, pH 7.8). Nossos resultados sugerem que a acidificação da água do mar, mas não as temperaturas mais altas, pode afetar a motilidade espermática no contexto das mudanças climáticas.

ABSTRACT

The selection of high-quality gametes is an essential requirement to consider in assisted reproduction programs. The development of biotechnological tools such as cryopreservation of gametes plays an important role in aquaculture production and in the formation of germplasm banks, which will later contribute to the genetic improvement of fish populations, mainly those in danger and that could be more affected by future climate changes. This thesis is being implemented under a co-tutorship agreement between Unesp and UPV. In the first phase of this thesis carried out at Unesp, we worked with a Neotropical species of high economic importance for the South American region. The Second phase carried out at the UPV worked with the European Eel (*Anguilla anguilla*), a species classified on the Red List of the International Union for Conservation of Nature (IUCN) as a "critically endangered" species. Part of this is due to the lack of information regarding native species with zootechnical potential. Siluriformes in general have ideal characteristics and arouse interest for fish farming, and within this group, the species *Pseudoplatystoma reticulatum* stands out, due to its desirable zootechnical characteristics for the development of reproduction in captivity. Biotechnologies such as gamete cryopreservation has gained prominence in fish farming worldwide, as it is a technique that aims to conserve the diversity and genetic integrity of species, as well as presenting advantages for the management of broodstock. Therefore, there is a need for studies aimed at the efficiency of production processes and artificial reproduction, which generate effective cryopreservation protocols, which contribute to the development of fish farming. In the first

phase, we sought to characterize the biochemical composition of the plasma and the seminal characteristics of the species. Evaluate the possible relationships between these parameters. In the composition of the seminal plasma, sodium ions (Na^+) were mainly observed and within the organic components total proteins and glucose stood out. Through principal component analysis (PCA) it was observed that motility had a strong positive correlation with motility time, sperm concentration and total protein. These analyzes served as the basis for the creation of a diluent solution used later in the cryoprotective substance. Then, the influence of seminal plasma as a constituent of the cryoprotective solution in the cryopreservation of *P. reticulatum* semen was determined. Three treatments were used: glucose 5% + Methanol 10% (T1), 30% natural seminal plasma (T2) and 30% artificial seminal plasma were added to this cryoprotectant based on the results of the biochemical components of the plasma determined. for the species in the previous experiment (T3). Sperm motility parameters, fertilizing capacity of cryopreserved semen, as well as DNA fragmentation were evaluated. The T1 treatment resulted in the best motility values followed by T2, and the fertilizing capacity of these two treatments was like the control, however, the T2 treatment showed less DNA damage. Through PCA it was shown that T1 had a better association with fertility and total and progressive motility. Finally, we evaluated the structures of the sperm subpopulations in each of the treatments used. Through multivariate analysis in two stages, it was possible to determine three sperm subpopulations in the cryopreserved semen of the species, SP1 (fast-linear), SP2 (fast-nonlinear) and SP3 (slow-linear). T1 presented the highest percentage of SP1, being confirmed by the effectiveness in protecting the cells

of this treatment in the cryopreservation process of the species. In the second phase that was carried out at the UPV, the general objective was to determine the effect of temperature and pH of seawater on sperm motility in the European eel. It was determined that the low pH of seawater (6.5-7.4) decreased the motility of eel spermatozoa compared to the control (pH= 8.2). When we studied the combined effect of the pH of the artificial seminal plasma and the pH of ASW (7.8 and 8.2), we did not find statistical differences in sperm motility and kinetics in relation to the pH of the artificial seminal plasma, but we did the pH of seawater. Higher total motility (MOT), FA and ME values were found at pH 8.2 than at pH 7.8. In contrast, seawater temperature did not affect sperm motility parameters or sperm longevity. To study the effect of the interaction between seawater temperature and pH on sperm motility, two temperatures were tested: 4 and 24 °C, and two pHs: 7.8 and 8.2. There were significant differences between temperature and pH in several kinetic parameters, such as MP, VCL, VSL, VAP, ME and SL, where the lowest values for MP, VCL, VSL and VAP were observed in samples activated at low temperature and Low pH (4 °C, pH 7.8). Our results suggest that seawater acidification, but not higher temperatures, may affect sperm motility in the context of climate change.

RESUMEN

La selección de gametos de alta calidad es un requisito indispensable a tener en cuenta en los programas de reproducción asistida. El desarrollo de herramientas biotecnológicas como la criopreservación de gametos, juega un papel importante en la producción acuícola y en la formación de bancos de germoplasma, que contribuirán luego en la mejora genética de las poblaciones de peces, principalmente aquellas en peligro y que pudieran estar más afectadas ante futuros cambios climáticos. Esta tesis está siendo implementada bajo un convenio de cotutela entre la Unesp y UPV. En la primera fase de esta tesis realizada en la Unesp, se trabajó con una especie neotropical de elevada importancia económica para la región Suramericana. La segunda fase realizada en la UPV se trabajó con la Anguila europea (*Anguilla anguilla*), una especie clasificada en la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (UICN) como especie "en peligro crítico de extinción". En la primera fase se buscó caracterizar la composición bioquímica del plasma y las características seminales de la especie para evaluar las posibles relaciones entre estos parámetros. El plasma seminal estuvo principalmente compuesto por iones de sodio (Na^+) y dentro de los componentes orgánicos sobresalieron las proteínas totales y la glucosa. A través del análisis de componentes principales (PCA) se observó que la motilidad tenía una fuerte correlación positiva con el tiempo de motilidad, la concentración de espermatozoides y las proteínas totales. Estos análisis sirvieron de base para la creación de una solución diluyente utilizada posteriormente en la sustancia crioprotectora. Luego se determinó la influencia

del plasma seminal como constituyente de la solución crioprotectora en la criopreservación de semen de *P. reticulatum*. Se utilizaron tres tratamientos: glucosa 5% + metanol 10% (T1), a este crioprotector se le agregó 30% de plasma seminal natural (T2) y 30% de plasma seminal artificial en base a los resultados de los componentes bioquímicos del plasma determinados para la especie en el experimento anterior (T3). Se evaluaron parámetros de motilidad espermática, capacidad fecundante del semen criopreservado, así como fragmentación del ADN. El tratamiento T1 resultó con los mejores valores de motilidad seguido del T2, y la capacidad fertilizante de estos dos tratamientos fue similar al control, sin embargo, el tratamiento T2 mostró menos daño en el ADN. Mediante el PCA se demostró que T1 tenía una mejor relación positiva con la fertilidad y la motilidad total y progresiva. Finalmente, evaluamos las estructuras de las subpoblaciones espermáticas en cada uno de los tratamientos utilizados. Mediante análisis multivariado en dos etapas, fue posible determinar tres subpoblaciones espermáticas en el semen crioconservado de la especie, SP1 (rápido-lineal), SP2 (rápido-no lineal) y SP3 (lento-lineal). T1 presentó el mayor porcentaje de SP1, siendo confirmado por la efectividad en la protección de las células de este tratamiento en el proceso de criopreservación de la especie. En la segunda fase que se está llevando a cabo en la UPV, el objetivo general fue determinar el efecto de la temperatura y el pH del agua de mar sobre la motilidad de los espermatozoides en la *Anguila europea*. Se determinó que el bajo pH del agua de mar (6.5-7.4) disminuyó la motilidad de los espermatozoides de anguila en comparación con el control (pH= 8.2). Cuando estudiamos el efecto combinado del pH del plasma seminal artificial y el pH de ASW (7.8 y 8.2), no encontramos diferencias estadísticas en la motilidad y cinética de los espermatozoides en relación con el pH del

plasma seminal artificial, pero sí el pH del agua de mar. Se encontraron valores más altos de motilidad total (MOT), FA (rápidos) y ME (médios) con un pH de 8.2 que con un pH de 7.8. En contraste, la temperatura del agua de mar no afectó los parámetros de motilidad de los espermatozoides o la longevidad de los espermatozoides. Para estudiar el efecto de la interacción entre la temperatura del agua de mar y el pH sobre la motilidad de los espermatozoides, se probaron dos temperaturas: 4 y 24 °C, y dos pH: 7.8 y 8.2. Hubo diferencias significativas entre la temperatura y el pH en varios parámetros cinéticos, como MP, VCL, VSL, VAP, ME y SL, donde los valores más bajos para MP, VCL, VSL y VAP se observaron en las muestras activadas a baja temperatura y pH bajo (4 °C, pH 7.8). Nuestros resultados sugieren que la acidificación del agua de mar, pero no las temperaturas más altas, pueden afectar la motilidad de los espermatozoides en el contexto del cambio climático.

RESUM

La selecció de gamets d'alta qualitat és un requisit indispensable a tenir en compte en els programes de reproducció assistida. El desenvolupament d'eines biotecnològiques com la criopreservació de gamets, juga un paper important en la producció aqüícola i en la formació de bancs de germoplasma, que contribuiran després a la millora genètica de les poblacions de peixos, principalment aquelles en perill i que poguessin estar més afectades davant de futurs canvis climàtics. Aquesta tesi està sent implementada sota un conveni de cotutela entre la Unesp i UPV. A la primera fase d'aquesta tesi realitzada a la Unesp, es va treballar amb una espècie neotropical d'elevada importància econòmica per a la regió. La Segona fase realitzada a la UPV es va treballar amb l'Anguila Europea (*Anguilla anguilla*), una espècie classificada a la Llista Roja de la Unió Internacional per a la Conservació de la Natura (UICN) com a espècie "en perill crític d'extinció". A la primera fase es va buscar caracteritzar la composició bioquímica del plasma i les característiques seminals de l'espècie. Avaluar les possibles relacions entre aquests paràmetres. A la composició del plasma seminal es va observar principalment ions de sodi (Na^+) i dins dels components orgànics van sobresortir les proteïnes totals i la glucosa. A través de l'anàlisi de components principals (PCA), es va observar que la motilitat tenia una forta correlació positiva amb el temps de motilitat, la concentració d'espermatozoides i les proteïnes totals. Aquestes anàlisis van servir de base per a la creació d'una solució diluent utilitzada posteriorment a la substància crioprotectora. Després es va determinar la influència del plasma seminal com a constituent de la solució crioprotectora en la criopreservació de semen de *P. reticulatum*. Es van utilitzar tres tractaments: glucosa 5% +

metanol 10% (T1), a aquest crioprotector se li va afegir 30% de plasma seminal natural (T2) i 30% de plasma seminal artificial sobre la base dels resultats dels components bioquímics del plasma determinats per a l'espècie a l'experiment anterior (T3). S'avaluaren paràmetres de motilitat espermàtica, capacitat fecundant del semen criopreservat, així com fragmentació de l'ADN. El tractament T1 va resultar amb els millors valors de motilitat seguit del T2, i la capacitat fertilitzant d'aquests dos tractaments va ser similar al control, però el tractament T2 va mostrar menys mal a l'ADN. Mitjançant la PCA es va demostrar que T1 tenia una millor associació amb la fertilitat i la motilitat total i progressiva. Finalment, avaluem les estructures de les subpoblacions espermàtiques a cadascun dels tractaments utilitzats. Mitjançant anàlisi multivariada en dues etapes, va ser possible determinar tres subpoblacions espermàtiques en el semen criopreservat de l'espècie, SP1 (ràpid-lineal), SP2 (ràpid-no lineal) i SP3 (lent- lineal). T1 va presentar el percentatge més gran de SP1, i va ser confirmat per l'efectivitat en la protecció de les cèl·lules d'aquest tractament en el procés de criopreservació de l'espècie. A la segona fase a la UPV, l'objectiu general va ser determinar l'efecte de la temperatura i el pH de l'aigua de mar sobre la motilitat dels espermatozoides a l'anguila europea. Es va determinar que el pH baix de l'aigua de mar (6.5-7.4) va disminuir la motilitat dels espermatozoides d'anguila en comparació del control (pH= 8.2). Quan estudiem l'efecte combinat del pH del plasma seminal artificial i el pH d'ASW (7.8 i 8.2), no trobem diferències estadístiques en la motilitat i la cinètica dels espermatozous en relació amb el pH del plasma seminal artificial, però sí el pH de l'aigua de mar. Es van trobar valors més alts de motilitat total (MOT), FA i ME amb un pH de 8.2 que amb un pH de 7.8. En contrast, la temperatura de l'aigua de mar no va afectar els paràmetres de motilitat dels espermatozous o

la longevitat dels espermatozous. Per estudiar el efecte de la interacció entre la temperatura de l'aigua de mar i el pH sobre la motilitat dels espermatozoides, es van provar dues temperatures: 4 i 24 °C, i dos pH: 7.8 i 8.2. Hi va haver diferències significatives entre la temperatura i el pH en diversos paràmetres cinètics, com MP, VCL, VSL, VAP, ME i SL, on els valors més baixos per a MP, VCL, VSL i VAP es van observar a les mostres activades a baixa temperatura i pH baix (4 °C, pH 7.8). Els nostres resultats suggereixen que l'acidificació de l'aigua de mar, però no les temperatures més altes, poden afectar la motilitat dels espermatozous en el context del canvi climàtic.

GENERAL INTRODUCTION

During the last decade, world fish production has been growing around 10% annually, mainly aquaculture production (Food and Agriculture Organization of the United Nations - FAO, 2020). This, in turn, has led to greater attention to fish reproduction research and in turn, knowledge about improving the quality of gametes becomes important. Along with this, the implementations of new technologies also increased, where cryopreservation began to play an important and viable role in industrial aquaculture. Cryopreservation has become an important tool for the conservation of many endangered species or long-term preservation and shipping of gametes in commercially important species including fish (Boryshpolets et al., 2020). Gamete quality and cryopreservation helps establish gene banks to reestablish populations using frozen samples and, in turn, mitigate the effects of climate change.

In the Neotropical region, Brazil presents itself as the country with the greatest diversity of fish species, with approximately 2,500 of the 4,475 species cataloged for the territory's watersheds. These particularities have aroused great interest both nationally and internationally, not only from the scientific community but also from companies linked to fish farming, thus stimulating research related to maintenance and development in captivity and promoting technological packages for native species with high value commercial (Buckup; Menezes; Ghazzi, 2007). In Brazil, the rapid growth of fish farming stands out, especially as intensive cultivation (Food and Agriculture Organization of the United Nations - FAO, 2020).

Enhancing the preservation of species diversity and genetic integrity can be achieved through the standardization of techniques for cryopreserving gametes, leading to the establishment of gene banks. Cryopreserved and thawed sperm is widely utilized as a crucial tool in global animal breeding programs. The potential benefits of extending this methodology to the aquaculture industry are similarly promising (Asturiano; Cabrita; Horváth, 2017). It provides a practical means to amplify the genetically effective population size and sustain genetic diversity, particularly for populations maintained in captivity (Robles; Santamaría; Casallas, 2005). One way to contribute to the conservation of diversity and genetic integrity of species is the standardization of methods or protocols for the cryopreservation of their gametes, which allows the creation of gene banks.

In this sense, seminal plasma characteristic must be evaluated, as it plays an essential physiological role in sperm maintenance and maturation, having a biochemical composition that supports and protects sperm viability, motility, and fertilization capacity, thus establishing an ideal environment for sperm storage (Ciereszko, 2008). The seminal plasma composition influences semen quality in general and plays a vital role in the sperm maturation and metabolism (Billard *et al.*, 1995; Bozkurt *et al.*, 2011; Ciereszko, 2008). The seminal plasma has specific characteristics that vary between species (Lahnsteiner *et al.*, 1998). In addition, detailed knowledge of seminal plasma components is essential to understand the events that lead to the production of good quality gametes and to identify crucial factors that may influence sperm function (Ciereszko; Glogowski; Dabrowski, 2011).

The gamete quality assessment is an essential component for developing effective cryopreservation protocols. To guarantee program must include assessments at all relevant stages throughout the process (Martínez-Páramo *et al.*, 2017). Standard methods of sperm quality assessment in fish include estimating sperm motility either subjectively or using sperm quality predictor software such as CASA (computer-assisted sperm analysis).

Despite the advantages of sperm evaluation which include a fast, accurate, and objective movement analysis for individual spermatozoa, its potential remains unexplored (Beirão *et al.*, 2011; Marinović *et al.*, 2021; Martínez-Pastor *et al.*, 2008; Gallego; Asturiano, 2018). Most researchers focused mainly on summarized statistics for the average values of the selected movement parameters and, in a way, considered only the spermatozoa within the same sample, forming a homogeneous population, and ended up failing to assess the intrinsic sperm variability of the samples (Beirão *et al.*, 2011; Gallego; Asturiano, 2018; Marinović *et al.*, 2021; Martínez-Pastor *et al.*, 2005; Martínez-Pastor *et al.*, 2011). The application of multivariate statistical analyses to the data produced by CASA has proven useful in classifying subpopulations (Martínez-Pastor *et al.*, 2011; Gallego *et al.*, 2015; Martínez-Pastor, 2022).

Among the species of Brazilian ichthyofauna, which stand out with potential for fish farming, we can mention *Pseudoplatystoma reticulatum*, belonging to the Siluriformes order, with the popular name of Surubim cachara (Kubitza, 1998). It is a species that has a high commercial value, being considered a noble product because it has tasty meat, with low fat content and the absence of intramuscular bones. These characteristics meet the current and future preferences of the fish market (Kubitza, 1998). However, due to overfishing and

environmental changes and/or destruction of their habitats, their populations are being significantly suppressed.

In this sense, the development of technologies that optimize its production in captivity becomes fundamental and the development of methodologies that allow the formation of a germplasm bank of the species is an eminent need to meet this demand. As part of the measures for the development of a protocol for cryopreservation of *P. reticulatum* semen, it is necessary to establish specific diluent solutions, mainly addressing the physiochemical characteristics of seminal fluid, thus allowing them to obtain effective method for semen cryopreservation gives species.

