IMPROVEMENT OF TECHNIQUES FOR SPERM EVALUATION AND CRYOBANKING IN EUROPEAN EEL

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A Dorotea y Angelines

"*In nature, nothing exists alone*"

— Rachel Carson

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SUMMARY

In the last decades, the European eel *Anguilla anguilla* has suffered a drastic decrease in the recruitment in most areas of their distribution range, leading the species to be included as critically endangered in the IUCN list. This situation, together with the high commercial importance of the species, evidence the need of taking actions such as development of reproduction in captivity and control of fisheries based on the complexity of their life cycle. One of the most interesting tools for its use in conservation biology is the sperm cryopreservation, which presents several advantages for this species such as the synchronization of gametes, selection of genetic lines or cryobanking.

However, the development of cryopreservation protocols necessarily requires good quality sperm, and it is also needed an accurate method to assess sperm quality both pre- and post-cryopreservation. On this last matter, fish sperm motility is considered one of the best quality biomarkers for sperm quality assessment in fish, and it can be evaluated subjectively or objectively using computer assisted sperm analysis (CASA-Mot) systems.

First, an experiment was conducted to evaluate the precision and accuracy of both methods for assessing sperm motility: the subjective method and the objective technique using CASA-Mot system. Moreover, it was tested whether the degree of expertise of the technicians in the case of the subjective method, has an effect on the accuracy of the motility estimation, and therefore there is an influence of the laboratory staff on the sperm motility assessment. Here we concluded that both the method and the technician expertise were key factors in order to accurately assess sperm motility in European eel, so the use of CASA-Mot together with qualified stuff is required to obtain reliable results.

Secondly, and alternative methods for European eel males maturation was evaluated by testing two different hormonal treatments: OVI, a recombinant α-choriogonadotropin; and VET, a human chorionic gonadotropin purified from female urine. After choosing the best hormonal treatment, the effect of three different doses was evaluated aiming for best performance and lowest cost on the treatment. The results of this experiment pointed at OVI

as the best hormonal treatment in terms on sperm quantity and quality in most of the weeks of treatment, and at a weekly dose of 1.5 IU/g fish, which also provide the greatest profitability, obtaining high quality sperm at a lower price.

In a third experiment, and using the knowledge acquired in the two first experiments (using the OVI hormonal treatment and CASA-Mot to assess sperm quality), a series of experiments were conducted to standardize the European eel sperm cryopreservation protocols available at the moment (using DMSO or methanol as cryoprotectant). The results indicated that the protocol using methanol was the best of them two in terms of sperm motility and velocity, sperm viability and preservation of DNA integrity.

Following this last standardized method, a fourth experiment was conducted aiming for improvement of the protocol in terms of volume (larger volumes) and sperm quality outcome. Moreover, a simple protocol for short-term storage was developed to complement the options to preserve sperm for different time periods. Of all the tested storing conditions, 1/50 dilutions at 4 ºC showed the best results, maintaining the motility compared to control for 3 days, and some sperm motility (12%) was still observed after 7 days. From the cryopreservation experiment, it was possible to scale up the cryopreserved volumes to 2 and 5 mL without losing sperm quality compared to lower volumes. Moreover, the protocol was further improved by supplementing the protocol with egg yolk as an additive, obtaining the highest cryopreserved sperm motilities (over 50%) ever reported in European eel.

The final chapter of this thesis consists of a historical review of all the work that has been carried out in cryopreservation of eel sperm. Here, it is described with detail the evolution of the different protocols developed since the early 2000's in the Japanese and European eels, and including the latest results presented in previous chapters, which put into perspective our advances in cryopreservation of eel sperm.

RESUMEN

En las últimas décadas, la anguila europea *Anguilla anguila* ha sufrido una disminución drástica de su población lo que ha llevado su inclusión como especie en peligro crítico en la lista roja UICN. Esta situación, junto con la gran importancia comercial de esta especie, evidencia la necesidad de tomar acciones como el desarrollo de la reproducción en cautividad y el control de la pesca. Una de las herramientas más interesantes para su uso en la biología de la conservación es la criopreservación de espermatozoides, que presenta varias ventajas para esta especie, incluyendo la sincronización de gametos, la selección de líneas genéticas o su uso para la creación de un criobanco.

Sin embargo, el desarrollo de protocolos de criopreservación necesariamente requieren esperma de buena calidad. Además, se necesita un método preciso para evaluar la calidad del esperma tanto antes como después de la criopreservación. Sobre esta última cuestión, la motilidad de los espermatozoides de los peces se considera uno de los mejores biomarcadores para la evaluación de la calidad de los espermatozoides en los peces, y se puede estudiar de forma subjetiva u objetiva utilizando sistemas "computer assisted sperm analysis" (CASA-Mot).

Primero, se realizó un experimento para evaluar la precisión y la exactitud de ambos métodos para estudiar la motilidad del esperma: el método subjetivo y la técnica objetiva que utiliza el sistema CASA-Mot. Además, se probó si el grado de experiencia de los técnicos en el caso del método subjetivo tiene un efecto en la precisión de la estimación de la motilidad y, por lo tanto, hay una influencia del personal del laboratorio en la evaluación de la motilidad del esperma. Aquí concluimos que tanto el método como la experiencia técnica eran factores clave para evaluar con precisión la motilidad del esperma en la anguila europea, por lo que se requiere el uso de CASA-Mot junto con material calificado para obtener resultados fehacientes.

En segundo lugar, se evaluaron métodos alternativos para la maduración de los machos de anguila europeos probando dos tratamientos hormonales diferentes: OVI, una gonadotropina recombinante; y VET, una gonadotropina purificada a partir de orina femenina. Después de elegir el mejor tratamiento hormonal de los dos, se evaluó el efecto de tres dosis diferentes con el

objetivo de obtener el mayor rendimiento al menor coste. Los resultados de este experimento apuntaron a OVI como el mejor tratamiento hormonal en una dosis semanal de 1.5 UI/g de pez, que proporciona la mayor rentabilidad, obteniendo esperma de alta calidad a menor precio.

En un tercer experimento, y utilizando los conocimientos adquiridos en los dos primeros experimentos, se realizaron una serie de experimentos para estandarizar los protocolos de criopreservación de esperma de anguila europea disponibles en ese momento (utilizando DMSO o metanol como crioprotector). Los resultados apuntaron al protocolo que utiliza el metanol como el mejor de ellos dos en términos de motilidad, velocidad y viabilidad de los espermatozoides y la preservación de la integridad del ADN.

Siguiendo este último método estandarizado, se realizó un cuarto experimento con el objetivo de mejorar el protocolo en términos de volumen (volúmenes de esperma más grandes) y de calidad espermática. Además, se desarrolló un protocolo simple de almacenamiento a corto plazo para complementar las opciones de preservar el esperma durante diferentes períodos de tiempo. De todas las condiciones de almacenamiento probadas, las diluciones 1/50 a 4 ºC mostraron los mejores resultados, manteniendo la motilidad en comparación con el control durante 3 días, y manteniendo cierta motilidad espermática (12%) después de 7 días. A partir del experimento de criopreservación, fue posible aumentar los volúmenes a 2 y 5 mL sin perder la calidad del esperma en comparación con volúmenes más pequeños. Además, el protocolo se mejoró aún más al complementarlo con yema de huevo como aditivo, obteniendo la mayor motilidad espermática criopreservada (más del 50%) jamás registrada en la anguila europea.

