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

Ph.D. Thesis

MALBELYS PADILLA SANCHEZ

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SÃO PAULO STATE UNIVERSITY "JÚLIO DE MESQUITA FILHO-UNESP INSTITUTE OF BIOSCIENCES, BOTUCATU
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MALBELYS PADILLA SANCHEZ

Thesis in co-tutelle submitted to the Institute of Biosciences of the Paulista State University - UNESP, Postgraduate Program in Biological Sciences (Zoology), Câmpus de Botucatu, SP and to the Polytechnic University of Valencia, Institute for Animal Science and Technology as part of the requirements to obtain the Ph.D. degree by both institutions.

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DEDICATION

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

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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 ºC, 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 ºC, 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 ºC, 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 ºC, 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 reproduccion asistida. El desarrollo de herramientas biotecnológicas como la criopreservación de gametos, juega un papel importante en la producción acuicola y en la formación de bancos de germoplasma, que contribuiran luego en la mejora genética de las poblaciónes 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 region 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 llevó 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 ºC, 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 ºC, 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 aqüicola 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 tenía 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 ºC, 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 ºC, 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:

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

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