On the other hand, climate change is causing important physiochemical changes, especially in seawater, such as an increase in temperature or a decrease in pH (acidification), which affect the reproduction of aquatic organisms, although in many cases the effects are unknown specific damages. Thus, the expected environmental changes could jeopardize the reproduction of fish in captivity, limiting their fecundity and, therefore, the future of aquaculture. However, it is not known what temperature and pH values could be critical to endanger the reproduction of the different aquatic species and the sustainability of their production.

The European eel was the species chosen for this objective of our study. It has been classified in the Red List of the International Union for Conservation of Nature (IUCN) as a "critically endangered" species and therefore, it may be one of the species greatly affected by the climate change. However, it is not known what temperature and pH values could be critical for the reproduction of the

European eel. It is necessary to develop tools to assess the actual physiological effects of anticipated environmental changes and use them to anticipate and mitigate their potential harmful effects. With these purposes, our study aims to determine the effect of temperature and pH of the European eel sperm extender and activation medium on motility performance.

Projects, grants and universities involved in this Thesis

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OBJECTIVES

The first phase of this doctoral thesis was developed in UNESP (Brazil) with the neotropical freshwater species with high commercial value *Pseudoplatystoma reticulatum*. Our study had the following objective:

- To determine the effects of the biochemical composition of seminal plasma as part of the cryoprotectant solution in determining an effective protocol for cryopreservation of *Pseudoplatystoma reticulatum* sperm.

As specific objectives we had:

- To characterize the seminal plasma composition and determine the relationships between the seminal plasma components and the semen characteristics of *Pseudoplatystoma reticulatum* in captivity.
- To describe the influence of seminal plasma composition on the cryoprotective substance of *Pseudoplatystoma reticulatum* sperm, using glucose and methanol in cryoprotective solutions with the addition of natural seminal plasma and artificial seminal plasma.
- To evaluate the quality and viability of the cryopreserved-thawed semen of the species.
- To verify the integrity of the DNA of cryopreserved-thawed spermatozoa.
- To evaluate the fertilizing capacity of the proposed cryogenic treatments.
- To evaluate subpopulation structure of the cryopreserved-thawed *P. reticulatum* sperm.

The second phase of this thesis was carried out at the UPV (Spain) with the European Eel (*Anguilla anguilla*). As main objective in the second phase we had:

- To evaluate whether the physicochemical variations of seawater influence the kinetic parameters of spermatozoa from European eels.

As specific objective we had:

1. Determine the effect of seawater pH and temperature on eel sperm extender and activation medium on motility and longevity.

CHAPTER 1

Relationship between seminal plasma composition and sperm quality parameters of the catfish *Pseudoplatystoma reticulatum*

ABSTRACT

Sperm quality is a fundamental parameter for the effective reproduction of fish in captivity and the development of reproductive techniques, such as semen cryopreservation. This study aimed to determine the composition of the seminal plasma of *Pseudoplatystoma reticulatum* and analyze the relationships between plasma components and sperm characteristics. Nine males were induced with carp pituitary extract in the reproductive period of the species (November and December/2019). Semen characteristics were evaluated: subjective sperm motility, motility, duration, released sperm volume, sperm concentration, pH, osmolality, and seminal plasma composition, including levels of calcium, chloride, sodium, magnesium, potassium, glucose, fructosamine, triglycerides, and total protein. To determine the relationship between seminal plasma components and sperm motility parameters, a principal component analysis (PCA) was performed. The seminal plasma of *P. reticulatum* is composed mainly of the Na⁺ ion and organic components such as protein and glucose. Through PCA, it was observed that sperm motility had a strong positive correlation with motility time, sperm concentration, and total protein and a negative correlation with osmolality and fructosamine.

INTRODUCTION

Brazil is the country with the greatest powers in the world for the development of fish farming due to its climate and diversity of species. The growth in recent years in the aquaculture sector has contributed to the decrease in fish catches in the natural environment (FAO, 2020). With the increase in the production of fish in captivity, it becomes necessary to develop effective protocols that

optimize artificial reproduction. In this sense, it is fundamental to obtain biological and technical knowledge that allows the evaluation of the reproductive parameters of the fish, allowing the development of biotechnology that will make it possible to optimize the reproduction in captivity of these species, such as protocols for manipulation and storage of gametes.

The use of high-quality gametes from both males and females is an essential prerequisite to achieving high fertilization success and hatching, both for aquaculture and scientific purposes (Bozkurt *et al.*, 2008; Yoshida; Asturiano, 2020). In the evaluation of fish semen quality, one of the most important criteria used in the literature is sperm motility, generally expressed as the percentage and duration of sperm motility after activation (Gallego; Asturiano, 2018; Lahnsteiner *et al.*, 1996).

Seminal plasma has a unique species-specific composition, containing substances that support sperm cells (Cierieszko; Glogowski; Dabrowski, 2011). In addition, it plays an important physiological role in sperm maturation, having a biochemical composition that supports and protects the viability, motility, and fertilizing capacity of sperm, creating an ideal environment for their storage (Bozkurt *et al.*, 2011; Cierieszko, 2008).

In fish, unlike other vertebrates, seminal plasma is composed mainly of mineral compounds (Na^+ , K^+ , Mg^{2+} and Ca^+), being characterized by low concentrations of proteins, as well as other organic substances, such as hormones and pheromones, cholesterol, glycerol, vitamins, free amino acids, sugars, citric acid and lipids (Cierieszko; Ottobre; Glogowski, 2000; Cosson, 2004; Linhart; Schleta; Slavik, 1991). The determination of seminal plasma

composition has a great influence on the biological quality of the semen. It can help to understand the design requirements to prepare the appropriate artificial seminal plasma solutions (Ciereszko, 2008).

Knowledge of the relationships between seminal characteristics and the chemical composition of sperm is a prerequisite for successfully assessing the reproductive capacity of different fish species (Ciereszko; Glogowski; Dabrowski, 2011; Hussain *et al.*, 2018). Also, these would provide knowledge for the preparation of artificial plasma solutions, which can be used for the dilution of semen for short-term storage or cryopreservation (Billard; Cosson, 1992). In the last years, the interest of researchers in studies on seminal plasma in fish has been growing, especially in publications that somehow cover correlations between seminal plasma components and sperm motility.

However, descriptions of plasma components and possible relationships between sperm motility parameters in species of neotropical fishes need further investigation. And, referring to fish species belonging to the Siluriformes order, some works related to this theme can be highlighted in species such as *Clarias gariepinus* (Steyn; Van Vuren, 1986), *Clarias macrocephalus* (Tan-Fermin *et al.*, 1999) and *Rhamdia quelem* (Borges *et al.*, 2005), however, they are still very few.

The catfish species *Pseudoplatystoma reticulatum*, popularly known as “Surubim cachara” (Silva *et al.*, 2015) is among the species of Brazilian ichthyofauna that stand out with potential for fish farming. In the literature, no previous published works are describing the composition of seminal plasma and its relationship with sperm physical parameters. As it is a species that has

high commercial value as it has characteristics that meet the fish consumer market (Kubitza, 1998). Its natural populations can be affected over time because of indiscriminate capture, as well as the destruction of their natural habitats.

Thus, it is essential to have in-depth knowledge of the seminal characteristics of the species, thus generating basic knowledge for future cryopreservation studies that could help to maintain the genetic viability of their populations. Given the above, the present study aimed to characterize the composition of seminal plasma and determine the relationships between its components and the characteristics of the semen of *P. reticulatum* in captivity.

MATERIALS AND METHODS

Fish handling

Nine adult males of *P. reticulatum* from the breeding stock of the company Pirai Fish-farming, located in Terenos, Mato Grosso do Sul, Brazil (20°25'57" S and 55°17'11" W) were used for the development of the present work. The experiments were carried out between the late spring and early summer (November and December) of 2019, corresponding to the peak of the species' reproductive season. All procedures used with the animals for the development of this experiment follow the standards approved by the Ethics Committee for the Use of Animals (CEUA-FEIS/UNESP 04/2021).

Sperm collection and analysis of seminal characteristics

After capturing the specimens of *P. reticulatum* in the excavated ponds, they were placed in a concrete tank for hormonal induction and semen collection.

Adult breeders have an average body mass of 2.5 kg and were identified with microchips. The animals were hormonally induced by applying a single dose of carp pituitary extract at 3 mg/kg of fish.

The semen was collected ($n = 9$) after 232 hours/degree ($t = 8$ hours; $T = 29$ °C) by gentle abdominal pressing and stored in graduated sterilized falcon tubes and kept in a styrofoam box at approximately 4 degrees until analysis. The semen obtained from each specimen and seminal volume (Sem. Vol.) was determined, considering the maximum volume obtained via abdominal massage until the beginning of the presence of blood, avoiding contamination with urine, feces, and blood.

Subjective sperm motility (Mot) was determined under light microscopy based on the scale proposed by Fribourgh (1966). Sperm was activated with 0.45% NaCl in a proportion of 1:10 (semen: activator). Motility duration (Mot.tm) was measured from sperm activation to observation of only 10% of motile sperm. The pH of fresh semen was measured using a pHmeter (Checker®). For osmolality (Osm), the semen was centrifuged at 3000 rpm for 15 minutes, the supernatant was removed and analyzed in a cryo-osmometer (OSMOMAT® model 030, Berlin, Germany).

To determine the sperm concentration (Conc), the semen was diluted in formalin-saline solution at a proportion of 1:1000 (semen: solution), and the count was performed in a Neubauer hemocytometer chamber, the results were obtained according (Kavamoto *et al.*, 1985).

Analysis of seminal plasma components

To obtain seminal plasma, the semen was centrifuged at 3000 g for 15 minutes. After centrifugation, the supernatant was transferred to sterile polyethylene tubes, properly identified, and stored in an ultra-freezer at -80 °C until biochemical analysis. Aliquots were thawed at room temperature (~25 °C) for biochemical measurements of seminal plasma. The tests for concentrations of calcium (Ca⁺), chlorides (Cl⁻), sodium (Na⁺), magnesium (Mg²⁺), potassium (K⁺), glucose (Glic), fructosamine (Frut), triglycerides (Trig), and total proteins (Tot. pro)., were performed using commercial kits (LABTEST® Diagnostica S.A.), following the instructions for each analysis (LEITE *et al.*, 2018). All analyzes were determined using a SpectraMax Plus 384 spectrophotometer.

Statistical analysis

Data on sperm characteristics and respective components of seminal plasma were presented through a descriptive analysis expressed as mean ± SEM (Standard Error of the Mean). To determine the relationships between seminal plasma components and sperm characteristics of the species, a Principal Component Analysis (PCA) was performed to reduce the redundancy of the observed variables and identify patterns in the dataset. First, the data were standardized, allowing variables measured on different measurement units to be compared. The first two eigenvectors with the highest percentage of accumulated variance were considered to construct the PCA graph. For this analysis, six individuals of the total analyzed were used. All analyzes were performed using the R Software, “FactorMineR” (R Core Team, 2020).

RESULTS

Sperm characteristics of *Pseudoplatystoma reticulatum*

Spermatological parameters of the sperm of *P. reticulatum* were found rather variable and they are shown in Table 1. The parameters of osmolality, motility, and motility time were where the greatest variability was found, with pH and concentration being the least variable.

Table 1. Sperm characteristics of *Pseudoplatystoma reticulatum* (n=9). Minimum values (Min), maximum values (Max), Mean value (Mean), Standard error of the mean (SEM).

Variable	Min	Max	Mean	SEM
Seminal volume (mL)	2.90	12.00	7.80	1.04
Sperm motility (%)	70.00	95.00	86.25	3.09
Time motility (s)	75.00	112.00	89.25	3.92
Concentration - (spz.10 ⁹ /mL)	12.70	18.10	15.71	0.72
pH	7.33	8.00	7.75	0.08
Osmolality (mOsm/kg)	173.66	276.00	211.62	11.01

Seminal plasma composition of *Pseudoplatystoma reticulatum*

The results of the analysis of the seminal plasma are shown in Table 2. The seminal plasma of *P. reticulatum* is mainly composed of the Na⁺ ion, followed by the Cl⁻, Ca⁺ and K⁺ ions, while the Mg²⁺ ion showed the lowest values. Within the organic components, total protein and glucose stood out; on the other hand, fructosamine and triglycerides had low values.

Table 2. Seminal plasma components of *Pseudoplatystoma reticulatum* (n=6). Minimum values (Min), maximum values (Max), Mean value (Mean), Standard error of the mean (SEM), Calcium (Ca⁺), Chlorides (Cl⁻), Sodium (Na⁺), Magnesium (Mg²⁺), Potassium (K⁺), Glucose (Glic), Fructosamine (Frut), Triglycerides (Trig), and Total proteins (Tot. pro).

Variable	Min	Max	Mean	SEM
Ca⁺ (mg/dL)	7.94	26.42	12.67	2.15
Cl⁻ (mM/L)	2.83	4.95	4.24	0.25
Na⁺ (mM/L)	108.78	140.97	124.87	3.81
Mg²⁺ (mEq/L)	0.36	2.14	1.15	0.24
K⁺ (mM/L)	0.01	6.09	3.15	0.75
Glic (mg/dL)	124.11	365.24	205.67	39.63
Frut (μmol/L)	23.93	131.63	64.75	15.67
Trig (mg/dL)	1.86	11.60	4.97	1.24
Tot. Pro (g/dL)	0.00	0.85	0.39	0.12

Correlation between seminal plasma components and seminal characteristics of *Pseudoplatystoma reticulatum*

In the principal component analysis (PCA) to determine the possible relationships between the seminal plasma components and the sperm characteristics of this species, the first two components were chosen: the first component (PC1) explained 47.1% of the data variance and the second component (PC2) explained 21.42%, totaling 68.52% of the total variation of the data (Figure 1).

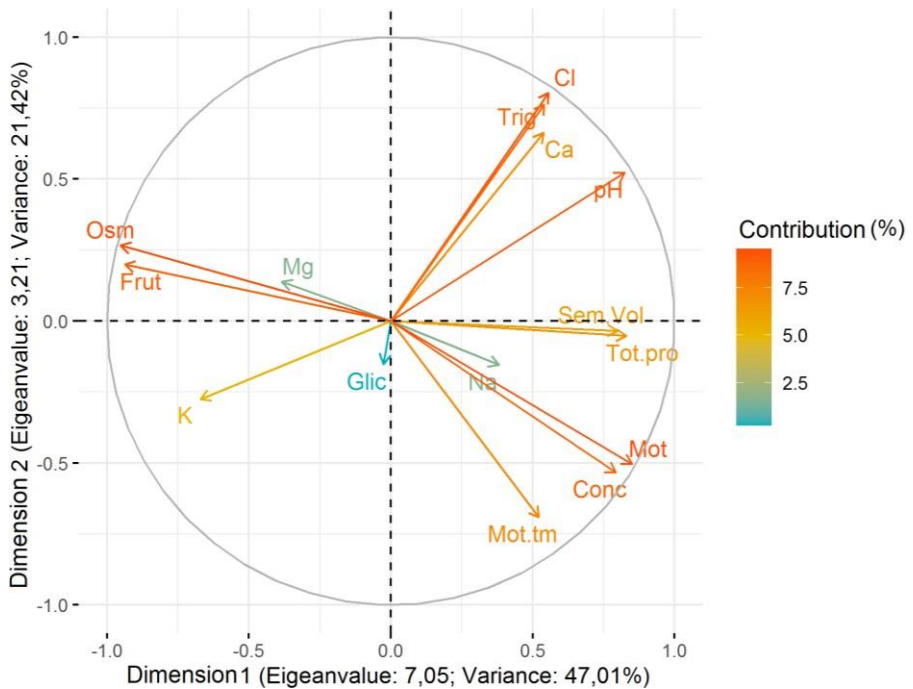


Figure 1. Representation of the first two axes obtained by PCA analyzing the relationships between the components of the seminal plasma and the sperm

characteristics of *Pseudoplatystoma reticulatum* (n=6). Total motility (Mot), time of motility (Mot. tm), Concentration (Conc) Calcium (Ca⁺), Chlorides (Cl⁻), Sodium (Na⁺), Magnesium (Mg²⁺), Potassium (K⁺), Glucose (Glic), Fructosamine (Frut), Triglycerides (Trig) and Total proteins (Tot.pro).

The first two axes are obtained through the PCA and represented in Figure 1, which can be observed in the colors from light blue to orange that indicate the percentage of contribution in the explained variance, with light blue for the lowest value and orange for the highest percentage.

The graph shows a cluster between variables such as Mot, Mot.tm, and Conc, indicating a strong positive correlation between them and Sem.vol and Tot.pro. On the other hand, Frut and Osm had a high positive correlation, but a negative relationship with Mot, Mot.tm, Conc, Tot.pt, and pH. The Cl⁻, Ca⁺, Trig, Sem.Vol, and Tot.pro variables had a strong positive correlation with pH, while this variable had a strong negative correlation with K⁺, which in turn had a positive correlation with Osm and Frut. The variables Glic, Na⁺ and Mg²⁺ contributed little to the variance of the analyzed data, but the Na⁺ and Mg²⁺ had a strong negative correlation between them.

DISCUSSION

Seminal plasma is an important component of fish semen, playing a vital role in sperm metabolism, function, survival, and motility (Navarro; Lemos; Ribeiro, 2019; Pinzón-Arciniegas; Mojica-Rodríguez; Cruz-Casallas, 2005). Numerous components of seminal plasma are directly linked to these functions. This study to determine the main plasmatic components of *P. reticulatum* semen and its

relationship with parameters of seminal quality is unprecedented and will serve as a basis for studies of the reproductive aspects of other fish species, in addition to providing important information for the development of extenders, extender immobilizers and cryoprotective solutions.