El último capítulo de esta tesis consiste en una revisión histórica de todo el trabajo llevado a cabo en criopreservación de esperma de anguilas, donde se describe en detalle la evolución de los diferentes protocolos desarrollados desde principios de siglo en la anguila japonesa y europea. En este, se incluyen los últimos resultados presentados en capítulos anteriores, lo que pone en perspectiva nuestros avances en criopreservación de esperma de anguila.

RESUM

En les últimes dècades, l'anguila europea Anguilla anguila ha sofert una disminució dràstica de la seva població el que ha portat la seva inclusió com a espècie en perill crític en la llista vermella UICN. Aquesta situació, juntament amb la gran importància comercial d'aquesta espècie, evidencia la necessitat de prendre accions com el desenvolupament de la reproducció en captivitat i el control de la pesca. Una de les eines més interessants per al seu ús en la biologia de la conservació és la criopreservació d'espermatozoides, que presenta diversos avantatges per a aquesta espècie, incloent la sincronització de gàmetes, la selecció de línies genètiques o el seu ús per a la creació d'un criobanc.

No obstant això, el desenvolupament de protocols de criopreservació necessàriament requereixen esperma de bona qualitat. A més, es necessita un mètode precís per avaluar la qualitat de l'esperma tant abans com després de la criopreservació. Sobre aquesta última qüestió, la motilitat dels espermatozoides dels peixos es considera un dels millors biomarcadors per a l'avaluació de la qualitat dels espermatozoides en els peixos, i es pot estudiar de forma subjectiva o objectiva utilitzant sistemes "computer assisted sperm analysis" (CASA-Mot).

Primer, es va realitzar un experiment per avaluar la precisió i l'exactitud de tots dos mètodes per estudiar la motilitat de l'esperma: el mètode subjectiu i la tècnica objectiva que utilitza el sistema CASA-Mot. A més, es va provar si el grau d'experiència dels tècnics en el cas del mètode subjectiu té un efecte en la precisió de l'estimació de la motilitat i, per tant, hi ha una influència del personal del laboratori en l'avaluació de la motilitat del esperma. Vam concloure que tant el mètode com l'experiència tècnica eren factors clau per avaluar amb precisió la motilitat de l'esperma en l'anguila europea, de manera que es requereix l'ús de CASA-Mot juntament amb material qualificat per obtenir resultats fefaents.

En segon lloc, es van avaluar mètodes alternatius per a la maduració dels mascles d'anguila europeus provant dos tractaments hormonals diferents: OVI, un gonadotropina recombinant; i VET, un gonadotropina purificada a partir d'orina femenina. Després de triar el millor tractament hormonal dels

dos, es va avaluar l'efecte de tres dosis diferents amb l'objectiu d'obtenir el major rendiment al menor cost. Els resultats d'aquest experiment van apuntar a OVI com el millor tractament hormonal en una dosi setmanal de 1.5 UI/g de peix, que proporciona la major rendibilitat, obtenint esperma d'alta qualitat a un preu millor.

En un tercer experiment, i utilitzant els coneixements adquirits en els dos primers experiments, es van realitzar una sèrie d'experiments per estandarditzar els protocols de criopreservació d'esperma d'anguila europea disponibles en aquest moment (utilitzant DMSO o metanol com crioprotector). Els resultats van apuntar al protocol que utilitza el metanol com el millor d'ells dos en termes de motilitat, velocitat i viabilitat dels espermatozoides i la preservació de la integritat de l'ADN.

Seguint aquest últim mètode estandarditzat, es va realitzar un quart experiment amb l'objectiu de millorar el protocol en termes de volum (volums d'esperma més grans) i de qualitat espermàtica. A més, es va desenvolupar un protocol simple d'emmagatzematge a curt termini per complementar les opcions de preservar l'esperma durant diferents períodes de temps. De totes les condicions d'emmagatzematge provades, les dilucions 1/50 a 4ºC van mostrar els millors resultats, mantenint la motilitat en comparació amb el control durant 3 dies, i mantenint certa motilitat espermàtica (12%) després de 7 dies. A partir de l'experiment de criopreservació, va ser possible augmentar els volums a 2 i 5 ml sense perdre la qualitat de l'esperma en comparació amb volums més petits. A més, el protocol es va millorar encara més al complementar-lo amb rovell d'ou com a additiu, obtenint la major motilitat espermàtica criopreservada (més del 50%) mai registrada a l'anguila europea.

L'últim capítol d'aquesta tesi consisteix en una revisió històrica de tot el treball dut a terme en criopreservació d'esperma d'anguiles, on es descriu en detall l'evolució dels diferents protocols desenvolupats des de principis de segle a l'anguila japonesa i europea. En aquest, s'inclouen els últims resultats presentats en capítols anteriors, el que posa en perspectiva nostres avenços en criopreservació d'esperma d'anguila.

GENERAL INTRODUCTION

1. The European eel

The European eel (*Anguilla anguilla*) is a member of the Elopomorpha superorder, and belong to the Anguilliforme order and the family Anguillidae. The family Anguillidae is formed by a single genus, *Anguilla*, that includes 19 species (Watanabe, 2003) all of which display a complex catadromous life cycle with long oceanic migrations ranging from few hundred to thousands kilometers depending on the species (Arai, 2014). Studies show that the genus *Anguilla* was originated in the deep ocean of tropical areas and radiated out from the tropics to colonize temperate regions. These species are now widespread, being present in temperate, subtropical and tropical areas all over the world, except in the western coasts of North and South America and in the South Atlantic (Inoue et al., 2010; Minegishi et al., 2005).

Eels are common in the traditional diets of many countries, especially in Europe and Asia, where the ability to provide high quality elvers can be highly profitable. In the late 1800's, the elvers were so plentiful that people used them to feed the pigs. Nowadays, elvers can be raised to eating size and sold again for a profit, and can quite literally worth their weight in gold. Although China is not one of the greatest consumers of eels, it is the country with most eel farms. There, it is common to buy elvers, grow them, smoke them and process them into "kabayaki", a popular dish highly appreciated in Japan (Schweid, 2002). This delicatessen is traditionally made using Japanese eel (*Anguilla japonica*), but imported eels from the United States (American eel) and Europe (European eel) were also used due to the scarcity of Japanese eels. However, since 2010, due to the decline of European eel catches, the EU imposed export restrictions, making illegal to sell European-caught eel to markets outside the EU (ICES, 2015). In the United States, although American eels (*Anguilla rostrata*) were consumed by the pilgrims from Europe, the nowadays catches are mostly used for export of elvers to Europe or Asia. In Europe, the European eel has been consumed in several countries for centuries. Already in the 11th century, eel fishery is mentioned in the English *Domesday book*, and as early as in the 15th century, in the first English-language cookbook, *The Forme of Cury*, there is a recipe for eel pie. Nowadays, eels are consumed in a wide variety of forms in many counties in

Europe. Smoked eels are common in North- and East-European countries. In Valencia, it is typical to eat the eels in *all i pebre*, a dish where eels are cooked in olive oil with paprika, potatoes and garlic. Elvers are also traditionally consumed in Europe, for instance in the Loire in France, in Comacchio in Italy or in the Basque country (Schweid, 2002).

1.1 European eel life cycle

The European eel, as all members of the *Anguilla* genus, is a catadromous species, with long oceanic migrations from continental waters to the ocean where they have their spawning areas. The longest spawning migration is cover by the European eel, with distances of 5000-8000 km from the coasts of Europe and North Africa to the spawning areas in the Sargasso sea (Arai, 2014; Schmidt, 1925). The life cycle of the European eel is complex and poorly understood (Figure 1). Although neither spawning adults nor eggs have ever been collected, marine expeditions conducted in the early 20th century by the Danish scientist Johannes Schmidt, pointed out that the spawning area of this species was located in the Sargasso Sea (Schmidt, 1923), in what is known as the panmixia theory and meaning that the whole species has one unique population. Although previous field observations, morphological traits and molecular data suggested that the complete homogeneity of the European eel population and the unique spawning location may have been an overstatement (van Ginneken and Maes, 2005), later findings after analyzing microsatellite loci in European eel larvae, indicated that the panmixia theory was the most plausible (Als et al., 2011).