The data obtained in the evaluation of the characteristics of the physical parameters of the species were variable among the individuals. Variations in seminal characteristics are related to physiological variations of the specimens, linked to their genetics and how they react to the “environmental conditions” to which they are subjected. Semen quality, particularly in aquaculture species, depends on several external factors, such as feeding regime, feed quality, temperature, and male reproductive season (Bozkurt *et al.*, 2008).

The seminal plasma in fish, in contrast to that of other vertebrates, is characterized by low concentrations of proteins and other organic substances, containing mainly mineral compounds such as Na⁺, K⁺, Ca⁺, and Mg²⁺ (Ciereszko, 2008). In the present study, the seminal plasma was composed of Na⁺ ions like that reported for *Clarias macrocephalus*, 164.4 ± 0.6 mM/L (Tan-Fermin *et al.*, 1999).

Usually in seminal plasma, the sodium concentrations are 10 times higher than potassium concentrations (Tan-Fermin *et al.*, 1999). In membrane permeability processes, ions play a fundamental role, according to the variation of their concentrations in the extra- and intracellular medium, some of these involved inhibitory effects on the initiation of sperm motility. The increase in Na⁺ may be related to the high secretory activity in the sperm ducts (Lahnsteiner; Patzner; Weismann, 1993), for *P. reticulatum* it seems to

be one of the fundamental ions in the maintenance of seminal plasma function. For other species of the order Siluriformes, similar concentrations to those obtained in this work were observed, i.e., *Clarias gariepinus* 125.25 ± 9.80 (Steyn; Van Vuren, 1986); *Clarias macrocephalus* 164.4 ± 0.6 (Tan-Fermin *et al.*, 1999), *Rhamdia quelem* 153.7 ± 2.4 (Borges *et al.*, 2005), but the deference may be due to differences in feeding conditions, age, environmental factors within the same species due to their reproductive processes. The Na^+ ion can act directly on the osmotic balance, motility, morphology, and metabolism of sperm (Rodríguez *et al.*, 2013).

Potassium (K^+) presented low concentration values in the present study, compared to other freshwater species, such as *Alburnus alburnus*, *Hypophthalmichthys molitrix*, *Clarias macrocephalus* (Lahnsteiner *et al.*, 1995; Rahman *et al.*, 2011; Tan-Fermin *et al.*, 1999). It has been reported that low concentrations of K^+ in seminal plasma may be associated with a high percentage of motility and better seminal quality in salmonids (Billard; Cosson, 1992). It has also been documented that in several species of the order Siluriformes, K^+ levels are generally low, but this variability is species-specific and not always low rates of this ion are related to low sperm quality since depending on the concentrations of the other ions which can also influence the motility mechanisms (Dziewulska, 2020).

Ca^+ and Mg^{2+} ions contribute significantly to the composition of fish sperm seminal plasma. These cations are effective as antagonists of the inhibitory effect of K^+ on the motility of the Na^+ ion (Billard; Cosson, 1992). In this study, the values of Ca^+ and Mg^{2+} were similar to those compared with other

siluriformes species (Borges *et al.*, 2005; Tan-Fermin *et al.*, 1999).

The concentration of glucose in the seminal plasma has a primordial function for the protection of spermatozoa, providing high energy during the process of spermiogenesis, as well as in sperm motility. Different sperm glucose concentrations may indicate differences in sperm metabolic energy from numerous fish species (Hussain *et al.*, 2018). In the present study, glucose showed a high concentration in seminal plasma, which coincides with what was detected for other fish species (Lahnsteiner *et al.*, 1995), which may suggest that *P. reticulatum* spermatozoa also have a need for higher amounts of energy for its functions, compared to a species of the same genus, *Pseudoplatystoma metaense*, for which (Ramírez-Merlano; Medina-Robles; Cruz-Casallas, 2011). In turn, the presence in seminal plasma of glucose and high levels of fructosamine, which is described as a glycated protein that is not commonly investigated in seminal plasma of animals, may indicate the inefficient use of glucose by the organism (Armbruster, 1987). In humans, high fructosamine levels may indicate infertility (Tomaszewski *et al.*, 1992). However, it is also known that fructosamine is consumed by superoxide dismutase (SOD), indirectly used to determine the activity of this antioxidant enzyme in the seminal plasma of dogs (Lopes *et al.*, 2011). Considering this information, together with the data obtained from the PCA analysis in this study, we can consider that fructosamine has a negative correlation with the parameters of sperm motility.

The PCA is an interesting tool, as it is a type of exploratory analysis that can help in understanding the results obtained presenting positive correlations between motility mainly with the concentration and motility time corresponding

to that reported for freshwater species such as *Rhamdia quelem*, *Salmo trutta*, *macrostigma*, *Barbus grypus* (Borges *et al.*, 2005; Bozkurt *et al.*, 2011; Güllü *et al.*, 2015).

However, in the case of motility and concentration, negative correlations have already been reported for species such as *Prochilodus lineatus* (Viveiros *et al.*, 2019), *Oncorhynchus mykiss* (Ciereszko; Dabrowski, 1994), *Cyprinus carpio* (Bozkurt *et al.*, 2009). It can be considered that a higher sperm concentration is not always related to a higher motility and fertilization rate (Williot; Kopeika; Goncharov, 2000).

It is understood that sperm concentration is species-specific and may be related to gonadal development and maturation, which in turn will depend on changes in temperature, nutritional quality, enzymatic activity, and age, which varies between individuals (Piros *et al.*, 2002; Shaliutina-Kolešová *et al.*, 2020).

Total proteins also play an important role in energy allocation in sperm, which may be the cause of a positive relationship with motility in the present study. Lahnsteiner; Mansour; Berger (2004) report that proteins in seminal plasma prolong sperm viability in *Oncorhynchus mykiss*. Lipids in seminal plasma are also used by spermatozoa as energy reserves while they remain in the spermatid ducts, and the synthesis of triglycerides may be a response to distinct physiological changes during artificial sperm storage (Lahnsteiner; Patzner; Weismann, 1993).

In the present study, triglycerides did not directly link with motility but showed a positive correlation with pH, Cl⁻, and Ca⁺. Specific physiological relationships between these variables should be studied in more detail in the future, however,

Lahnsteiner *et al.* (1998) reported that low triglyceride concentrations may indicate inadequate energy sources, which may reduce motility rates and fertilizing capacity in *O. mykiss*. Cl^- and Ca^+ ions also showed no apparent relationship with motility in the present study.

The pH was not correlated with motility in this study. However, other authors have reported the effects of pH on sperm motility in several fish species, suggesting its importance in seminal plasma characteristics and membrane potential as well as motility (Baradaran Noveiri *et al.*, 2019; Billard; Cosson, 1988; Gallego *et al.*, 2014; Pérez; Gallego; Asturiano, 2020; Silva Pinheiro *et al.*, 2020). According to previous studies, during the passage of sperm from the testis to the spermatic duct, an increase in external pH may be responsible for the acquisition of motility in some salmonid fish (Morisawa; Morisawa, 1986, 1988) and, therefore, the pH of the seminal fluid can also affect the final maturation of sperm (Lahnsteiner *et al.*, 1998).

Several authors have reported the importance of the Na^+ ion in the beginning of sperm motility. For this reason, it is widespread to observe activating solutions that contain Na^+ , i.e. for *Esox lucius* L. (Hadi-Alavi *et al.*, 2009) *Prochilodus lineatus* (Viveiros *et al.*, 2009), *Brycon insignis* (Orfão *et al.*, 2011), *P. lineatus* and *B. orbignyanus* (Viveiros *et al.*, 2019). In the present study, sodium contributed little to the variance of the data, but it also had a trend in the graph very close to motility, so it could be positively related to this variable.

Knowledge of the physical and chemical constituents of sperm and seminal plasma is a prerequisite for success in assessing the reproductive capacity of different fish species. It is important to emphasize that the composition of

seminal plasma can vary not only according to the characteristics of each species but also due to other external factors such as age, reproductive period, nutrition, and many others.

CONCLUSION

The seminal plasma of *P. reticulatum* has the predominant ions and sugars in its composition, Na⁺ and glucose, respectively, in addition to proteins. Was observe positive relationships between motility and the parameters of motility time, sperm concentration, total protein, and negative relationships with osmolality and fructosamine.

CHAPTER 2

Seminal plasma as part of the extender in cryopreservation of *Pseudoplatystoma reticulatum* semen: effect on sperm motility and subpopulations

ABSTRACT

The cryopreservation of semen from neotropical fish is an important tool for aquaculture, being necessary to establish cryogenic protocols for most native species. This research aimed to determine the influence of seminal plasma as a constituent of the cryoprotective solution on the cryopreservation sperm and of sperm subpopulation structure of the cryopreserved-thawed *Pseudoplatystoma reticulatum* semen. As cryoprotective solutions were used: T1 = 5% glucose + 10% methanol; T2 = T1 + 30% of *P. reticulatum* natural seminal plasma e; T3 = T1 + 30% artificial seminal plasma. The data were analyzed by the sperm analysis program (CASA), and a Principal Component Analysis (PCA) was applied. DNA damage was evaluated using the comet assay. There was a statistically significant difference in the variables of Total Motility (MOT) and Progressive Motility (PM) between T1 and T3, with no significant difference between these and T2. The fertilization test showed statistical differences between T3 and Control (fresh sperm), T2 and T1. In the evaluation of DNA, the three treatments showed significant differences, with T2 being the most effective in protecting against DNA damage. The PCA analysis showed that the FERT and seminal quality variables MOT and MP better represented T1. Three subpopulations were present for the cryopreserved-thawed sperm, SP1 (fast-linear), SP2 (fast-non-linear), and SP3 (slow-linear). The simple combination of methanol (10%) and glucose (5%) was the most effective treatment for maintaining fast and linear subpopulations. The treatment supplemented with seminal artificial plasma did not show effective results in the protection of the spermatozoa of the species Therefore, we can

suggest T1 whit a high degree of protection in the cryopreservation of *P. reticulatum* sperm.

INTRODUCTION

Brazil has the greatest diversity of fish in the Neotropical region since approximately 2,500 of the 4,475 species are registered and cataloged in hydrographic basins. This characteristic has aroused great interest, both nationally and internationally, from the scientific community and companies linked to fish farming, thus stimulating research on the maintenance and production of Neotropical species in captivity. Given this scenario, fish farming in the national territory has grown rapidly in recent years, especially concerning intensive cultivation (Buckup; Menezes; Ghazzi, 2007; FAO, 2020).

Beforehand, the fish semen cryopreservation technique is an important tool in the production of aquatic organisms, both for the development of genetic improvement programs and for the conservation of genes in germplasm banks (Carolsfeld *et al.*, 2003; Martínez-Páramo *et al.*, 2017; Tiersch, 2008). The application of this technique constitutes a practical method to increase or genetically adequate size of the populations and maintain their genetic diversity, especially of the species kept in captivity (Robles; Santamaría; Casallas, 2005).

Many factors can alter the physiological state of the cells subjected to the cryopreservation process. Therefore, the success of the application of this technique will depend on the preservation of the structural and metabolic characteristics of the spermatozoa, as well as maintaining their ability to move and fertilize the female gamete (Watson, 2000).

The leading cause of damage to cells during the cryopreservation process is the formation of intracellular gel crystals since this damage can be remedied using cryoprotective substances, according to the seed characteristics of the species (Robles; Santamaría; Casallas, 2005). Likewise, the determination of the most suitable composition of the extender and the selection of the type and concentration of the cryoprotectant is fundamental stages in the development of a cryopreservation protocol to meet the specific needs of the sperm of each species (Ciereszko *et al.*, 2014; Lahnsteiner *et al.*, 1996).

Simple extenders that contain only permeable cryoprotectants and sugars as non-permeable cryoprotectants have been used successfully to cryopreserve salmonid sperm (Ciereszko *et al.*, 2014). The use of a cryoprotective solution including glucose and methanol is effective in increasing the number of motile spermatozoa after seminal thawing in various species of fish, such as *Coregonus lavaretus*, *Salvelinus alpinus* L., *Oncorhynchus mykiss* (Ciereszko, 2008; Ciereszko *et al.*, 2013, 2014; Mansour; Richardson; Mcniven, 2006). In catfish there are reports that methanol was the most suitable internal cryoprotective substance for freezing semen, citing species such as *Clarias gariepinus* and *Pseudoplatystoma corruscans* (Carolsfeld *et al.*, 2003; Viveiros; So; Komen, 2000).

Research in different species of teleost fish confirms the importance of adding seminal plasma to the composition of freezing solutions as an antioxidant system, which in turn minimizes the effect of damage to sperm cells during the cryopreservation (Ciereszko *et al.*, 1999; Figueroa *et al.*, 2013, 2015; Martínez-Páramo *et al.*, 2013; Nynca *et al.*, 2014; Shaliutina-Kolešová *et al.*, 2020).

Researchers have also reported that the incorporation of seminal plasma in a freezing medium causes minor damage caused by the cryopreservation process to the DNA of the gametes (Figueroa *et al.*, 2013, 2015).

Currently, research has a growing interest in the study of sperm subpopulations. The theory that sperm samples are not homogeneous and that various subpopulations coexist in the same sample has been accepted among the scientific community (Beirão *et al.*, 2011). In this sense, the study of sperm cell subpopulations is relatively recent and still little explored. Still, it represents an essential source of new information on male fish gametes' biology and intrinsic variations (Martínez-Pastor *et al.*, 2005).

The kinetic characteristics of spermatozoa represent a more adequate criterion for determining subpopulations. The assessment of sperm motility was further enhanced with the application of computer-assisted sperm analysis software (CASA) (Gallego *et al.*, 2017; Gallego; Asturiano, 2018; Marinović *et al.*, 2021). This tool has enabled a fast, accurate, and objective movement analysis of spermatozoa, but although the application of this system is quite broad, its potential is rarely fully utilized (Beirão *et al.*, 2011; Marinović *et al.*, 2021; Martínez-Pastor *et al.*, 2008).

Currently, there are no published works with information on cryopreservation protocols for this species, which guarantees seminal quality and success in the fertilization process with the use of thawed semen, which in turn allows its practical application, both in production and in genetic banks.

However, in the neotropical region, the subject has been little explored, with only one work in the literature so far, published by Gallego *et al.* (2017) for

Colossoma macropomum, who identified and characterized sperm subpopulations through kinetic parameters in fresh spermatozoa, evaluating the changes caused by the cryopreservation process, and evaluating the correlation of these subpopulations with fertilization rates.

Pseudoplatystoma reticulatum belongs to the family Pimelodidae of the Order Siluriformes and is one of the priority species for the growth of national fish farming due to its desirable commercial and zootechnical characteristics, such as fast growth and efficient feed conversion (Inoue *et al.*, 2009). For this species, there are few studies on seminal evaluation, one on seminal characterization in spawning (Shiro Júnior, 2013) and another on the evaluation of the effects before and after hormonal induction with carp pituitary on seminal quality in captivity (Streit Junior *et al.*, 2012).

This research aims to determine seminal plasma's influence as a cryoprotectant solution's constituent on the cryopreservation sperm and subpopulation structure of the cryopreserved-thawed *P. reticulatum* sperm.

MATERIALS AND METHODS

Breeder management

For the study, eight male and one female specimen of *P. reticulatum* belonging to the breeding stock of Pirai Fish Farm, located in the municipality of Terenos, Mato Grosso do Sul, Brazil (20°25'57" S and 55°17'11" W) were used. The collection was carried out between December 2020 and January 2021. All adult breeders were previously marked with a microchip and had an average body mass of 2.5 kg. All procedures used with the animals to develop this experiment

are within the standards approved by the Ethics Committee for the Use of Animals FEIS UNESP (CEUA-FEIS/UNESP 04/2021).

Sperm collection and quality evaluation

Adult male specimens of *P. reticulatum* were reared in two tanks maintained at 25 °C. The hormonal inducer was administered in a single dose of crude extract of carp pituitary (EBH) (Danubio Aquaculture, Brazil) at 3 mg/kg of live weight. For dilution of hormone was used 0.5 mL/kg of saline solution (0.9% NaCl) dose. After 232 hours/degrees (t = 8 hours; mean T = 29 °C), the semen was extruded using abdominal massage in the anteroposterior direction of the body, avoiding contamination with urine, feces, and blood. It was collected in sterile graded tubes, and the initial motility was evaluated to choose those samples showing more than 80% of motile sperm for the freezing process.

Seminal plasma extractions and artificial seminal plasma compositions

The semen was centrifuged twice at 3000 rpm for 15 minutes. After centrifugation, the supernatant (seminal plasma) was collected and transferred to sterile polyethylene tubes for later use in the cryoprotectant medium. The artificial plasma was composed of NaCl (7.35 mM), CaCl₂ (0.06 mM), KCl (0.09 mM), MgSO₄ (0.04 mM), NaH₂PO₄ (0.04 mM), glucose (2 mM), pH (7.7), Osmolality (269 mOsmol/kg). This solution was made based on previous studies (unpublished personal data) of the composition of the seminal plasma of the species.

Sperm cryopreservation

For the freezing process, were selected four *P. reticulatum* males. As cryoprotectant solutions were tested: T1: 5% glucose + 10% methanol (as a control solution previously tested); T2: T1 + 30% of seminal plasma of the species (Figueroa *et al.*, 2013, 2015), and T3: T1 + 30% of artificial plasma.