The complexity of their life cycle includes several metamorphoses. First, the larvae hatched as pre-leptocephalus and then develops into a leptocephalus larvae, which has a laterally compressed body and looks like a leaf with a small head. These larvae are transported by the oceanic currents, and after one year drifting, they reach the continental coasts, where they metamorphose into glass eels. They swim into the coastal waters and upstream, where they are established in their final habitat and spend most of their lives as yellow eels (van den Thillart et al., 2009). This growth phase is the longest and commonly lasts between 2 and 25 years (Tesch and White,

2008), although, specific individuals have been reported to live much longer depending on the habitat and growing conditions, such as water temperature. Finally, yellow eels undergo a final metamorphosis into silver eel in a process called silvering.
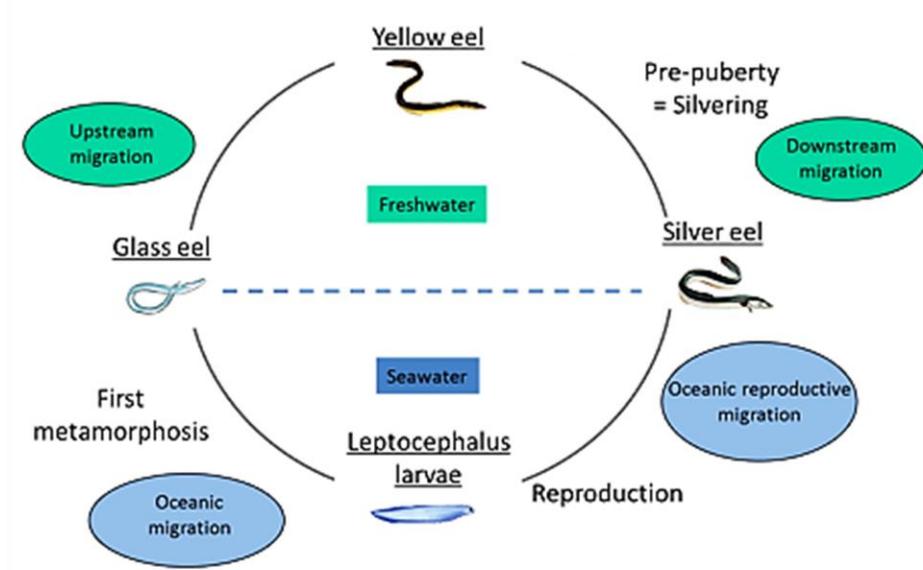
Figure 1. *European eel life cycle.*

Silvering is a puberty related event, which marks the beginning of sexual maturation, migration and the reproductive phase (Dufour et al., 2003). During this phase, the eels suffer several morphological changes such as enlargement of eyes, darkened skin or increased skin thickness, within others (Aoyama and Miller, 2003). The migration period towards the spawning areas of the silver eels was thought to last for 6-7 months (van Ginneken and Maes, 2005). However, a study using electronic tagging techniques in silver eels they manage to map their oceanic migration, and they observed that silver eels adopted a mixed migratory strategy, with some individuals reaching the spawning areas fast enough for spawning, whereas others arrive much later, in time for the following spawning season (Righton et al., 2016).

Silver eels are still sexually immature when they start their reproductive migration, with sexual maturation occurring during the migration period

towards the reproduction site in the ocean. External environmental factors seems to trigger the development of the gonad during their 5000-8000 Km migration, although it is still unknown which environmental stimuli are responsible for the final maturation of the eels (van Ginneken and Maes, 2005).

1.2 Conservation status

Since the early 80's, the population of European eel has continuously declined. The eel stock has decreased as much as 95-99% of its levels in 1960-80 (ICES, 2013). This drastic reduction has led to include the species on the Red List of Threatened Species, by the International Union for Conservation of Nature (IUCN), as "Critically Endangered" (Jacoby and Gollock, 2014), which is the last category before extinction (IUCN, 2019). The reasons behind the decline of the stock are uncertain but probably are a sum of several impacts, including overexploitation, water pollution, migration barriers, habitat loss, climate change and infection with the swimbladder parasite *Anguillicola crassus* (Castonguay et al., 1994; Dekker, 2003; Kirk, 2003; Knights, 2003; Székely et al., 2009).

The critical situation of the European eel population has forced the European Union to take actions, publishing a regulation in 2007 establishing measures for the recovery of the stocks (Council Regulation 1100/2007, 18th September 2007). This regulation is mandatory for all the state members on the EU and recommended to elaborate a management plan for the European eel. The plans necessary included measurements for habitat restoration, fishery restrictions and restocking. Moreover, export restrictions were imposed and European eels caught in the EU cannot be exported to non-EU countries.

2. Artificial maturation

In eels, dopaminergic inhibition in addition to a deficient stimulation of gonadotropin-releasing hormone (GnRH), block their sexual maturation as long as the reproductive oceanic migration is not performed (Dufour et al., 1988; Dufour et al., 2003; van Ginneken and Maes, 2005). Therefore, eels are blocked in a pre-pubertal stage and do not mature spontaneously in

captivity. To induce an artificial full maturation in eels, costly hormonal treatments are required that last for several weeks in males and even months in females (Lokman and Young, 2000; Mylonas et al., 2017; Ohta et al., 1996; Oliveira and Hable, 2010; Pedersen, 2003).

2.1 Hormonal treatments

The traditional hormonal treatment with gonadotropins to induce maturation in European eel males typically consists of weekly injections of human chorionic gonadotropins (hCG) (Gallego et al., 2012; Pérez et al., 2000) and has been used as the preferred method to obtain high quality sperm in European eels (Palstra and van den Thillart, 2009). However, the are several formats in which hCG have been successfully administered in European eel (Gallego et al., 2012).

The first hormonal treatments for European eel gonadal maturation consisted in the administration of urine from pregnant women intraperitoneally injected (Fontaine, 1936). By the end of the XX century, several companies developed the technology to isolate hCG from urine of pregnant women, making available commercial treatments that simplify the maturation treatments, and allowing to standardize the process (Dollerup and Graver, 1985; Khan et al., 1987). Later, many studies focused in optimizing and standardizing the hormonal treatment based on purified hCG, developing a protocol consisted in weekly intraperitoneal injections of 1.5 IU/g fish, that succeed in gonadal maturation and production of good sperm quality (Asturiano et al., 2006). In more recent years, through the development of recombinant DNA technology, new recombinant hCG (hCG$_{rec}$) was developed and a protocol using this hormonal treatment became the preferred alternative yielding good results in eel maturation and sperm quality (Gallego et al., 2012). However, the application of heterologous hormonal treatments with hCG have been associated to low rates of fertilization and hatching (Tanaka et al., 2002a).

2.2 Sperm quality in fish

The ultimate biomarker for assessing sperm quality is its fertilization capacity of mature oocytes and production of viable embryos (Cabrita et al., 2008; Fauvel et al., 2010). However, the fertilization capacity of the sperm is dependent on multiple factors, including quantifiable traits that are directly correlated to fertilization success and therefore can be used as alternative biomarkers. In fish sperm, several traits have been used as biomarkers for good sperm quality including sperm density (Sørensen et al., 2013), motility (Gallego et al., 2013b), morphometry (Alavi et al., 2015), energy content (Dzyuba and Cosson, 2014), and membrane and DNA integrity (Fauvel et al., 2010). Regardless of these sperm quality biomarkers, motility is currently considered the best sperm quality biomarker in fish, due to its facility of analysis and its high correlation with fertilization and hatching rates (Gallego and Asturiano, 2018b; Suquet et al., 1992).