The semen was diluted in a proportion of 1:5 (semen: extender), packed in 0.5 mL PVC straws (n= four males; 6 straws/male), which were arranged in a steel tray and frozen in liquid nitrogen vapor at 3 cm above the liquid nitrogen level (-185 °C), inside an isothermal styrofoam box with a freezing time of 10 min., then immersed in liquid nitrogen. Subsequently, they are placed in racks and stored in a cryobank (Taylor-Wharton, XT21-A111M). The thawing process was carried out in a water bath at 37 °C for 10 s, avoiding contact of the semen with water.

Cryopreserved-thawed sperm motility analysis

Sperm activation was performed by adding 30 µl of NaCl solution (0.45%) and 0.1 µl of semen through a Makler™ camera (Sefi Medical Instruments Ltd, Israel). The analyses were performed within 5 s of activation and then every 5 s until completed 30 s. Three replicates were recorded for everyone.

Motility parameters of cryopreserved-thawed semen were measured using the ISAS® CASA (Computer Assisted Sperm Analysis) system (ISAS® Integrated Semen Analysis System, Proiser, Valencia, Spain) coupled to a UB200i phase contrast microscope (UOP/Proiser) with a 10x negative phase contrast objective. Images were captured with an ISAS 782C camera (Proiser, Spain)

and recorded at 25 frames per second (fps).

The following parameters were measured: total motility (MOT, %), progressive motility (PM, %), curvilinear velocity (VCL, $\mu\text{m/s}$), linear velocity (VSL, $\mu\text{m/s}$), average speed (VAP, $\mu\text{m/s}$), linearity coefficient (LIN, %), coefficient of straightness (STR, %), average spermatozoa spatial trajectory oscillation (WOB, %), the amplitude of lateral head displacement (ALH, μm), beat crossing frequency (BCF, Hz). The following parameters were considered for software calibration: (STR) >80% to consider progressive spermatozoa and VCL >10 $\mu\text{m/s}$ for motile cells.

Fertilization test

To analyze the fertilizing capacity of cryopreserved-thawed semen of *P. reticulatum*, a mature female was induced with two doses of carp pituitary extract (EBH). First one of 4.5 mg/kg live fish, and a similar one 8 hours later. Four males were selected for semen collection for fertilization control and received a single dose of 3 mg EBH/kg of live fish, coinciding with the second dose of the females. Afterward, the eggs were removed from the female by abdominal massage in the anteroposterior direction of the body. Four replicates were tested for each treatment, each male being replicated.

The number of oocytes per sample was established by the number of non-hydrated oocytes, averaging 1g of sample, corresponding to 1804 ± 156 oocytes/g. For fertilization with cryopreserved-thawed semen, one straw of thawed semen was used for 1 g of oocytes (~1800 oocytes), establishing a ratio of 7.5×10^4 sperm/oocyte. The semen was thawed in a water bath at 37 °C for

10 s. A 0.45% NaCl solution was used to activate the spermatozoa. The fertilized and hydrated eggs were taken to the incubators and, after 8 hours, were fixed in 10% buffered formalin for later determination of the fertilization percentage.

DNA integrity

The DNA integrity of cryopreserved-thawed spermatozoa was determined using the comet assay, according to the protocol proposed by Carneiro-Leite *et al.* (2020) and Klaude *et al.* (1996). For this purpose, slides were prepared with a thin layer of *Normal Melting Temperature Agarose* (NMA), for which the slides were dipped in the agarose, the excess was removed, left to dry, and then stored in the refrigerator. Then, 10 μ l of diluted semen and 120 μ l of *Low Melting Temperature Agarose* (LMA) were added to a microtube, then three drops of this solution were placed on the slides previously prepared with agarose, then covered with a coverslip and taken to the refrigerator for about 30 minutes to solidify the (LMA). After this period, the coverslips were removed and the slides placed in a glass vat, covered with the lysis solution pH 10 (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, Lauryl 35 mM), and kept immersed for at least one hour at 4 °C. Subsequently, the slides were placed in pH 13 electrophoresis solution (200 mM EDTA, 10 N NaOH), and kept for 30 minutes at 4 °C; then electrophoresis was performed for 20 minutes at 25 V and 300 mA.

After this process, the slides were covered with neutralization solution pH 7.5 (0.4 M Tris) for 5 minutes (3x), then dried and fixed by immersion in absolute

ethanol, cooled for 5 minutes, and dried again. The slides were stained with 100 µl of ethidium bromide solution for analysis. Each slide was covered with coverslips and analyzed in 40x objective with a fluorescence microscope. A hundred cells were counted for each treatment. The recorded images were analyzed with the Open Comet add-in (v1.3.1) of the Image J processing platform (Gyori *et al.*, 2014).

The parameter used to compare DNA damage in different treatments was the percentage of tail DNA provided by the program, which is the percentage of DNA (% DNAt) migrated in the tail compared to the head ($DNA\ Tail = 100 - DNA\ Head$).

Sperm subpopulations analyses

For the study of selected sperm subpopulations, data of all moving spermatozoa in cryopreserved-thawed sperm analyzed by the CASA system were imported for a single data set or data matrix that represented a total of 9116 observations according to the methodology proposed by Martínez-Pastor *et al.* (2005), with a modification for the definition of resulting clusters, in the present study using the NbClust of software R with 30 indexers.

Subpopulation analysis was performed in four sequential steps: principal component analysis (PCA) using the eight motility variables, with the main components being selected with an eigenvalue higher than one (Kaiser criterion); nonhierarchical clustering analysis (k-means) using the first two principal components, using Euclidean distances to calculate cluster centers.; hierarchical clustering analysis, using the average linkage method (UPGMA);

and selection of the final number of clusters to define the best 15 clusters, we used 30 indexers proposed by Charrad *et al.* (2014) on package NbClust of software R.

Statistics

The descriptive analysis of all variables evaluated in the study was performed considering the mean and standard deviation values. The data were submitted to the Shapiro-Wilk normality test, and values greater than three times the interquartile range were considered *outliers* and removed from the analyses. The variables, which had repeated measures, were analyzed using the General Linear Model (GLM).

In the fertility and DNA fragmentation variables, the treatment effect was evaluated in an Analysis of Variance (ANOVA - one way), and homoscedasticity was met according to Levene's test. Contrasts within treatments were obtained using the Bonferroni test. Principal component analysis (PCA) was based on the correlation matrix between variables. The first two eigenvectors with the highest percentage of accumulated variance were considered for graph construction. The *ellipses* indicate the grouping degree of the evaluated groups based on a coefficient of 0.95. All analyses were performed in R Software (R Core Team, 2020).

In the descriptive analysis of the motility data, it was expressed by half of the median and interquartile interval values, and the absence of normality was observed using the Shapiro-Wilk test. The subpopulations and their structure were determined using the Kruskal-Wallis test, and the difference between the

groups was verified using the Bonferroni multiple comparison test. All the analyses were carried out in Software R (R Core Team, 2020), adopting a significance level equal to 5%.

RESULTS

Cryopreserved-thawed sperm motility analysis

The results of the characteristics of the motility variables analyzed by the CASA program is presented in Table 1. Here was a significant statistical difference between treatments T1 and T3 regarding the variables MOT and MP, while the rest of the variables showed no differences between treatments.

Table 1. Descriptive analysis and effect of treatments on motility variables (mean \pm SE) in cryopreserved-thawed *Pseudoplatystoma reticulatum* sperm. Glucose 5% + Methanol 10%; T2: T1 + 30% seminal plasma from the species; T3: T1 + 30% artificial plasma; total motility (MOT, %), progressive motility (PM, %), curvilinear velocity (VCL, $\mu\text{m/s}$), linear velocity (VSL, $\mu\text{m/s}$), average velocity (VAP, $\mu\text{m/s}$), linearity coefficient (LIN, %), straightness coefficient (STR, %), average spermatozoa spatial trajectory oscillation (WOB, %), lateral head displacement amplitude (ALH, μm), beat crossing frequency (BCF, Hz). Within the rows, values with different letters indicate that the values differ significantly ($p < 0.05$).

Variables	T1	T2	T3
MOT (%)	27.23 ± 9.44 ^a	20.99 ± 4.44 ^{ab}	15.70 ± 4.63 ^b
MP (%)	11.47 ± 3.61 ^a	13.06 ± 5.61 ^{ab}	8.67 ± 2.62 ^b
VCL (µm/s)	62.02 ± 11.19	59.87 ± 8.53	59.2 ± 10.84
VSL (µm/s)	52.88 ± 13.43	53.62 ± 11.19	51.38 ± 12.50
AP (µm/s)	57.69 ± 12.36	58.25 ± 9.91	55.27 ± 11.83
LIN (%)	85.87 ± 4.76	86.12 ± 4.21	85.90 ± 7.18
STR (%)	90.75 ± 2.68	91.35 ± 2.60	92.38 ± 3.78
WOB (%)	93.32 ± 2.29	93.59 ± 1.96	92.85 ± 4.24
ALH (µm)	1.48 ± 0.21	1.45 ± 0.15	1.39 ± 0.11
BCF(Hz)	7.83 ± 0.57	8.18 ± 0.56	8.33 ± 0.41

Figure 1 (A-B) show the significant statistical difference between treatments T1 and T3. T2 did not show statistical differences with treatments T1 and T3 but obtained the highest results for MP, Figure 1 (B).

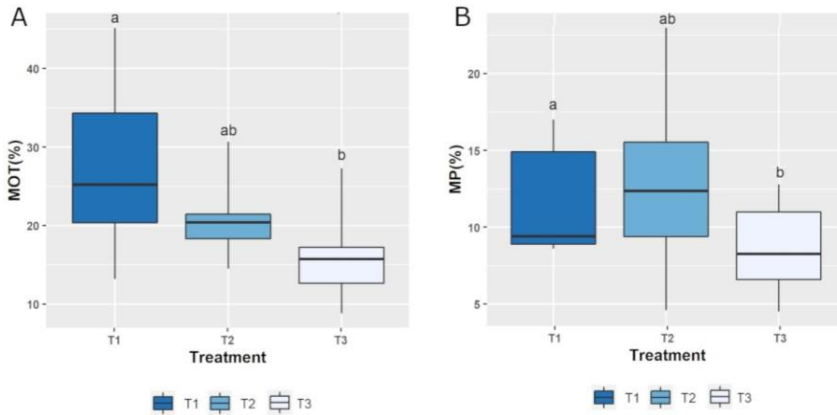


Figure 1. Evaluation of Total Motility (MOT)- A and Progressive Motility (MP)- B, among the three treatments analyzed for *Pseudoplatystoma reticulatum*. T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma of the species; T3 - T1 + 30% artificial plasma. Values with different letters differ significantly ($p < 0.05$).

Fertilization test

In the effects of treatments on fertilization, there was a statistical difference between T3 with the control and treatments T1 and T2. However, treatments T1 and T2 had no statistical differences and showed the best fertilization rates, like the control (Figure 2).

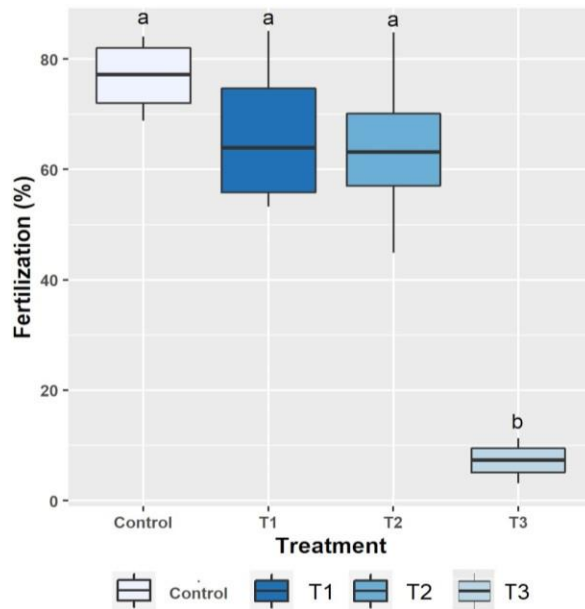


Figure 2. Comparative analysis of the effect of cryoprotective treatment on the fertilization variable with cryopreserved-thawed semen (4 replicates/treatment) of *Pseudoplatystoma reticulatum*. Control - fresh semen; T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma of the species; T3 - T1 + 30% artificial plasma. Values with different letters differ significantly ($p < 0.05$)

DNA integrity

The results obtained in the analysis of the comparison of DNA damage in the three treatments represented by the percentage of DNA in the tail (% DNAt) showed statistically significant differences (Figure 3). T2 provided the lowest rate of DNA damage with an average of $7.99 \pm 2.92\%$, followed by T1 ($11.12 \pm 4.07\%$), and, finally, T3 provided the highest rate of DNA damage in cryopreserved-thawed *P. reticulatum* spermatozoa ($25.48 \pm 7.27\%$).

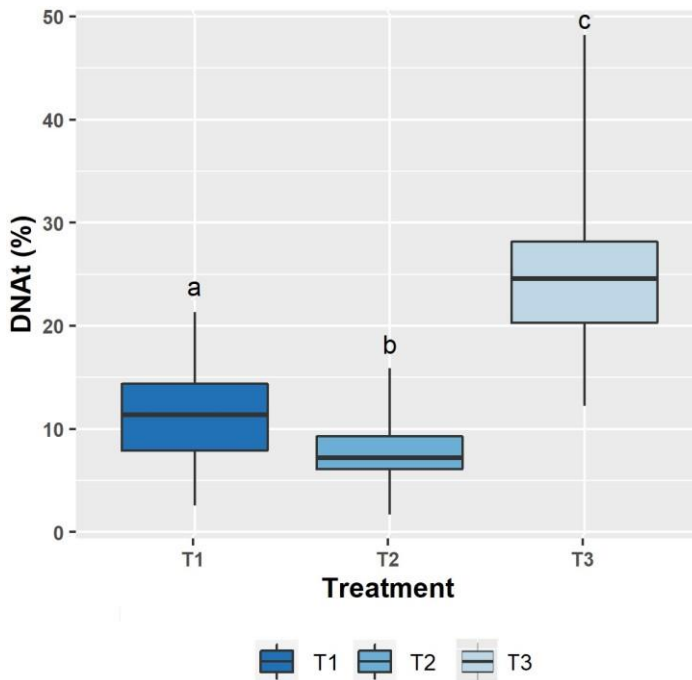


Figure 3. Comparison of the treatments analyzed in the DNA fragmentation variable of *Pseudoplatystoma reticulatum*. T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma of the species; T3 - T1 + 30% artificial plasma; percentage of DNA migrated in the tail compared to the head DNAt. Values with different letters differ significantly ($p < 0.05$).

The principal component analysis (PCA) was based on the correlation matrix between the 10 analyzed variables of the species motility parameters and the fertilization variable. The first two eigenvectors with the highest percentage of cumulative variance and a value ≥ 1 were considered for a graphical representation.

The first component showed eigenvector of 6.16, explaining the highest percentage (61.30%) of the total variables analyzed, while the second component obtained an eigenvector 2.57 explaining the second highest percentage of the total variance analyzed (25.70%), in total both components explained 87% of the accumulated variance.

The correlation of the variables shown in PC1 varies from -0.74 to 0.93; in PC2, they range from -0.49 to 0.74. The parameters VSL, LIN, VAP, VCL, STR, and WOB were positively related to PC1, unlike ALH, which had a negative relationship with this component, in PC2 the variables of FERT, MOT, and MP were represented better with a positive relationship, and BCF negative relationship (Figure 4).

The ellipses in Figure 4 separate the three treatments at a 95% confidence level. The ellipse of T1 does not present any overlap region with the ellipse of T3, showing an evident separation between these treatments, whereas T2 presents overlap with the other treatments, showing greater overlap with T3.

As can be seen in Figure 4, the vectors that had the greatest influence on the separation of group T1 from group T3 were: FERT, MOT, MP, and ALH. Group T2 is in a central position, being influenced by all vectors. The variables with a strong positive correlation between them can be grouped, for example, FERT, MOT, and MP, and the variables VCL, VAP, and VSL. We can also observe a positive relationship, especially of the variable VCL with FERT MOT and MP. On the other hand, WOB, LIN, STR, and BCF had a strong positive correlation with each other but a strong negative relationship with ALH.

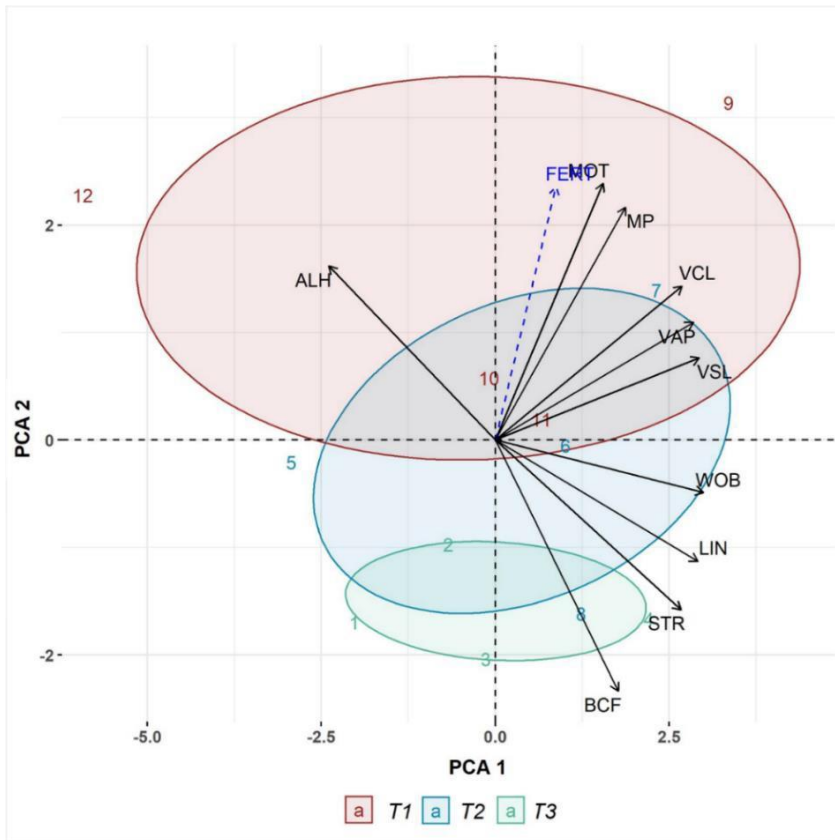


Figure 4. Scatter plot of the first and second principal components of the 14 analyzed parameters, with 95% confidence ellipses grouped by the three treatment groups (T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma of the species; T3 - T1 + 30% artificial plasma). In blue the fertilization variable. T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma from the species; T3 - T1 + 30% artificial plasma; total motility (MOT), progressive motility (MP), curvilinear velocity (VCL), linear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR), mean spermatozoa spatial trajectory oscillation (WOB), lateral head shift amplitude (ALH), beat crossing frequency (BCF), fertilization (FERT): discontinuous line.