The analysis of motility of fish sperm has been traditionally performed subjectively, where a technician evaluates the sperm motility though an observation under the microscope and estimating the percentage of motile spermatozoa, usually by assessing a motility score corresponding to an arbitrary criteria from 0 (immotile) to 5 (most spermatozoa motile) (Rurangwa et al., 2004). However, it has been reported that this kind of subjective evaluation used in human and animal sperm may lead to variations of 30 to 60% in the same ejaculates (Verstegen et al., 2002). Yet, the particular variation depending on the observer, have not been studied in fish sperm. This inconsistency make the results obtained with subjective evaluation difficult to compare between laboratories or experiments, and sometimes can make the results not reliable (Rosenthal et al., 2010).

An objective analysis is also possible using a tracking software developed for the analysis of spermatozoa movement. This objective method is known as computer-assisted sperm analysis system, or CASA-Mot, and it is consistent of a microscope, a camera and an image analysis software. CASA-Mot not only analyzes the percentage of motile spermatozoa but also makes possible to analyze other kinetic parameters, therefore the use of this tool have been widely applied in multidisciplinary studies, from broodstock management or

cryopreservation to ecology or toxicology (Gallego and Asturiano, 2018b; Kime et al., 2001).

The development of CASA-Mot systems started in the 70s in mammalian sperm studies, and in the 90s the method was adapted for it use in fish spermatozoa studies (Perchec et al., 1995; Toth et al., 1995). To date, these systems have been used and validated in a wide range of animal groups such as marine invertebrates, birds, marine mammals, reptiles or even insects (Al-Lawati et al., 2009; Lüpold et al., 2008; Montano et al., 2012; Riesco et al., 2017; Tourmente et al., 2011).

One of the main advantages of the use of CASA-Mot for analysis of sperm is the possibility of analyze several kinetic parameters. The two most common parameters used for fish sperm analysis are spermatozoa motility (MOT) and spermatozoa progressive motility (pMOT) (Kime et al., 2001), which can provide a general overview of the quality of the sperm (Rurangwa et al., 2004). MOT indicates the percentage of spermatozoa moving in a sperm sample, whereas pMOT indicates the percentage of spermatozoa that swims in an essentially straight line. However, CASA-Mot analyses provide many other parameters that are useful to obtain a more complete analysis of sperm quality. These parameters include the curvilinear velocity (VCL), that is the speed at which the spermatozoa moves in its real trajectory, the straight line velocity (VSL), that is obtained dividing the distance between the start and the end of the movement in a straight line by the time elapsed, and the average path velocity (VAP) which is the speed of the spermatozoa through its spatial average trajectory (Figure 2).

Other interesting parameters analyzed by CASA-Mot include straightness (STR) which indicates the linearity of the spatial average path of the spermatozoon, or the beat cross frequency (BCF) that is the average rate at which the curvilinear sperm trajectory crosses its average path.

Another interesting feature is that CASA-Mot can discriminate different spermatozoa groups according to its VAP in "fast", "medium" and "slow" spermatozoa. This feature provides valuable information of the different subpopulations in a sperm sample (Gallego et al., 2015). It is noteworthy that most of the parameters evaluated by CASA systems have been positively

correlated with spermatozoa fertilization success in several fish species (Table 1).

3. Cryopreservation of fish sperm

Cryopreservation is the conservation of biological material in liquid nitrogen (LN) at very low temperatures (-196 ºC) that may potentially preserve its viability indefinitely (Bakhach, 2009). Cryopreservation of fish gametes has many potential applications in aquaculture, conservation biology or cryobanking. The use of cryopreserved sperm for aquaculture purposes includes improvement of broodstock management in hatcheries, selection of genetic lines, or avoiding gamete synchronization problems. This last application is of particular interest in eels, since gamete production of male and female must be synchronized. Moreover, sperm cryopreservation may be used to provide biological materials for research scholars to perform comparative experiments, to promote exchange of genetic material for use in breeding and genetic studies, or to allow the cryobanking of genetic resources of endangered species (Asturiano et al., 2017; Cloud and Patton, 2009).
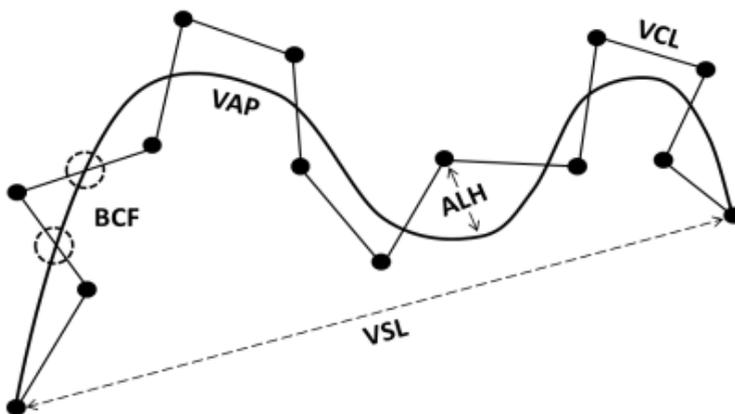
Figure 2. *Diagram of the kinetic parameters analyzed by CASA-Mot. The black dots represent the recorded trajectory of the head of a motile spermatozoon. VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BFC, beat/cross frequency (from Gallego and Asturiano, 2018).*

Table 1. *Correlation coefficients between the most used sperm kinetic parameters and fertilization rate (FR) or hatching rate (HR) in several fish species.*

Species	Motility		Velocity		Reference
	FR	HR	FR	HR	
Clarias gariepinus				0.81	(Rurangwa et al., 2004)
Colossoma macropomum	0.67		0.78		(Gallego et al., 2017)
Cyprinus carpio		0.53		0.54	(Linhart et al., 2000)
Hippoglossus hippoglossus	0.55				(Ottesen et al., 2009)
Pagrus major	0.88	0.88			(Liu et al., 2007)
Prochilodus lineatus			0.80		(Viveiros et al., 2010)
Sander vitreus			0.48		(Casselman et al., 2006)
Sparus aurata		0.77			(Beirão et al., 2011)
Takifugu niphobles	0.70	0.68	0.82	0.81	(Gallego et al., 2013b)

The development of a new cryopreservation protocol is normally dependent on several factors, such as the extender solution, the cryoprotectants, packaging and the cooling and thawing rates. All these factors are species dependent and therefore need to be developed for each species individually (Cloud and Patton, 2009). The use of extender is necessary to maintain the sperm inactive before freezing and are normally designed mimicking the seminal plasma, whereas the cryoprotectant is used to protect the integrity of the spermatozoa through the freezing and thawing process. The most common cryoprotectants are dimethyl sulphoxide (DMSO), ethylene, propylene glycol, glycerol and methanol. However, in marine fish species DMSO generally gave the best results.

The freezing and thawing rates are also critical for the development of a cryopreservation protocol, with cooling rates normally ranging between 5 and 99 ºC/min in marine fish species and thawing rates ranging between 1-40 ºC/min (Suquet et al., 2000).

Another important factor is the container used for packaging the sperm, which normally varies between small freezing straws of 0.25-0.5 mL to larger cryotubes of 5 mL or more. However, as mentioned above, all these factors are critical to develop a cryopreservation protocol, and need to be assessed individually for each species (Cloud and Patton, 2009; Suquet et al., 2000).

3.1 Origins of eel sperm cryopreservation

The first eel sperm cryopreservation protocol was developed for Japanese eel by Tanaka et al. (2002a). They used DMSO at 10% v/v as cryoprotectant, which is the most common cryoprotectant used in sperm of marine fish species (Gallego and Asturiano, 2018a), and it was used together with a designed extender solution that included NaCl, $NaHCO_3$ and soya lecithin. Since DMSO caused a hypertonicity in the medium that caused the activation of sperm motion (Horváth et al., 2005), the $NaHCO_3$ in the extender solution reduced that activation (Tanaka et al., 2002b). Moreover, the protocol consisted in the use of 2 mL cryovials, cooled in LN vapor for 5 min, 2 mm above the LN surface, before immersion and storage. The thawing consisted in immersion in water at 40 ºC for 70 s.

Using this protocol, Tanaka et al. (2002a) obtained good post-thaw sperm motility values (37-46%), and was further used for fertilization trials where they managed to fertilize Japanese eel oocytes using cryopreserved sperm. However, the hatchability of the fertilized eggs was lower than eggs fertilized with fresh sperm.

Years later, Müller et al. (2017) and Koh et al (2017) developed in parallel new sperm cryopreservation protocols for Japanese eel. Both protocols used methanol as cryoprotectant, which is osmotically inert and do not activate the sperm, and used similar extender solutions, ASP and ASP30 respectively. These extender solutions were both designed to be iso-ionic with the seminal plasma, yet differed slightly between each other and Koh´s protocol added FBS to the media. Although both protocols resulted in good post-thawing sperm motility, they differed in other aspects such as straw size or cooling and thawing rates.

Since these protocols consisted in small volumes of sperm, the most recent work of Nomura et al. (2018) adapted the protocol of Koh et al. (2017), to a larger-scale to be used for fertilization programs. In this work, they used 5 mL straws and adapted the cooling rate to that volume, and the cryoprotectants and extenders were as described in the latest protocol (Koh et al., 2017). Using this protocol, they obtained very good results in the

fertilization trials, with no differences in egg hatching and survival rated when using fresh and cryopreserved sperm.

3.2 European eel sperm cryopreservation

Cryopreservation of the European eel sperm was developed shortly after the first cryopreservation protocols for Japanese eel. Two independent research groups established their own cryopreservation protocols in Spain and Hungary. These protocols differed in most aspects, from the rearing conditions of the eels to the type of cryoprotectants applied into their experiments.

Our group developed in Spain a protocol (Asturiano et al., 2003; 2004) mimicking the protocol previously developed for Japanese eel (Tanaka et al., 2002a) using DMSO as cryoprotectant (10%) and different extenders. In follow up studies, they obtained the best results using sperm diluted 1:5 in Tanaka extender (extender used for Japanese eel (2002a)) or P1 extender (extender designed to be iso-ionic with the European eel seminal plasma (Pérez et al., 2003)), with 10% DMSO, reaching post-thaw sperm motilities of 32 - 36% (Asturiano et al., 2003; 2004).

In parallel, the group from Hungary (Müller et al., 2004) developed a cryopreservation protocol using a modified Kurokura solution as extender and 10% methanol as cryoprotectant. The dilution rate used was 1:8:1 of sperm:extender:methanol, obtaining similar results to those of the Spanish protocol (36% motile spermatozoa). Following this study, Szabó el al. (2005) conducted a series of experiments to test different extenders and cryoprotectants (DMSO and methanol). The best results were obtained using DMSO (10%) or methanol (10%) with Tanaka extender. The samples cryopreserved using the protocol with methanol could be further diluted 1:9 in Tanaka's medium to reduce the toxicity of the cryoprotectant. However, this was not a possibility when using DMSO as cryoprotectant, probably explained by the change in osmolality of sperm following dilution (Horváth et al., 2005). Following Müller's and Szabó's protocols, using methanol 10% as cryoprotectant and Tanaka extender, Müller et al. (2012; 2018) successfully used cryopreserved European eel sperm in fertilization trials

with Japanese eel eggs, obtaining hybrid larvae. However, the hatching rate was very low.

Alternatively to methanol based protocols, valuable results were obtained following protocols using DMSO in terms of percentage of spermatozoa motility, viability and spermatozoa head size (Asturiano et al., 2007; Garzón et al., 2008; Marco-Jiménez et al., 2006). However, the use of this cryoprotectant still increased the medium osmolality resulting in inducing spermatozoa motility activation and premature ATP consumption. To avoid this drawback, Peñaranda et al. (2009) tested different combinations of pH and $NaHCO_3$ concentrations, that resulted in the development of an improved medium based on the P1 medium, but containing 100 mM $NaHCO_3$ and pH 6.5 that partially prevented the activation effect of DMSO. With this protocol, they obtained post-thaw spermatozoa motility values close to 40%, which is well sufficient for fertilization trials, that were conducted by Asturiano et al. (2016). In this work, they successfully produced fertilized eggs and larvae. However, the fertilization and hatching rate were again low.

Despite the fact that cryopreservation protocols developed by the groups from Spain and Hungary were proved to succeed in fertilization trials, they differed in many aspects and a need of standardization of the protocol was evident. Moreover, following the improvements obtained in Japanese eels (Nomura et al., 2018), research should focus in scaling up in volume the protocols together with the use of additives for improving sperm cryopreservation outcomes for large scale fertilization programs.

4. Projects, grants and companies involved in this Thesis

All the experiments carried out in this thesis were funded by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 642893: IMPRESS (Improved production strategies for endangered freshwater species).

In addition, complementary training was conducted during short-term scientific missions (STSM) at Nofima AS (Tromsø, Norway) for 3 months and in the University of South Bohemia (Vodñany, Czech Republic) for 1 month. These STSM were funded with grants from COST Office (Food and Agriculture

COST Action: *Assessing and improving the quality of aquatic animal gametes to enhance aquatic resources. The need to harmonize and standardize evolving methodologies, and improve transfer from academia to industry*; AQUAGAMETE).

OBJECTIVES

The main objective of this thesis was to develop different techniques and protocols for European eel sperm cryopreservation, for improve the control of the reproduction in captivity of European eel. The specific objectives include:

- To demonstrate the intrinsic variation in the subjective evaluation of sperm motility and the convenience of use CASA-Mot systems to assess sperm quality

- To study the effect of different doses and hormonal treatments as alternative maturation methods for male European eels.

- To compare and standardize the sperm cryopreservation protocol for European eel sperm and evaluate the epigenetic effects of the available cryopreservation methods.

- To develop a simple and effective protocol for short-term storage of European eel sperm, where temperature, dilution and stirring during storage were tested.

- To adapt the available cryopreservation protocols of European eel sperm to larger volumes for its implementation in large-scale fertilization trials.

- To improve the latest sperm cryopreservation protocols for European eel sperm by testing the effect of the additives BSA, FBS and egg yolk.