Sperm subpopulations analyses

Using the principal components analysis (PCA), the matrix composed of total data of 9116 spermatozoa was processed. It resulted in two principal components (PC) with eigenvalues above 1, representing a total of 81.36% of the cumulative variance of the variables measured by CASA. These PCs are used later for the analyses of clusters. PC1 explained 50.24% and was positively correlated with the variables VSL, VAP, LIN, STR, WOB, and BCF, the other hand, PC2 explained 31.12% and showed a strong positive relationship with VCL and ALH variables.

Immediately later, the analysis began the second steps through two PCA results that allowed to reduce the size of the total data analyzed. Using non-hierarchical analyses, the proposals for divergent clusters were determined according to the largest Euclidean distance between the data using the k-means model, resulting in 15 clusters (Figure 5).

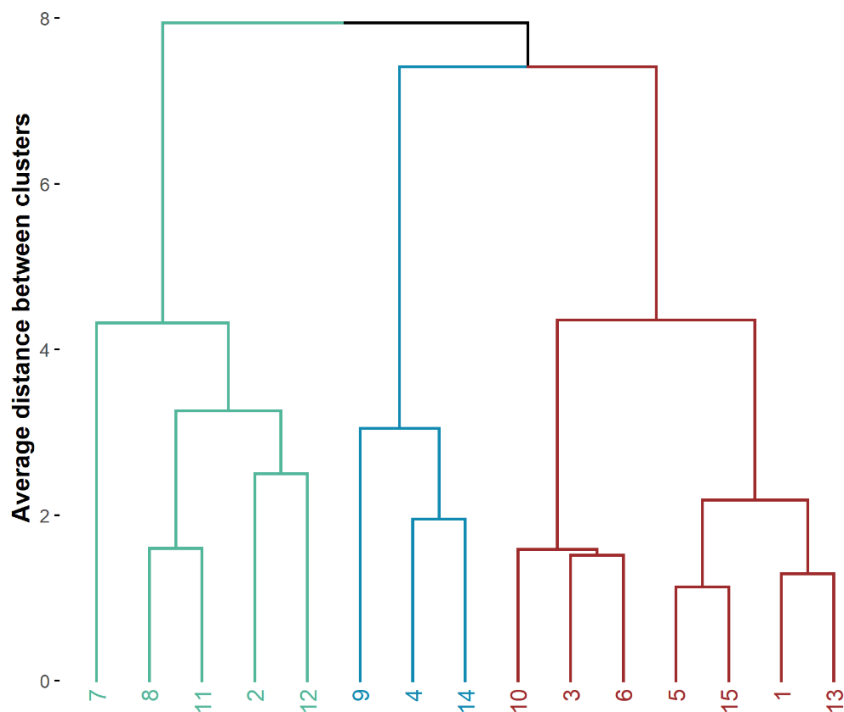


Figure 5. Dendrogram resulting from the hierarchical clustering of 15 PCA-derived clusters and the subsequent non-hierarchical grouping of motility data (steps clustering procedure). The final division was differentiated by NbClust resulting in 3 clusters (subpopulations). lines green-SP1, blue-SP2, vermelha-SP3.

In the third stage, the resulting centroids of the 15 clusters of the first stage were analyzed using hierarchical analysis (average linkage method, UPGMA). The final grouping was suggested by 6 indexes (Marriot, trcovw, TraceW, Ball, PtBiserial, SDindex) of 30 evaluated by the NbClust package of software R, resulting in three final groupings as the most efficient model prediction, three subpopulations (Figure 5).

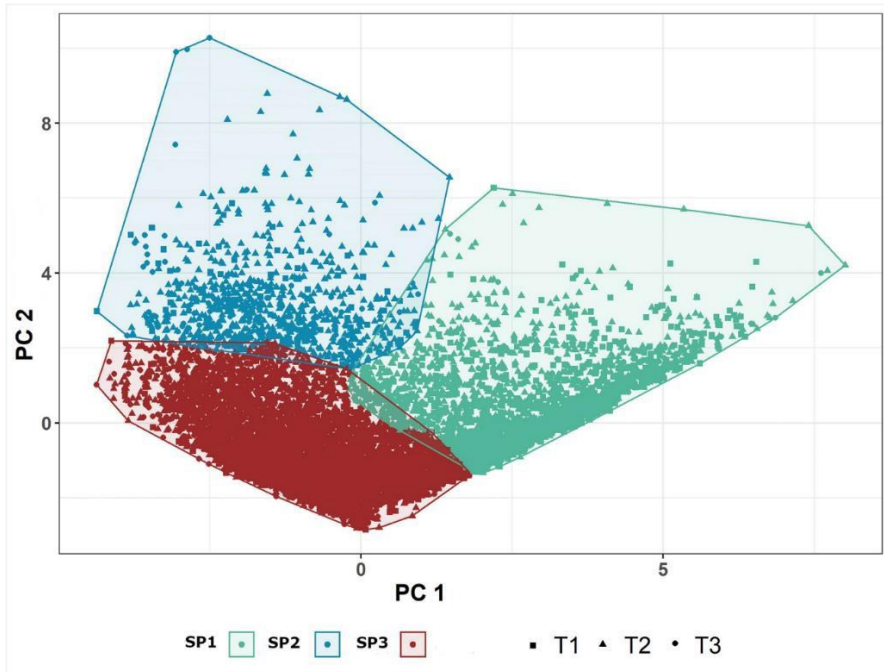


Figure 6. Analysis of principal components of sperm motility parameters with polygons representing the subpopulations: SP1(fast-linear), SP2 (fast-non-linear), and SP3 (slow-linear), defined by the cluster procedure T1 - glucose 5% + Methanol 10%; T2 - T1 + 30% seminal plasma from the species; T3 - T1 + 30% artificial plasma. T1 - glucose 5% + Methanol 10%; T2 – T1 + 30% of seminal plasma of the species; T3 – T1 + 30% artificial plasma.

Figure 6 shows the graph resulting from the PCA with the distribution of the three subpopulations well defined after the analysis of clustering. Subpopulation 1 (SP1) represented 26.62% of the total population and was characterized by spermatozoa with relatively high values of velocities (VCL, VSL, VAP) and linearity (LIN, STR), and low ALH, and consequently were considered as fast-linear. Subpopulation 2 (SP2) presented less percentage, with 9.23%, and had moderate velocity spermatozoa (with high VCL but low VSL and VAP), low linearity (LIN, STR), and high ALH, and were considered fast-non-linear. Finally, subpopulation 3 (SP3) was more frequent and was characterized by low-velocity values (VCL, VSL, VAP), moderate linearity (LIN, STR), and high ALH, and cells were considered slow-linear. Thus, these variables differ statistically among the three identified subpopulations (Table 2).

Table 2. Descriptive statistics of the 8 variables analyzed by CASA for each subpopulation SP1 (fast-linear), SP2 (fast-non-linear) and SP3 (slow-linear). Total n = 9116 spermatozoa. Data are shown as mean (interquartile interval). Values with different letters differ significantly ($p < 0.05$).

Variables	SP1	SP2	SP3
Number Spz (%)	2426 (26.62)	842 (9.23)	5848 (64.15)
VCL ($\mu\text{m/s}$)	78.4 (38.70) ^a	88.05 (30.17) ^b	26.50 (19.20) ^c
VSL ($\mu\text{m/s}$)	65.30 (37.00) ^a	16.95 (20.30) ^b	13.50 (13.10) ^c
VAP ($\mu\text{m/s}$)	73.00 (38.27) ^a	44.40 (26.00) ^b	20.10 (15.40) ^c
LIN (%)	91.10 (20.55) ^a	18.35 (20.40) ^b	57.20 (39.80) ^c
STR (%)	95.00 (12.10) ^a	38.10 (32.47) ^b	76.85 (30.70) ^c
WOB (%)	96.10 (8.90) ^a	50.00 (20.85) ^b	80.00 (25.20) ^c
ALH (μm)	1.6 (0.90) ^a	4.40 (1.40) ^b	1.50 (1.00) ^c
BCF (Hz)	8.00 (4.00) ^a	5.00 (3.90) ^b	2.45 (4.00) ^c

The proportion of spermatozoa from each subpopulation varied according to the treatments applied (Figure 7). In T1 samples, most of the spermatozoa belong to SP1 and SP3 (45.75% and 50.48, respectively), with only 3.77% to SP2. The percent of SP3 was an increment in T2 (63.03%) and decrease in spermatozoa of the SP1 category (23.86%), but an increase of SP2 (13.11%).

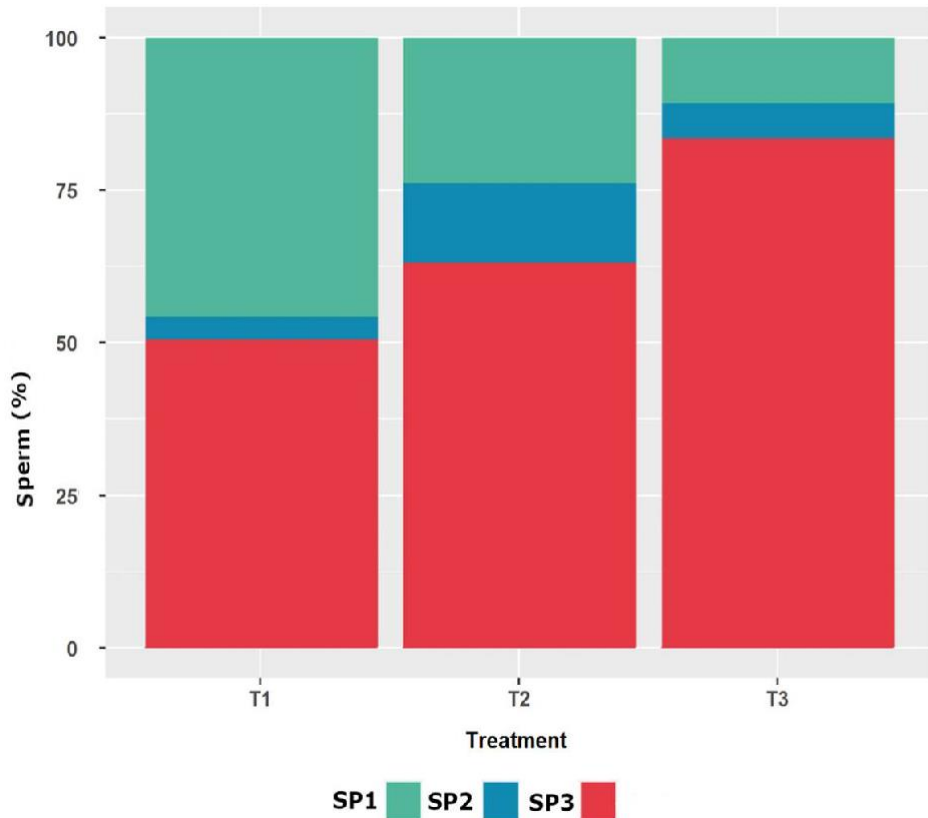


Figure 7. Percentage of each subpopulation of sperm SP1 (fast-linear), SP2 (fast-non-linear) and SP3 (slow-linear) for each treatment (T1 – glucose 5% + Methanol 10%; T2 - T1 + 30% of seminal plasma of the species; T3 – T1 + 30% of artificial plasma).

In T3 treatment the distributions were the majority of the SP3 subpopulation with 83.50% of the total motile spermatozoa, SP1 and SP2 (10.75% and 5.75, respectively) with low values. All treatments were significant differences in SP1 and SP3, for SP2 differences were not significant in treatments T1 and T3.

DISCUSSION

In seminal plasma, numerous proteins have already been identified, allowing the characterization of the mechanisms involved in the protection of spermatozoa, especially against oxidative stress and microbial attack (Ciereszko *et al.*, 2017). The results obtained in this study report for the first time the evaluation of the influence of seminal plasma characteristics on the cryopreservation of *P. reticulatum* semen with the use of methanol and glucose as the base cryoprotective freezing medium.

It is established that extenders and cryoprotectants are essential and play a vital protective role in cryopreservation during freezing and thawing, but there is a need for species-specific and detailed analysis. In this context, methanol has been a cryoprotectant with positive results in the cryopreservation of several species of Siluriformes (catfish), such as *Leiarus marmoratus* (Borges *et al.*, 2020), *Clarias gariepinus* (Viveiros; So; Komen, 2000; Kovács *et al.*, 2010; Viveiros *et al.*, 2001), *Pseudoplatystoma corruscans* (Carolsfeld *et al.*, 2003), *Ictalurus punctatus* (Christensen; Tiersch, 2005), *Pelteobagrus fulvidraco* (Pan *et al.*, 2008).

In the present study, the combination of methanol 10% + glucose 5% (T1) and with addition of natural seminal plasma from *P. reticulatum* (T2) showed to be more effective in protecting cells than when seminal artificial plasma was added to the cryoprotectant substance (T3), obtaining the best values of total motility, with values between

~20-27% and progressive motility between ~11-13%. Superior results were found for *P. fulvidraco* (Pan *et al.*, 2008), using 10% methanol and obtaining

motility rates of $65\pm 5\%$ post thawing, besides using Ringer's solution as diluent. For *P. corruscans*, higher motility values (60-80%) were also reported using 10% methanol + glucose + coconut water powder as a cryoprotectant (Carolsfeld *et al.*, 2003). Muchlisin; Hashim; Chong (2004) tested different cryoprotectants for *Mystus nemurus*, obtaining the best motility results (58%) with 10% methanol. On the other hand, similar results to our study were found for *P. metaense* (Ramírez-Merlano; Medina-Robles; Cruz-Casallas, 2011), with motility rates of $\sim 23\%$ combining methanol 12% with egg yolk. Other authors, such as Linhart *et al.* (2005) found values lower than those found in the present study (12.1%), with the use of 10% methanol in a period of exposure to the cryoprotectant of one hour, it is worth noting that this same combination obtained the highest velocity rate (105 $\mu\text{m/s}$) like the control. In our study, the rest of the velocity variables analyzed by CASA did not show statistical differences between the treatments tested, but the use of 10% methanol seems to provide adequate protection for the maintenance of seminal quality in *P. reticulatum*.

High fertilization rates were obtained using methanol as a cryoprotectant in several works with Siluriformes (catfish), such as *C. gariepinus* (Viveiros; So; Komen, 2000; Kovács *et al.*, 2010; Viveiros *et al.*, 2001) and *Pseudoplatystoma fulvidraco* (Pan *et al.*, 2008). In the latter study, high fertilization rates were linked to high motility rates. For the species *Pseudoplatystoma metaense* the resulting fertilization percentage was higher than 70% (Ramírez-Merlano; Medina-Robles; Cruz-Casallas, 2011).

The analysis of the fertilizing capacity of cryopreserved-thawed semen is very useful to understand the effectiveness of cryopreservation protocols. In this

study the treatments T1 and T2 provided high fertilization rates compared to the control, coinciding with the treatments with the best motility rates, while T3 obtained low fertilization values and was the treatment with the lowest motility rates. Thus, it can be stated that fertilization success may be conditioned by sperm motility rates, which on his hand, indicates an adequate physiological state of the sperm cells, as well as enough ATP levels that help trigger important processes during fertilization.

Cierieszko *et al.* (2014), using a simple methanol-glucose freezing medium found high effectiveness in cryopreserved-thawed semen for *Oncorhynchus mykiss*, obtaining high fertilization rates similar to the control. This method also appears to be effective for other species of the family such as *Salmo trutta m. fario* L. (Nynca *et al.*, 2014), in this case, uses 0.18 M glucose and 9% methanol. The present study ratifies the importance of the combination of the base cryoprotectants (methanol 10% + glucose 5%) used in the experiment, providing protection and energy necessary for the success of the fertilization process in the species *P. reticulatum*.

Thus, the high fertilization rates in the present study with thawed semen from treatments T1 and T2, with mean motility rates between 20-27%, could be influenced by some of the factors mentioned above, making the fertilization process successful for these two treatments.