CHAPTER 1

Subjective and objective assessment of fish sperm motility: when the technique and technicians matter

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Abstract

Fish sperm motility is nowadays considered the best sperm quality biomarker in fish, and can be evaluated both by subjective and computerized methods. With the aim to compare the precision and accuracy of both techniques, fish sperm samples were assessed by subjective methods and by a computer assisted sperm analysis (CASA-Mot) system, and simultaneously by three different technicians with different degrees of expertise on the sperm quality analysis. Statistical dispersion parameters (CV, coefficient of variation; and RG, range) were estimated in order to determine the precision and accuracy of the techniques and the influence of laboratory staff on sperm motion assessments. Concerning precision, there were not much significant differences between the technical support staff (high, medium, and low experimented technician), and statistical dispersion parameters were quite similar between them independent of the technique used and the sperm motility class analyzed. However, concerning accuracy, experimented technician reported subjective motility values very closed to the values provided by the CASA-Mot system, only 10 percentage points away from the data provided by a CASA-Mot system. However, medium and low experimented technicians often overestimate the CASA-Mot values, and amplitudes up to 30 percentage points were detected in several sperm assessments. To sum up, both the technique (subjective or objective) and the technician (degree of expertise) became key factors in order to reach accurate motility estimations, so the use of both qualified staff and novel CASA-Mot systems seems to be a critical requirement for obtaining satisfying results in fish species with similar motility patterns.

1. Introduction

Over the years, a relatively high number of sperm parameters have been used to assess sperm quality in fish (Fauvel et al., 2010). These sperm biomarkers have so far been documented in scientific articles, and several traits such as osmolality, plasma composition, sperm density, or sperm morphology have been linked to the ability of sperm to fertilize the ova (reviewed by Cabrita et al. 2014). However, sperm motility is currently considered the most useful tool for assessing sperm quality in fish, and high correlations have been reported between sperm motility and fertilization or hatching rates in several fish species such as pufferfish (*Takifugu niphobles*; Gallego et al. (2013b)), rainbow trout (*Oncorhynchus mykiss*; Bozkurt andSecer (2006)), red seabream (*Pagrus major*; Liu et al. (2007)) or tambaqui (*Colossoma macropomum;* Gallego et al. (2017)).

Nowadays, sperm motility evaluation can be done by two different ways in the laboratory: (i) the subjective way, in which a technician (more or less experienced) makes an evaluation of sperm motility through a simple observation under the microscope; and (ii) the objective way, in which sperm analysis systems, particularly CASA-Mot (computer-assisted sperm analysis) system, integrate the successive positions of the heads of moving spermatozoa in every frame video-taped for calculating their trajectories and kinetic characteristics.

A subjective evaluation method has been the most used technique to evaluate sperm motility over the history, but some problems have emerged from this method (Rurangwa et al., 2004). First drawback is focused on the own limitation of the human eye, through which we can only provide a coarse evaluation of (i) the percentage of motile spermatozoa and (ii) the sperm motility duration. In addition, this type of evaluation depends on the observer's experience, and several aspects such as sperm density, sperm velocity, and drift can cause over- or underestimations (Hala et al., 2009). Therefore, the low reproducibility of this subjective assessment, which can result in variations of 30 to 60% of CV (coefficient of variation) from the same sample, often makes difficult to interpret and compare the results intra- and inter-labs (Rosenthal et al., 2010; Verstegen et al., 2002).

By contrast, the gradual appearance and popularization of CASA-Mot systems have made possible to estimate a higher number of sperm motion parameters not given by subjective evaluation (spermatozoa velocities, motion pattern models, sperm subpopulations, etc.) and do it in an objective, sensitive, and accurate way (Kime et al., 2001). Nevertheless, it is important to consider that CASA-Mot systems are not ready-to-use devices, and they also depend largely on technical and biological settings which need to be standardized for enhancing the comparability of data produced by different research groups (Boryshpolets et al., 2013; Gallego et al., 2013a). In addition, CASA-Mot systems are not available for many research groups due to the initial investment necessary to purchase the complete equipment (software, high-resolution camera, etc.), so half of the scientific studies carried out during the last years have not used a CASA-Mot systems for the spermatozoa motion assessment (Gallego and Asturiano, 2018b).

In this scenario, technique and technicians could have an important role for obtaining credible assessments of spermatozoa kinetic features, so the aim of this study was to compare the precision and accuracy of both subjective and objective techniques and, simultaneously, the influence of laboratory staff previous experience on sperm motion assessments.

2. Materials and methods

2.1 Fish handling and sperm collection

Thirty adult European male eels from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Spain), were moved to the Aquaculture Laboratory of the Universitat Politècnica de València (Spain). The fish were distributed in two 150-L aquaria (approximately 15 males per aquarium) keeping a constant temperature of 20 °C and covered to reduce light intensity and fish stress. During 1 week, the eels were gradually acclimatized from fresh- water to seawater (salinity = 37 ± 0.3 g/L). Later, they were anesthetized once a week with benzocaine (60 ppm) for injecting 1.5 IU g^{-1} fish of recombinant human chorionic gonadotropin (Ovitrelle, Merck S.L., Madrid). Fish were fasted throughout the trial and they were handled in accordance with the

European Union regulations regarding the protection of experimental animals (Dir 86/609/EEC).

From the seventh week of hormonal treatment, sperm samples were weekly collected by abdominal pressure 24 h after the administration of the hormone (following the protocol described by Pérez et al. 2000), and taking special care to avoid the contamination with feces, urine, and seawater. Samples were diluted 1:9 (sperm:extender) in P1 medium (Peñaranda et al., 2010b) and kept in plastic tubes at 4 °C until sperm kinetic analyses, which were carried out during the next 2 h after sperm collection.

2.2 Experimental design

Each of the samples was evaluated according to Fig. 1 by three different techniques: (i) by subjective way (human eye) directly through the ocular lens (eyepieces) of the microscope, (ii) by subjective way (human eye) using a computer monitor connected to the microscope, and (iii) by an objective way using a CASA-Mot system. The main difference between the two subjective assessments was that sperm sample observed directly through the eyepieces was done in a bright-field microscopy (dark cells on bright background) with a great wide field of view; while the assessment through the screen (monitor) was done in a dark-field (bright cells on dark background) with a smaller wide field of view. In addition, these three assessing methods were carried out by three different technicians with different degrees of expertise on the sperm quality analysis: (i) a high experimented technician (high ET; a postdoctoral researcher) with years of experience on sperm motility assessment, (ii) a medium experimented technician (medium ET; a pre-doctoral student) whose thesis is focused on issues related to sperm motion analysis, and finally (iii) a low experimented technician (low ET; a grade student) with very little experience on the sperm quality analysis. It is important to remark that the dispersion parameters (see the "Statistical analysis" section) used in this study were estimated analyzing the same sample through three consecutive sperm activations for each technique.
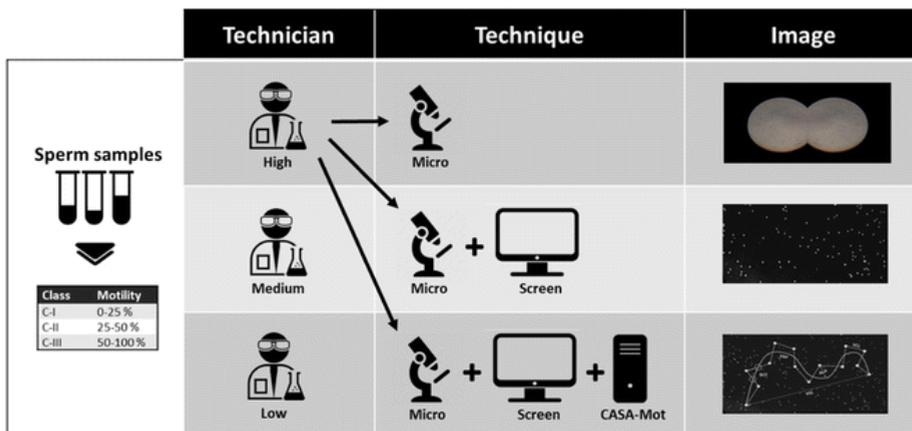
Fig 1. *Experimental design for carrying out the motility assessments through the three different techniques (microscope, screen, and CASA-Mot system) and three technicians with different degrees of experience (high, medium, and low). Each sperm sample was assessed consecutively by the three methods and the same observer in order to avoid differences between the different evaluation methods. Samples were evaluated in different orders with every technique to avoid the observer's preconception on the grade of motility of the sample from the technique used previously*

2.3 Sperm motility assessment both by subjective and objective methods.

Each sample was activated by mixing 0.5 µL of P1-diluted sperm (see the "Fish handling and sperm collection" section) with 4.5 µL of artificial seawater (Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH level was adjusted to 8.2). All the motility analyses (both by subjective and objective methods) were performed by triplicate.

In relation to the subjective method, technicians estimated the sperm motility (percentage of motile spermatozoa) by both (i) looking directly through the eyepieces of the microscope and (ii) looking directly through a computer monitor. Spermatozoa were considered motile presenting any type of movement (progressive or non- progressive) according the World Health Organization (WHO) criteria in the 5th edition (2010).

In addition, technicians were asked to classify every sample as fast (spermatozoa with fast progressive movement), medium (spermatozoa with medium forward movement), or slow (spermatozoa with slow forward movement or non-progressive movement) depending on the motion (estimated subjectively) of swimming spermatozoa. Finally, objective assessments were done immediately after subjective evaluation using a CASA-Mot sys- tem, and several kinetic parameters such as total motility (MOT, %), progressive motility (pMOT, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and average path velocity (VAP, µm/s) were recorded for further analysis. Several manuscripts have reported high correlations between these parameters with fertilization and hatching rates in several fish species, so they become good biomarkers to predict and sperm quality and carrying out sperm studies (Gallego and Asturiano, 2018b).

In order to perform an in-depth analysis of the results, sperm samples were classified into three classes based on the percentage of motile spermatozoa provided by the CASA-Mot system: class I (C-I) = 0–25% of motile cells; class II (C-II) = 26–50% of motile cells; and class III (C-III) = 51–100% of motile cells.

2.4 Setting used on CASA system.

Kinetic sperm analysis was carried out by the motility module of ISAS®v1 (Proiser R+D, S.L.; Paterna, Spain) using an ISAS® 782M camera recorder capturing 60 frames per second (fps). Between 200 and 600, spermatozoa were captured in each field adjusting the brightness and contrast in the CASA-Mot settings in relation to the microscope light with the aim to reach spermatozoa clearly defined (Gallego et al., 2013a). Range size particles were defined between 2 and 20 µm and spermatozoa were considered immotile if their VCL was lower than 10 µm/s.

2.5 Statistical analysis

For evaluating the variability on the dataset, several measures of dispersion such as the coefficient of variation (CV, %) and the absolute range (RG, difference between the smallest value and the largest value of a series) were estimated both for each method and for each technician (observer).

In order to evaluate the accuracy, the amplitude (difference between the subjective evaluation and the motility values provided by a CASA-Mot system) was estimated. Coefficients of correlation (*r*) between the subjective and objective assessments were also obtained for high, medium, and low experimented technicians (ET) among different sperm motility classes (C-I, C-II, and C-III). Finally, box plots were created in order to assess the ability of each technician to appreciate the velocity of swimming spermatozoa.

Data expressed in percentages were transformed using the arcsine transformation, and Shapiro-Wilk test was used to check the normality of data distribution. One-way analysis of variance (ANOVA) was used to analyze the data and significant differences between treatments were detected using the Tukey multiple range test (*P* < 0.05). Statistical analyses were performed using the statistical pack- age SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Precision of techniques & technicians

The precision for both techniques and technicians was evaluated through CVs and RG values (see Figs. 2 and 3, respectively). CVs were quite similar between technicians independent of the technique used and the sperm motility class analyzed (Fig. 2), and statistical differences were only found assessing samples from C-II and C-III through a subjective motility analysis (Fig. 2a, b).

Regarding the absolute range (RG, defined as the difference between the smallest value and the largest value registered in the same motility assessment), a similar pattern than that obtained in CVs was found. However, trends in RG showed that high ET showed smaller RGs than medium and low ETs independently of the technique applied and the sperm motility class analyzed (Fig. 3). Nevertheless, statistical differences were only found assessing samples from C-II and C-III through a subjective motility analysis (Fig. 3a, b).
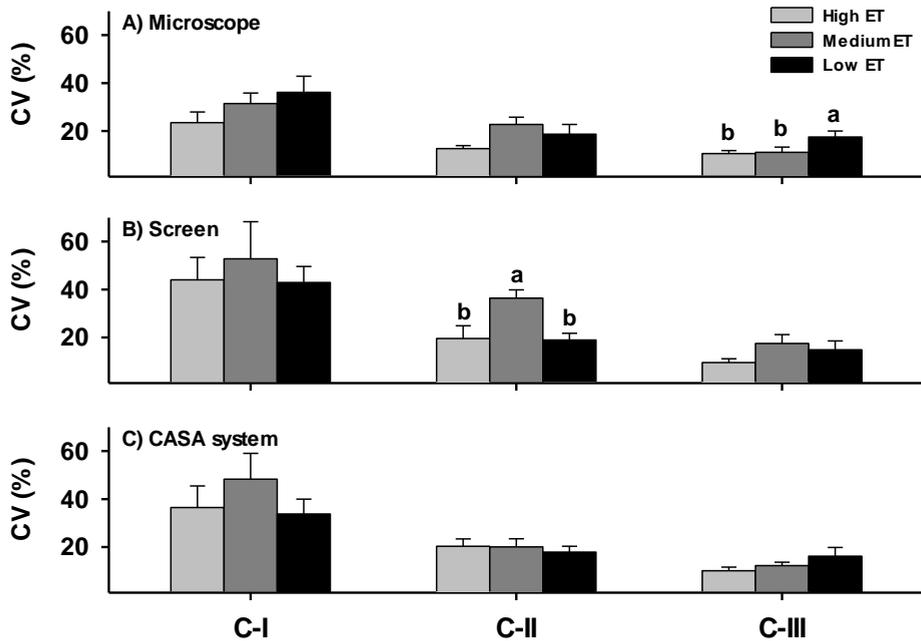
Figure 2*. Coefficients of variation (CVs) obtained by High, Medium, and Low experimented technicians (ETs) among different sperm motility classes (C-I, C-II and C-III). Sperm motility was assessed through (A) the eyepieces of the microscope, (B) the computer monitor (screen), or (C) by a CASA system. Different letters indicate statistical differences (P ≤ 0.05) between different technicians.*
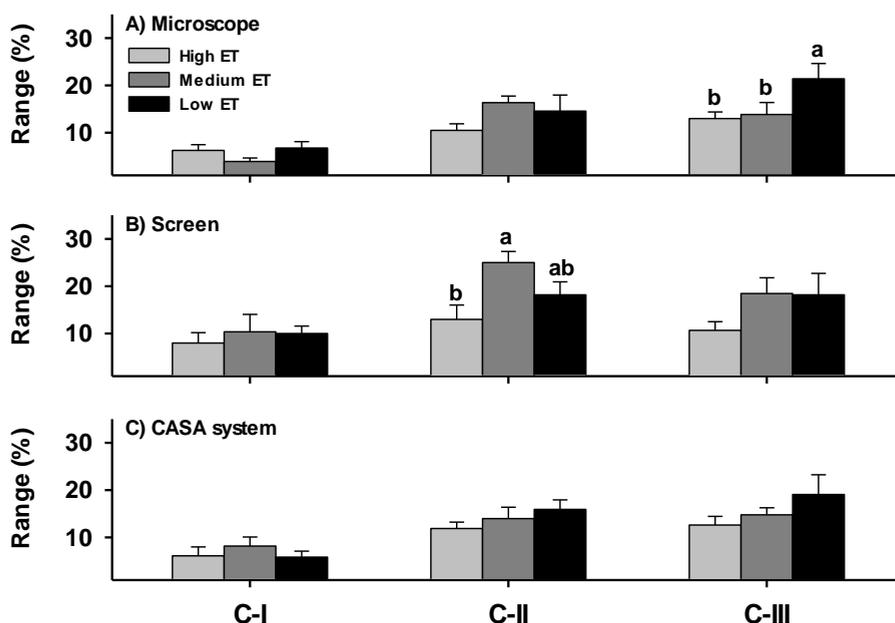
Figure 3. Absolute ranges (RGs, difference between the smallest value and the largest value of a series) obtained by High, Medium, and Low experimented technicians (ET) among different sperm motility classes (C-I, C-II and C-III). Sperm motility was assessed through (A) the eyepieces of the microscope, (B) the computer monitor or screen, or (C) by a CASA system. Different letters indicate statistical differences (P ≤ 0.05) between different technicians.