Principal component analysis (PCA) is a multivariate technique that has recently been used by several researchers to reduce the dimensionality of the data in CASA (Caldeira *et al.*, 2018; Gallego *et al.*, 2017; Martínez-Pastor *et al.*, 2011). Analysis using PCA consists of replacing the variables in a multivariate data set with estimated uncorrelated derived variables (linear

combinations of the initial variables) called principal components. This is a useful tool in the exploratory analysis of data and can assist in the understanding of the results obtained. In the present study, the PCA explained in the first two components the 87% of the total variation of the data, being able to group in the first component the variables related positively with velocities, linearity, and negatively with ALH. So, it can be cataloged as velocity and movement component. However, in the second component a positive relationship was observed with the variables total and progressive motility, FERT, and negative with BCF, so we defined this component as progressivity. The created ellipses showed a greater positive relationship of the second component with T1, while it was negative with T3, and these treatments can be clearly separated. On his hand, T2 was better related to the first component, linked to the characteristics of speed and movement, and also had a lesser positive relationship with the progressivity component. Thus, through this analysis we can define that T1 is more effective for fertilization, along with an increase in total and progressive motility and a decrease in BCF.

Since fish spermatozoa velocity decreases rapidly with time, the duration of progressive movement will also have a significant influence on the ability of spermatozoa to enter the oocyte (Kime *et al.*, 2001). For *C. gariepinus* (Rurangwa *et al.*, 2001) the positive relationships between progressive sperm velocities and fertilization, present the highest values for the correlation with VCL ($r=0.8$), likewise for other species, such as *Prochilodus lineatus* (Viveiros *et al.*, 2010), *Cyprinus carpio* (Linhart *et al.*, 2000), also present similar correlations.

Gallego *et al.* (2017) evaluated the changes after the cryopreservation process

of *Colossoma macroporum* sperm subpopulations where it positively relates total and progressive motility rates ($r=0.6$), however the highest rates were with VCL and VAP ($r > 0.7$). The results of the research coincide with the results obtained in the present study where the PCA analysis showed positive correlations of the FERT variable with MOT, MP and within the velocities FERT showed the highest with positive relationship with VCL, thus, one can support the hypothesis that high curvilinear velocities, in some cases, provide the spermatozoa to find the micropyle more easily (Tuset *et al.*, 2008).

DNA integrity is an important indicator in the transmission of genetic information to future generations (Contreras *et al.*, 2017; Sariözkan *et al.*, 2013). It has been reported that fish seminal plasma contains a variety of substances that protect against damage and preserve spermatozoa characteristics, including motility, capacitation, survival and longevity (Ciereszko *et al.*, 1999; Dietrich *et al.*, 2014; Figueroa *et al.*, 2013, 2015; Martínez-Páramo *et al.*, 2013). This study reaffirms previously exposed, by evaluating the fragmentation of DNA, the treatment with the addition of seminal plasma (T2) was shown to be more effective in protecting spermatozoa, showing significant differences with the other two treatments, perhaps this is the best treatment for the protection of DNA and consequently better progeny development. These results may provide interesting data for future research on the development of offspring.

Dietrich and Ciereszko (2018) also state that the composition of seminal plasma and its stabilizing effect on sperm make it a natural additive to decrease the detrimental effects of cryopreservation. Research with *Clarias gariepinus* reported that cryopreserved-thawed spermatozoa resuspended in common carp seminal plasma exhibited higher motility values than normally thawed

spermatozoa (Müller *et al.*, 2019). For *Cyprinus carpio*, seminal plasma fractions were added to the cryoprotectant substance obtaining significantly higher post-thaw motility rates and less DNA damage compared to spermatozoa frozen with an extender alone (Shaliutina-Kolešová *et al.*, 2020). However, the results of the treatment without seminal plasma addition (T1) were the second best, becoming effective also in the protective capacity for sperm cells. Considering that this treatment obtained high fertilization rates, we could think about the possibility exposed by Cabrita *et al.* (2005) that depending on the level (~40%) and nature of the damage, these can be repaired by the DNA repair mechanisms of the oocytes during early embryogenesis, and therefore, this damage is irrelevant in the fertilization process.

The multivariate clustering method is beneficial for identifying sperm subpopulations. The proposal by Martínez-Pastor *et al.* (2005) was applied in this study where the number of clusters in the final dendrogram was evaluated after the hierarchical analysis in the clustering process using pseudo-F, CCC and pseudo-t² statistical tests. Unlike what these authors proposed for the analysis of final clusters, our study evaluated these based on the NbClust program of the R software, which offers 30 indexers to validate this last number of clusters in a more rigorous test. This variant of the original method can be recommended for use in future research in the area.

In cryopreserved-thawed sperm, three subpopulations of *P. reticulatum* were found (SP1, SP2, and SP3), based on the kinetic criteria. Other fish species have also been reported such as *Sparus aurata* (Beirão *et al.*, 2011), *Oncorhynchus mykiss* (Kanuga *et al.*, 2012), *Anguilla anguilla* (Gallego *et al.*,

2015), and *Cyprinus carpio* (Marinović *et al.*, 2021). For *Colossoma macropomum* (Gallego *et al.*, 2017) find three subpopulations for fresh sperm, but in cryopreserved sperm, only two subpopulations have been identified, reaffirming the deleterious effect of the cryopreservation process on spermatozoa. In our study we were not able to carry out the analysis of subpopulations in the fresh semen samples, but to minimize this effect we made sure to choose all those samples with more than 80% motility, which allows us to affirm that the cryopreservation process could have caused modifications in the physiological performance of spermatozoa linked to the different treatments used.

For other fish species such as *Barbus balcacinus* and *Solea senegalensis*, four sperm subpopulations have been described (Kojadinović *et al.*, 2020; Martínez-Pastor *et al.*, 2008). Although the multivariate analysis method used to identify *Barbus balcacinus* subpopulations did not follow the same two-step clustering procedure, this method is the most widely used for fish. Therefore, it can be concluded that it is common in groups determined by three or four subpopulations, even according to different classification methodologies.

The deleterious effects of the cryopreservation process on spermatozoa can seem evidenced by the high percentage of SP3 cells. This subpopulation is characterized by presenting spermatozoa with low values of speed, moderate linearity, and high ALH, indicating a compromised metabolism of these spermatozoa that in a short period could totally lose motility, similar to the described for *C. carpio* (Marinović *et al.*, 2021).

Several studies have suggested that the subpopulations of spermatozoa characterized by high velocities and linearity, as in the case of SP1, are mostly

linked to the event of the fertilization process (Gage *et al.*, 2004; Gallego *et al.*, 2017).

In our study, T1 was more effective and protective than the other two treatments tested, managing to maintain a greater quantity of fast and linear sperm cells, characteristics that may be directly linked to fertilization success (Gallego *et al.*, 2015; Marinović *et al.*, 2021) In the case of T2 and T3, there is evidence of progressive loss of speed, perhaps due to the presence of spermatozoa with some flagellar or membrane damages, that induce the displacement with erratic movements.

The present study contributed new information for the species, which will help in the improvement of sperm analysis in fish during the cryopreservation processes. Likewise, the investigations focused on the study of subpopulations of spermatozoa should replace the traditional statistical approach considering only average values, providing new guidelines on the importance of precision in evaluating sperm quality.

CONCLUSION

Natural seminal plasma supplementation in our study provided interesting results for motility, fertilization, and low DNA fragmentation rates. However, the treatment supplemented with seminal artificial plasma did not show effective results in the protection of the spermatozoa of the species, so in future work with the species, it is necessary to integrate knowledge about the protein profile of the plasma of the species and evaluate the addition of some proteins to the composition of the seminal artificial plasma, for the success of a more effective application.

Despite the T2 was shown to be more effective in terms of DNA protection, the T1 treatment also obtained low fragmentation rates and although both treatments presented high fertilization rates, we consider, based on the results obtained through PCA, that T1 was more effective overall. On the other hand, the results obtained in the analysis of subpopulations demonstrate the influence of the two treatments used for the maintenance of sperm quality after the cryopreservation process, being T1 more effective in the maintenance of fast and linear subpopulations, evidencing a high degree of cell cryoprotection through the cryopreservation process.

For these reasons, we can consider T1 as the most effective treatment for the cryopreservation of sperm of this species. In addition, the use of a simple freezing medium favors the producer, being more economically viable as an effective protocol for the species in captivity. The results obtained in this study are relevant and may constitute an effective protocol for the cryopreservation of *P. reticulatum* sperm.

CHAPTER 3

Effect of temperature and pH on the sperm motility of the European eel: in the context of climate change

ABSTRACT

The current climate change situation could bring critical effects for marine species, especially those already considered endangered. It is necessary to develop tools to anticipatedly assess the physiological effects of such environmental change. With this purpose, our study aims to determine the effect of a range of seawater temperatures and pHs on sperm motility in the European eel (*Anguilla anguilla*). Low seawater pH (6.5-7.4) decreased the eel sperm motility in comparison to the control (pH= 8.2). We also studied the combined effect of the pH of the artificial seminal plasma (the plasma where the sperm cells are suspended) with the pH of Artificial Sea Water (ASW, pH 7.8 or and 8.2). We did not find statistical differences in sperm motility and kinetic parameters caused by the artificial seminal plasma pH. However, seawater pH induced significantly higher values of total sperm motility (MOT), and the percentage of fast (FA) spermatozoa with a pH of 8.2 in comparison with a pH of 7.8. In contrast, the seawater temperature did not affect sperm motility parameters or sperm longevity. To study the effect of the interaction between seawater temperature and pH on sperm motility, two temperatures: 4 and 24 °C, and two pHs 7.8 and 8.2, were tested. There were significant differences between temperature and pH in several kinetic parameters and, in general, the lowest values were observed in the samples activated at the low temperature and the low pH (4 °C, pH 7.8). Our results suggest that seawater acidification, but not high temperatures, can affect sperm motility in the context of climate change, at least in the case of the European eel.

INTRODUCTION

Climate change is inducing changes to the environmental conditions of ocean and coastal ecosystems, including ocean warming, acidification and deoxygenation. Populations of many marine fish species will be affected, and under suboptimal conditions, they will become extinct (Coley et al. 2022). Fish populations are becoming more vulnerable to short-term natural climate variability, and scientists predict that the potential global catch could decrease by as much as 16-25% by the end of the century (Cheung et al. 2018).

Regarding reproduction, most fish species show external fertilization: their spermatozoa are delivered in the external environment (marine or freshwater), what represent both a drastic environmental change and a source of chemical signals controlling the motility function (Cosson 2019). When the spermatozoa are released into the surrounding water, they experience an osmotic shock, which could be either hypoosmotic (in freshwater spawners) or hyperosmotic (in seawater spawners) (Pérez 2020) and such an osmotic shock induces the initiation of sperm motility. Sperm motility is a key requirement to assess the quality and fertilizing capacity of the sperm (Billard et al. 1995).

Changes in the external medium can regulate sperm motility by interaction with factors such as temperature, pH, and several others. Because motility in fish spermatozoa is triggered externally, it highly depends on the environment where the reproduction occurs (Dzyuba and Cosson 2014); therefore, we can expect the spermatozoa to be strongly influenced by the environmental temperature (Lahnsteiner and Caberlotto 2012).

Knowledge of the physiological mechanisms by which high water temperature disrupts fish reproduction could contribute to understanding potential alterations of captive fish breeding, and to assist in their prediction (Merino et al. 2023). Increased water temperature can generate negative effects on sperm function, altering the motility time and sperm velocity, increasing the rate of cell metabolism, and causing a mismatch of energy resources. These effects could promote changes in the movement of the sperm, jeopardizing their quality and fertilizing capacity (Cejko et al. 2016; Fenkes et al. 2017).

pH is an important environmental factor in seawater chemistry and in cell physiology (Pérez et al. 2020) and it is known that climate change is decreasing the pH of marine waters. It is estimated that current mean seawater average pH is around 8.1, but it could decrease to values of 7.8 by the end of the century (Coley et al. 2022). In those aquatic species showing external fertilization, the pH of the activating medium to which they release spermatozoa also influences the initiation of sperm motility (Alavi and Cosson 2005). In several fish species, such as sturgeons (Gallis et al. 1991), pufferfish (Takai and Morisawa 1995) or Japanese eel (Tanaka et al. 2004) it has been proven that alkaline waters (pH 7.4-9.0) favor the activation of motility sperm, while acidic waters (pH 6.0-7.2) reduce or inhibit it.

The European eel has been classified on the Red List of the International Union for Conservation of Nature (IUCN) as "critically endangered" and therefore, may be one of the species greatly affected by climate change. However, it is not known which temperature and pH values could be critical and might endanger the reproduction of the European eel. This is a species with a peculiar life cycle in which pubertal individuals undertake, apparently in 6–7 months, a

transatlantic migration to the spawning areas in the Sargasso Sea (Tesch 1978), during which they face important environmental changes (salinity and temperature) that modulate the endocrine control of their sexual maturation (Tesch 1978; Mazzeo et al. 2016). It is necessary to develop tools to assess the actual physiological effects of anticipated environmental changes and use them to anticipate and mitigate their potentially harmful effects. With these purposes, our study aims to determine the effect of environmental temperature and pH on the motility performance of the European eel sperm.

MATERIAL AND METHODS

Fish maintenance and hormonal treatment

A total of 135 male eels (mean body weight 105.7 ± 4.2 g) in three batches of 45 males (2021, 2022) were transported to our facilities at the Universitat Politècnica de Valencia (Spain) from the local eel farm Valenciana de Acuicultura, S.A. (Puzol, Valencia). Each fish batch was distributed in three 96-L aquaria (approximately 15 male eels per aquarium) equipped with separate recirculation systems, thermostats, and coolers, and covered with black panels to reduce light intensity and fish stress. The animals were gradually acclimatized to seawater (salinity 37 ± 0.3 g/L) over the course of 1 week and were then maintained in seawater at 20 °C until the end of the experiment.

Once the fish were in seawater, the hormonal treatment with recombinant human chorionic gonadotropin (hCGrec; Ovitrelle, Merck S.L., Madrid) was initiated as described in previous studies (Gallego et al. 2013; Pérez et al. 2020). Once a week, the animals were anesthetized with benzocaine (60 ppm) and weighed before receiving an intraperitoneal injection of hCGrec diluted in

NaCl 0.9%, at a dose of 1.5 IU/g fish.

The fish were fasted throughout the experiment and handled in accordance with the European Union regulations concerning the protection of animals used for scientific purposes (Dir 2010/63/UE) and with the recommendations provided by the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The Experimental Animal Ethics Committee from the Universitat Politècnica de València approved the applied protocols, and final permission (2023-VSC-PEA-0039) was granted by the local government (Generalitat Valenciana).

Sperm collection and sampling

Sperm samples were collected once a week, from the 6th week of hormonal treatment until weeks 18 (batch 1), 24 (batch 2), and 20 (batch 3). The samples were collected 24 h after the administration of the hormone to obtain maximum sperm quality (Pérez et al. 2000). The sperm was collected in 15 ml Falcon tubes by applying gentle abdominal pressure, fish anesthetization (benzocaine, 60 ppm). The genital area was previously cleaned with distilled water, and dried, in order to avoid sample contamination by feces, urine and seawater. The sperm samples were kept refrigerated (4 °C) until the motility analyses were performed within the first hour after collection.

Sperm motility evaluation

The standard sperm diluent used in this work was P1, an artificial seminal plasma isosmotic and isoionic with the European eel seminal plasma (in mM: NaCl 125, NaHCO₃ 20, MgCl₂ 2.5, CaCl₂ 1, KCl 30; osmolality 325 mOsm/kg

and pH adjusted to 8.5) (Peñaranda et al. 2010). The sperm motility activation was undertaken described by Gallego et al. (2013), by mixing 1 μl of diluted sperm (dilution 1/25 in P1) with 4 μl of artificial seawater (ASW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was prepared in a SpermTrack-10® chamber, with a depth of 10 mm (Proiser R+D, S.L.; Paterna, Spain) and observed using a Nikon Eclipse 80i microscope, with a 10x lens (Nikon phase contrast 10 - 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after mixing the sperm with ASW, using a high-sensitivity HAS-220 video camera (using a frame rate of 60 fps) and the ISAS software (Proiser R+D, S.L.; Paterna, Spain), a computer-assisted sperm analysis (CASA-Mot) system.

For each motility test, samples were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 °C in a water bath until the sperm motility evaluation. Only the best samples (>60% total motility) were selected for the experiments. The sperm motility parameter considered in these studies were total motility or percentage of motile cells (MOT, %), and spermatozoa were considered immotile if their VCL was <10 $\mu\text{m/s}$. Other kinetic parameters were also explored: progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast spermatozoa (FA; showing an average path velocity, VAP>100 $\mu\text{m/s}$); curvilinear velocity (VCL, $\mu\text{m/s}$), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; straight line velocity (VSL, $\mu\text{m/s}$), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position; VAP ($\mu\text{m/s}$), defined as the time/average of a sperm head along its spatial

average trajectory; straightness (STR, %), defined as the linearity of the spatial average path (VSL/VAP); ALH, amplitude of the lateral movement of the sperm head, and cross beating frequency (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory crosses its average path trajectory.

Experiments

Experiment 1. Effect of seawater pH on sperm motility and longevity of the sperm

This experiment was performed in 3 sessions, with 25 sperm samples from 25 fish in total. Sperm samples were first diluted in P1 as has been described previously. The pH of artificial seawater (ASW) was adjusted to the next pHs (± 0.02): 6.5, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2 and 9.5. It is considered that a pH of around 8.1 is the standard pH of natural seawater, and our aim was to test the effect of lower pHs such as those that might occur due to climate change. The sperm motility of each sample at each pH was measured in triplicate (3 activations per sample), with 2 videos captured for each triplicate.

As there were no statistical differences in the sperm motility of the samples activated with ASW at pH of 7.6, 7.8, 8.0, and 8.2, we wanted to know if there were differences in the total time of motility (or longevity) between those pHs. We activated the sperm samples with ASW at pHs 7.6, 7.8, 8.0, and 8.2 and registered the total time of motility with a timer, until the spermatozoa moved less than 10%. This test was performed in two sessions with a total number of 14 samples (initial motility >60%).

Experiment 2. The combined effect of seawater pH and diluent pH on sperm motility

To evaluate the possible interaction between the diluent pH, which imitates the seminal plasma composition, being 8.5 in the European eel (Pérez et al. 2003), and the seawater pH, we assayed the dilution of sperm in diluent P1 adjusted to 3 pHs: 8.5 (considered as the control only in this experiment), 8.0 and 7.5. The dilution was 1:25 (v/v). Samples (n= 16) were incubated at the different pHs of the extender for 1 h at 4 °C. Samples were evaluated later activating them with ASW at two different pHs: 7.8 and 8.2. The pH value of 7.8 was chosen since it is the seawater pH value expected for the year 2100 as a result of climate change (Hartin et al. 2016).

Experiment 3. Effect of the seawater and extender temperature on sperm motility and kinetic parameters

This experiment was performed in a single session, with 10 sperm samples with good motility (>60%). Each sample was diluted 1:25 in P1 and divided into two subsamples: one was maintained at 4 °C, and the other maintained at room temperature, approximately 23 °C. Samples were incubated at those temperatures for 1 h. The sperm activation of the sample maintained at 4 °C was undertaken with ASW also maintained at 4 °C, while the sample maintained at 23 °C was activated with ASW at that temperature. Motility and kinetic parameters were then measured.

Experiment 4. Effect of the seawater temperature on sperm longevity

To evaluate the effect of the seawater temperature on sperm longevity, this parameter was measured as described above. We selected 10 sperm

samples with good motility (>60%) for the analyses. Each sample was diluted 1:25 in diluent P1 (pH 8.5). Diluted samples were incubated at 4 or 23 °C for 1 h, and later activated using ASW at the same temperatures.

Experiment 5. The combined effect of pH and seawater temperature on sperm motility

In this experiment, sperm samples with good motility (>60%) were selected and two ASW and diluent temperatures were tested: 4 and 24 °C (RT), as well as two pH values in the seawater: 7.8 and 8.2, resulting in 4 experimental combinations. Then, aliquots of ASW at 7.8 and 8.2, as well as the diluted samples, were maintained either at 24 °C or in the refrigerator at 4 °C for 1 h. This test was performed with a total number of 12 samples in three sessions. The sperm motility was evaluated, as previously described, with a CASA-mot system.

Statistical analyses

Each variable was first analyzed to check its normality using the asymmetry and Kurtosis coefficients as reference parameters. The abnormal variables were transformed to their logarithm in basis 10, and normality was checked again. When the experiment had 2 variables, a two-way ANOVA was performed first, and according to the results, a single ANOVA was then performed to evaluate the differences between the experimental treatments. The experiments with a single variable were analyzed by a one-way ANOVA. The means comparison was undertaken with the Duncan multiple range test. For all the tests, the differences were considered significant when p-value <0.05.

All the statistical procedures were performed with the software Statgraphics

Plus® 5.1 (Statistical Graphics Corp., Rockville, MO, USA) and GraphPad Prism 9.3.0 (GraphPad Software, San Diego, California USA, www.graphpad.com). Results are presented as the mean \pm standard error.

RESULTS

Experiment 1. Effect of seawater pH on sperm motility and longevity in eel sperm

A trial was performed to test if a specific seawater pH generated any effect on the eel sperm motility in comparison to the control (pH= 8.2). Figure 1 shows how kinetic sperm values were higher in the pH range of 7.8-8.2 of ASW. When ASW became more acidic, kinetic values, including total motility (MOT) decreased. Also, at a high pH (9.5), there were lower MOT, MP, FA, VCL, VSL, and VAP values than those found with the pH 7.8-8.2. Thus, when ASW pH is between 7.8-8.2, there is a higher percentage of motile spermatozoa (MOT), a higher percentage of fast spermatozoa (FA), which move with higher velocities (VCL, VSL, VAP), and have a higher displacement in the water (progressive motility, MP).

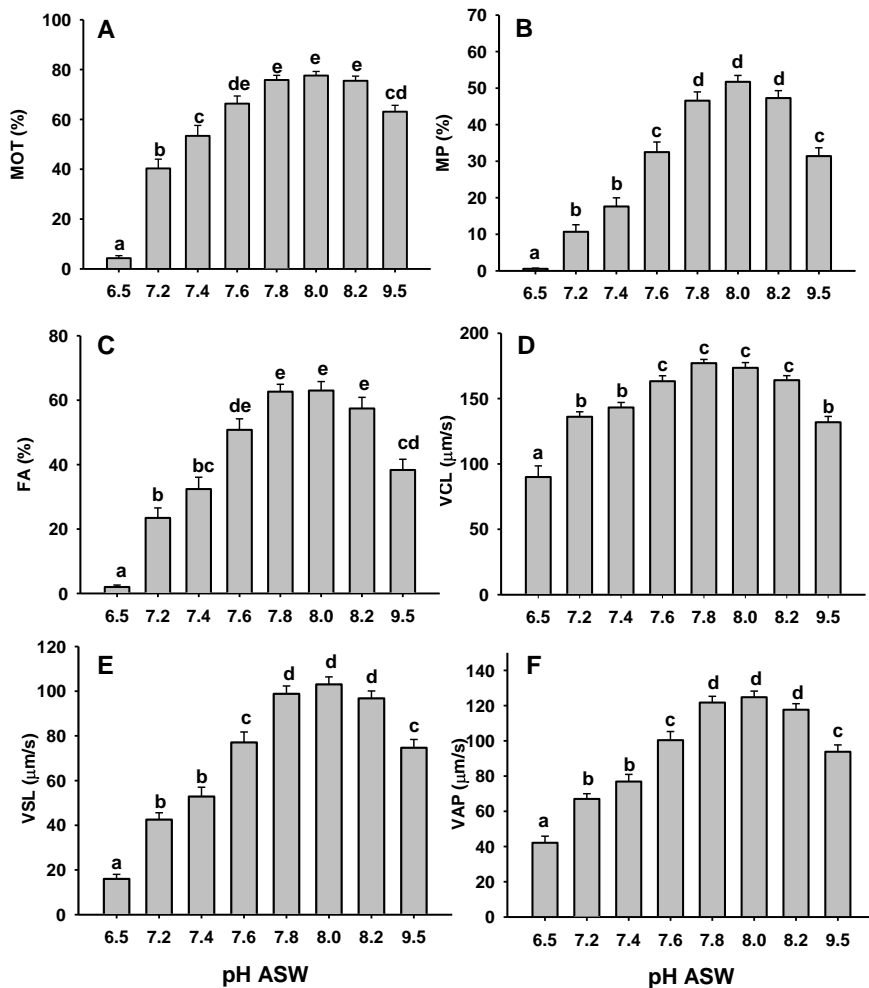


Figure 1. Effect of artificial seawater pH in the total motility (MOT), progressive motility (MP), percentage of fast spermatozoa (FA), curvilinear velocity (VCL), straightline velocity (VSL), and average path velocity (VAP). Values are represented as means \pm standard error. $n = 23$. Different letters indicate significant differences between means ($p < 0.05$).

Regarding other parameters, a progressive increase in LIN, STR, and WOB values could be observed (Fig. 2) when the pH increased from 6.5 to 7.8, without a decrease at the high pH (9.5). The ALH and the BCF were deeply reduced at the low pH (6.5) in relation to the other pH values. The maximum BFC was observed at pH 7.6-8.2, being reduced both at lower and higher pHs.

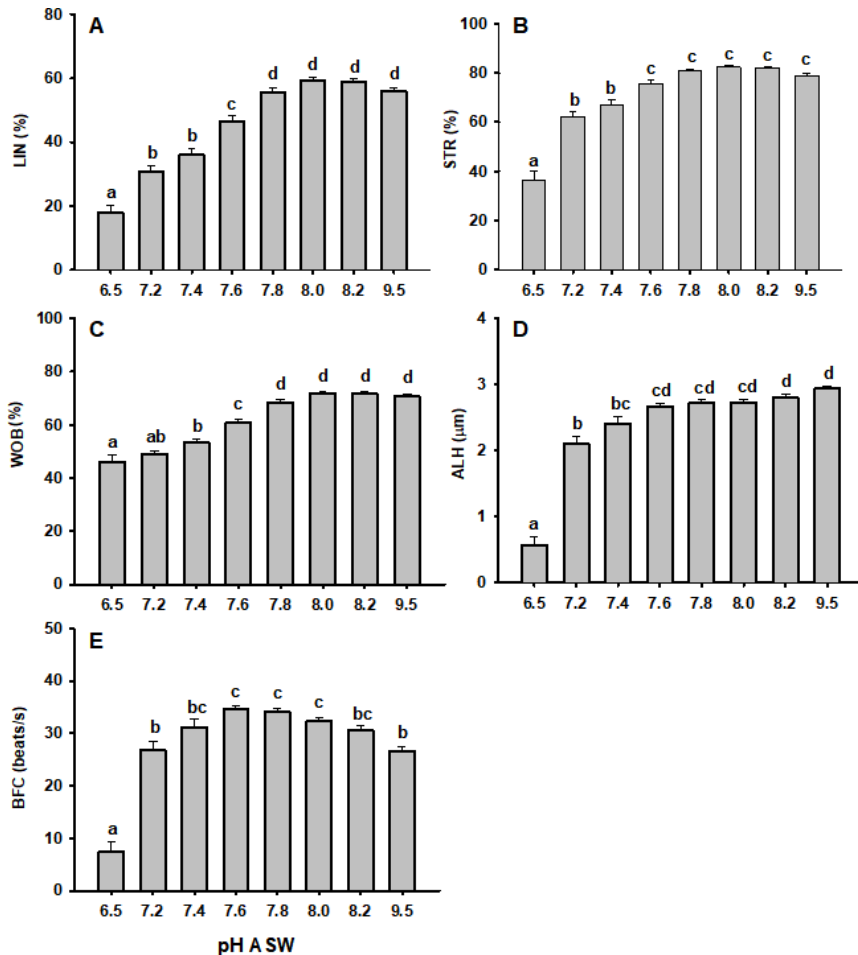


Figure 2. Effect of artificial seawater pH in the linearity index (LIN), straightness (STR, %), oscillation index (WOB), lateral movement of the sperm head (ALH), and cross beating frequency (BCF). Values are represented as means \pm standard error. n = 23. Different letters indicate significant differences between means ($p < 0.05$).

Figure 3 shows the longevity of sperm motility at different pHs. A gradual but not significant increase in longevity was observed when the ASW pH increased from 7.6 to 8.2.

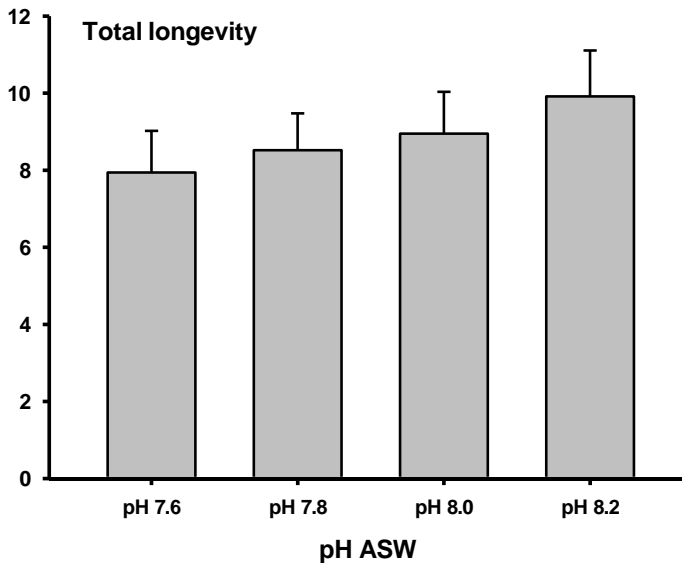


Figure 3. Total longevity of the eel sperm motility (min) activated at different pH of the artificial seawater. Values are represented as means \pm standard error. n = 10.

Experiment 2. The combined effect of seawater pH and diluent pH on sperm motility

The aim of this trial was to check the potential interactions between the pH of the artificial seminal plasma and the ASW pH on the eel sperm motility. In Figure 4, it could be observed that in general, there were no statistical differences in the sperm motility and kinetic parameters in relation to the artificial seminal plasma pH, but seawater pH had a significant effect, showing higher values of total motility (MOT), FA, and ME with a pH of 8.2 than with a pH of 7.8.

The straightline velocity (VSL, Figure 4F) was affected by the extender pH, with higher velocity in samples diluted at pH 8.0 and activated with ASW at pH 8.2, although the differences were significant only with the samples diluted in extender at pH 7.5 and activated with ASW at 7.8.

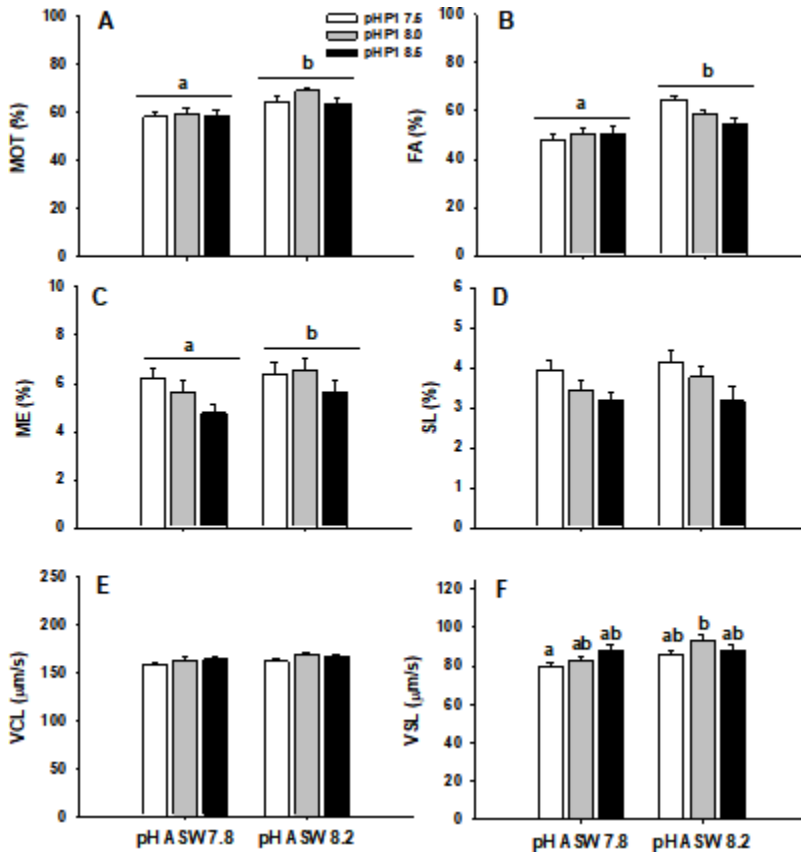


Figure 4. The combined effect of the extender pH with the ASW pH in the total motility (MOT), the percentage of fast (FA), medium (ME) and slow (SL) spermatozoa, the curvilinear velocity (VCL) and the straight line velocity (VSL). Values are represented as means \pm standard error. $n = 16$. Different letters indicate significant differences between means ($p < 0.05$).

Figure 5 shows the effects of the combined extender pH and the ASW pH on other kinetic parameters. There was a significant interaction between the extender pH and the ASW pH in VAP and WOB, which lowest values were observed in the samples diluted with the extender at pH 7.5 and then activated

with ASW at pH 7.8. The LIN, ALH, and BCF values were only affected by the ASW pH, with lower values of LIN and ALH at pH 7.8 than at 8.2, but higher values of BCF at ASW at pH 7.8 than at 8.2.

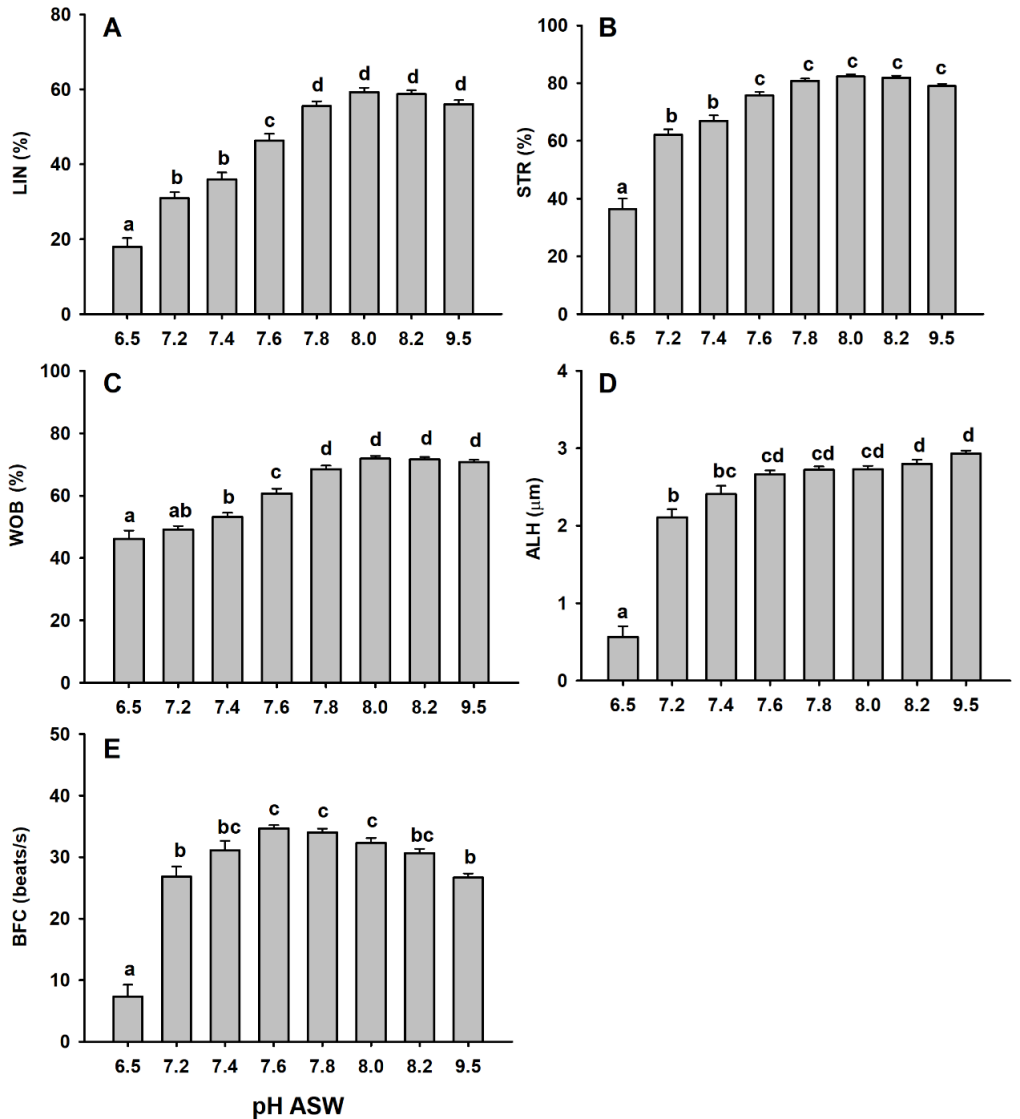


Figure 5. The combined effect of the diluent pH with the ASW pH in the average path velocity (VAP), the linearity index (LIN), oscillation index (WOB), lateral movement of the sperm head (ALH), and cross beating frequency (BCF). Values are represented as means \pm standard error. $n = 16$. Different letters indicate significant differences between means ($p < 0.05$).

Experiment 3. Effect of seawater temperature on sperm motility and kinetic parameters

In this experiment, sperm activation was performed at two temperatures: 4 °C (control) and 23 °C. It should be noted that both the samples and the ASW were maintained at the same temperature. No significant differences were found in MOT, MP, FA, VCL, VSL, VAP, ME, SL, LIN, STR, WOB, and ALH (supplementary data). However, there was an effect of the temperature on the BCF, which was lower ($p < 0.01$) in the samples activated at a high temperature, 23 °C (Figure 6).

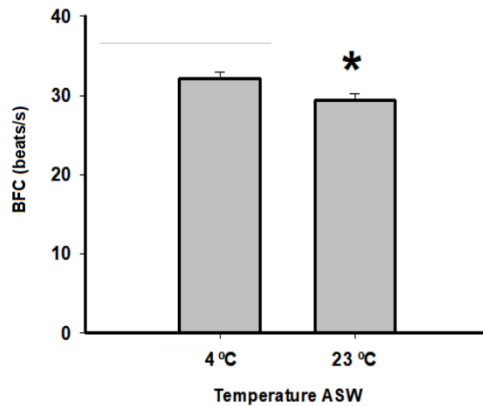


Figure 6. Effect of seawater temperature (ASW) in the beat cross frequency of

eel sperm. Values are represented as means \pm standard error. $n = 10$. Asterisks indicate significant differences between means ($p < 0.05$).

Experiment 4. Effect of seawater temperature on sperm longevity

This experiment checked the effect of different ASW temperatures (4 and 23 °C) on sperm longevity. Although decreasing trend longevity was observed with the highest temperature, no significant differences were found between the two tested temperatures (data not shown).

Experiment 5. Combined effect of pH and seawater temperature on sperm motility

With the aim of studying the effect of the interaction between seawater temperature and pH on sperm motility, combinations of two temperatures: 4 and 24 °C, and two pH: 7.8 and 8.2, were tested. Total motility was not affected by temperature or pH (Figure 7A). However, as can be observed in Figure 7 B, D, E and F, there were significant interactions between temperature and pH in several kinetic parameters, such as MP, VCL, VSL, VAP, in which the lowest values were observed in the samples activated at 4 °C and pH 7.8. Accordingly, the highest values for ME and SL were also observed in the samples activated at 4 °C and pH 7.8 (Figure 7D, E).

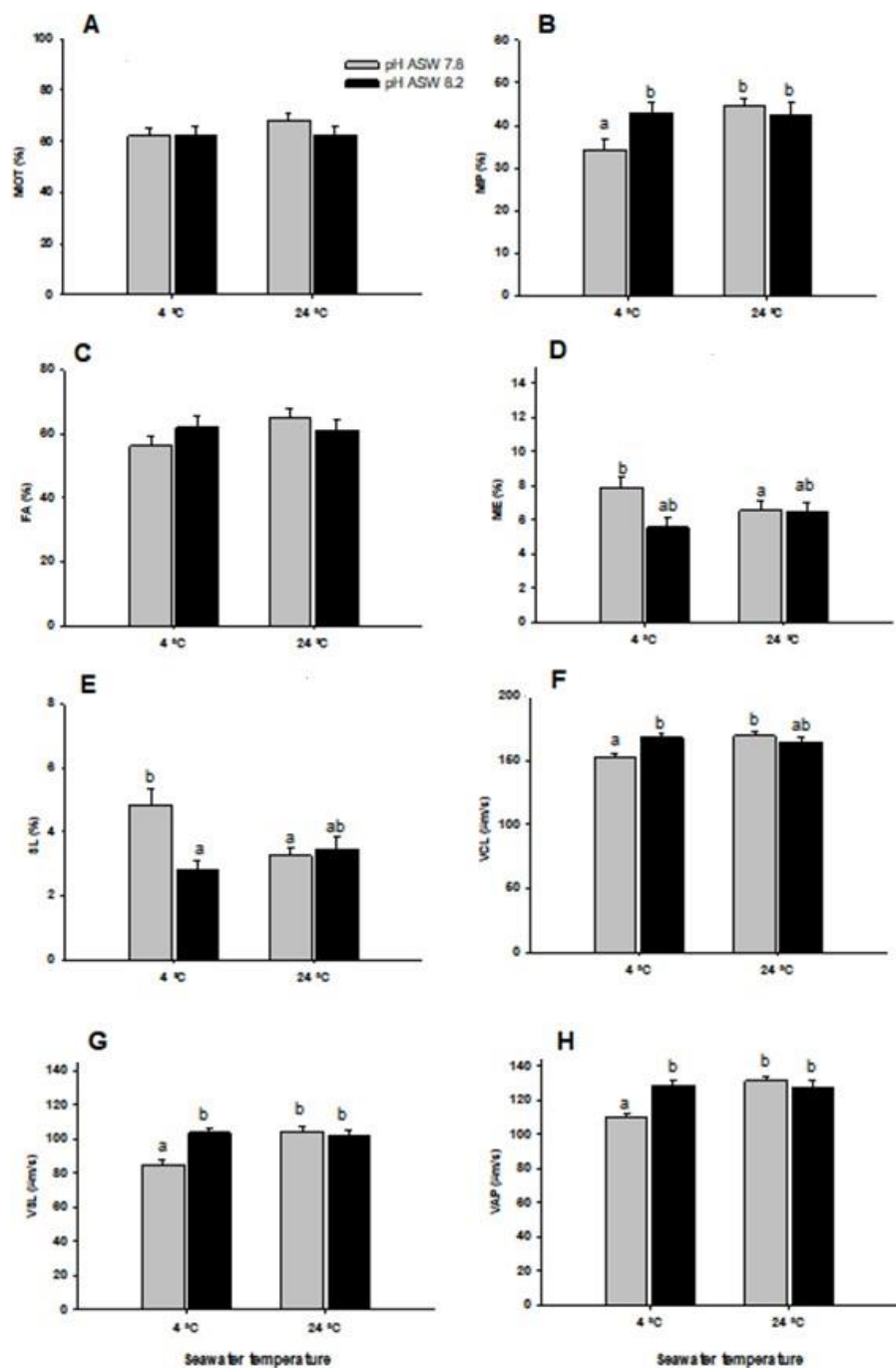


Figure 7. Effect of temperature and pH of seawater (ASW) on the total motility (MOT), progressive motility (MP), the percentage of fast (FA), medium (ME), and slow (SL) spermatozoa, the curvilinear velocity (VCL) and the straight line velocity (VSL). Values are represented as means \pm standard error. $n= 12$. Different letters indicate significant differences between the means ($p < 0.05$).

As for MP, other kinetic parameters (LIN, STR, and WOB) showed their lowest values in the samples activated at 4 °C and pH 7.8 (Figure 8). Regarding the lateral displacement of the head (ALH), it was influenced by the seawater pH, lower values for ASW at pH 7.8 than at 8.2 (Figure 8D).

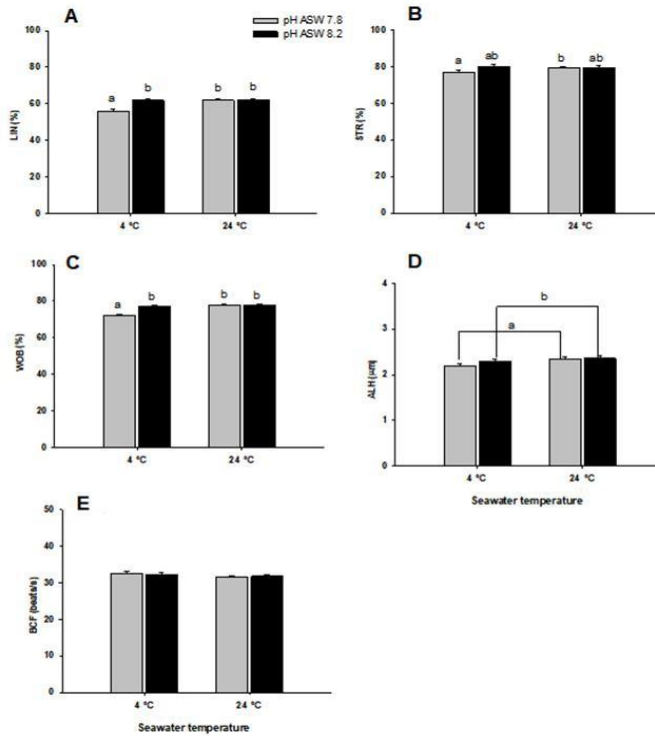


Figure 8. Effect of seawater temperature and pH (ASW) on the linearity index (LIN), oscillation index (WOB), lateral movement of the sperm head (ALH), and cross beating frequency (BCF). Values are represented as means \pm standard error. $n = 12$. Different letters indicate significant differences between the means ($p < 0.05$).

DISCUSSION

Due to climate change, sea acidification may mean a decrease of pH from current levels (pH 8.2) to values of 7.7-7.8 in the coming years (Hartin et al. 2016). As a consequence, marine organisms may suffer serious effects, such as a decrease in sperm motility, which is considered one of the main indicators of seminal quality (Gallego and Asturiano 2018, 2019). Eel sperm motility, as well as the sperm motility of many other marine species, could be affected by such a change, as motility was reduced at acidic pHs (Gallego et al. 2013; Gallego et al. 2017; Pérez et al. 2020).

The results in this study indicate that the pH range of 7.8-8.2 in seawater is optimal for maintaining a good European eel sperm movement capacity, producing high values of motility and velocities (MOT, MP, FA, VCL, VSL, and VAP), although some evidence (experiment 2) indicates that a pH of 7.8 induces small but significantly lower motility than a pH of 8.2. The present results agree with the linear regression found by Pérez et al. (2020) between seawater pH and sperm motility, where the lowest values were found at the low pH and the highest at the high pH tested, 8.2. According to the results obtained, it seems that European eel spermatozoa can tolerate the acidification of seawater as long as the levels are below pH 7.8.

Moreover, similar results were found for the Japanese eel (Tanaka et al. 2004), where acidification of the activating medium to levels of 7.7 decreased sperm motility to a great extent. Furthermore, studies carried out with salmonids (Ciereszko et al. 2010) and common trout (*Salmo trutta m. trutta* L.) (Dziewulska and Domagała 2013) also suggest that the low pH of the activating

medium affects sperm motility. The reason for the decrease in sperm motility at low seawater pHs could be that dynein, the motor of the flagellum, cannot act at low pH, as was observed in the steelhead trout (*Oncorhynchus mykiss*) (Woolsey and Ingermann 2003). Another possibility is that a lower extracellular pH in the seawater, perhaps inhibits an ion channel such as the potassium ones, the proton pump or the ion exchangers necessary for the motility initiation.

For instance, a potassium channel involved in sperm motility, the CNKG, has been described in zebrafish, and it is activated by alkalinization (Fechner et al. 2015). The change of membrane potential is another factor that influences fish sperm activation, and it was demonstrated that resting membrane potential was dependent on pH, besides K^+ and Na^+ , in rainbow trout (*Salmo gairdneri*) (Catti et al. 1990). In mammals, a proton pump seems to be involved in sperm motility (Escoffier et al. 2020). Thus, a low seawater pH could inhibit the ionic exchanges and the subsequent membrane potential change necessary for the motility activation.

Also, a decrease in the pH of seawater can limit the metabolic activity of gametes and reduce the mitochondrial membrane potential of spermatozoa, hindering flagellum motility (Catti et al. 1990; Alavi and Cosson 2005; Ciereszko et al. 2010; Dziewulska and Domagała 2013; Castro-Arnau et al. 2022). The alkalinization of the medium is a necessary process for the activation of sperm movement in most fish (Alavi and Cosson 2005). Our results showed no difference in the effect of the pH of the extender (artificial seminal plasma) on sperm kinetics, but seawater pH significantly affected motility, showing better kinetic parameters for pH 8.2 than 7.8. In the case of the European eel, sperm

motility could be affected by such a change, as motility was reduced at acidic pHs, similar to our previous results (Gallego et al. 2014; Vilchez 2017; Pérez et al. 2020), as well as what has been observed with the sperm motility of the Japanese eel (Tanaka et al. 2004).

Previous research has reported that internal pH influences spermatozoan maturation and the motility of ejaculated spermatozoa in several fish species (Catti et al. 1990; Gallis et al 1991; Ciereszko et al. 2010; Dziewulska and Domagała 2013; Kutluyer 2018; Castro-Arnau et al. 2022). Studies undertaken by Pickering and Pottinger (1987), demonstrated that sperm exposure to a reduced pH for brown trout (*Salmo trutta*) shows elevated ammonia levels in combination with significantly increased plasma cortisol levels, which can lead to poor sperm quality.

Pérez et al. (2020) observed in European eel that the change in the intracellular pH of sperm cells at activation is linearly dependent on the pH of the diluent medium; this can favor the progressive movements and rectilinear velocity of the spermatozoa when they are activated with seawater with pH 8.2 as found in our present study.

The temperature requirements for reproduction reveal a certain vulnerability in the face of climate change, and since motility in fish spermatozoa is triggered externally, it is highly dependent on environmental conditions (Dzyuba and Cosson 2014). The results of several studies suggest that the temperature of the medium used in motility activation is directly involved in the sperm motility characteristics of many fish species with either internal or external fertilization (Dadras et al. 2017; Fenkes et al. 2017; Merino et al. 2023), such as carp (*Cyprinus carpio*) (Cejko et al. 2016), or brown trout (Fenkes et al. 2017), to

mention just a few examples.

In the present study the effect of the seawater temperature on sperm motility and kinetic parameters was evaluated, and according to the results obtained, the different temperatures examined (4 and 24 °C) did not significantly affect the motility of the European eel sperm. In fact, the only kinetic parameter that presented significant differences was BCF, which was lower at the high temperature, indicating a negative effect. The effect of seawater temperature on sperm longevity was not observed. Similar results were found for gilthead seabream (*Sparus aurata*) spermatozoa, where it was demonstrated that the initial motility parameters were not affected by a temperature range of 4–22 °C (Lahnsteiner and Caberlotto 2012).

When we combined the effect of pH and seawater temperature on sperm motility, we observed a trend in which the lowest pH (7.8) combined with the lowest seawater temperature (4 °C), caused a reduction of the European eel sperm motility. Higher temperatures seem to favor the sperm quality of the species when combined with a more alkaline pH of the activating medium (pH 8.2). Although seawater pH can influence the initiation and duration of sperm motility, according to our results, the pH in the range 7.8-8.2 does not appear to affect the duration of movement of the European eel spermatozoa, which is possibly more resistant than other species to pH variation.

Studies on different fish species, mostly from temperate or cold waters, indicate there is an inverse relationship between environmental temperature and the duration of sperm motility, which could be the result of expenditure of limited energy stores available for motility (Alavi and Cosson 2005; Dadras et al. 2017). For Nile tilapia (*Oreochromis niloticus*) spermatozoa, environmental

temperature induces an increase of velocity without a decrease of the percentage motility at an extremely wide range of temperatures (5–50 °C). Flagellar beat frequency would be associated with an increasing ATP utilization and generation rate with increasing temperatures (Dzyuba et al. 2019).

CONCLUSION

Our results indicate that the pH 8.2 and a temperature of 4 °C is adequate to be used as standard in the European eel sperm activating medium. Temperature does not appear to affect the duration of sperm movement, but the combination of low temperature and low pH induced lower sperm motility. The present results indicate that in the context of climate change, the European eel sperm could experience pHs lower than the standard (up to 7.8) and higher temperatures than in the spawning area. That is, it can move perfectly at 23-24 °C, while the supposed temperature in the spawning area is 20 °C (van Ginneken and Maes 2005). Since these factors can modulate reproductive development, oocyte production in females may also be affected. The present study may serve as a basis for future protection plans for the species and mitigate the effects of climate change on their populations.

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