3.2 Accuracy of techniques & technicians

The ability of technicians to carry out an accurate subjective evaluation was measured as the difference (amplitude) between the CASA-Mot motility values and the subjective estimations (Figs. 4 and 5). Concerning subjective motility assessments carried out through the eyepieces of the microscope, high ET obtained subjective motility values relatively closed to CASA-Mot motility values, presenting over- or under- estimations of only around 10 percentage points throughout all the sperm motility classes (Fig. 4a). However, although medium and low ETs had acceptable amplitude values in C-I class, overestimation of values was the common trend in samples belonging

to C-II and C-II classes, with subjective sperm motility values 25 percentage points higher than the motility assessed by a CASA-Mot system (Fig. 4b, c).
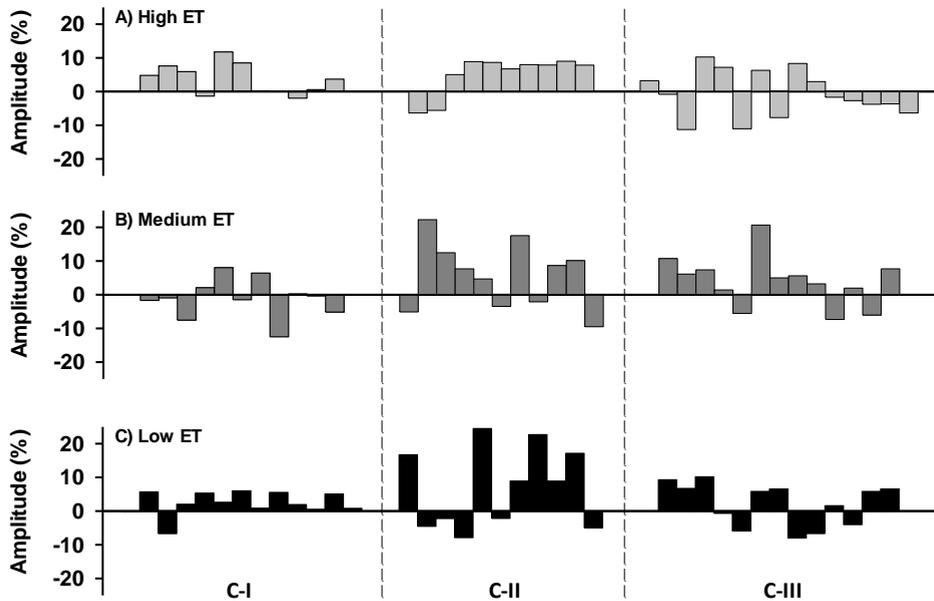
Figure 4. *Differences (amplitude) between the sperm motility values provided by a CASA system and the sperm motility assessments carried out through the eyepiece of the microscope by a High (A), Medium (B), and Low (C) experimented technicians (ETs).*

Concerning subjective motility values obtained through the computer monitor (screen), high ET also obtained subjective motility values relatively closed to real motility values assessed by a CASA-Mot system, presenting once again over- or underestimations of around 10% along all the sperm motility classes (Fig. 5a). Medium ET was able to estimate good subjective values (relatively closed to CASA-Mot motility values) of the samples belonging to C-I and C-II classes, but underestimations (up to 16%) were the common pattern on the C-III class (Fig. 5b). Finally, low ET was not able for estimating subjective motility values closed to CASA-Mot assessments, and high overestimations were the common trend in all the sperm classes,

reaching amplitude values up to 25 and 31% in C-I and C-II classes, respectively (Fig. 5c).
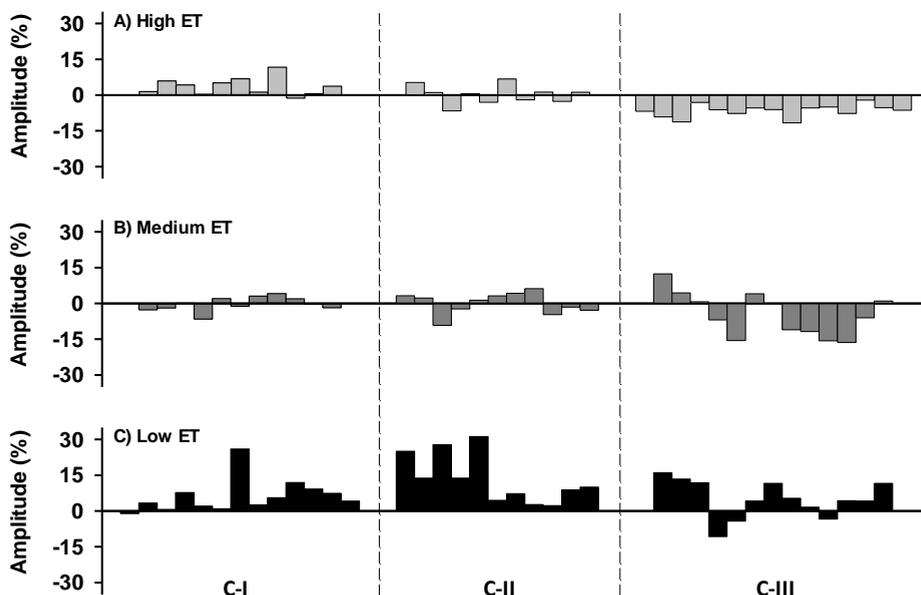
Figure 5. Differences (amplitude) between the sperm motility values provided by a CASA system and the sperm motility assessments carried out through the computer monitor by a High (A), Medium (B), and Low (C) experimented technicians (ETs).

Coefficients of correlation provided in Table 1 show that although all technicians showed relatively high *r* values among C-I and C-III classes (> 0.8 and > 0.7, respectively), high ET was the only technician able to reach acceptable *r* values in samples belonging to C-II class. In this sense, medium and low ETs presented low *r* values (0.42 and 0.57, respectively) between the subjective microscope assessments and CASA-Mot estimations.

CHAPTER 1

Table 1. *Coefficients of correlation (r) between the sperm motility values assessed subjectively by eyepieces of the microscope (micro) and through the computer monitor (screen) with the sperm motility values provided by a CASA system. r were estimated for High, Medium, and Low experimented technicians (ET) among different sperm motility classes (C-I, C-II and C-III).*

|			High ET		Medium ET		Low ET	
			MOT Screen	MOT CASA	MOT Screen	MOT CASA	MOT Screen	MOT CASA
C-I	MOT	Micro	0.88	0.92	0.87	0.78	0.88	0.88
	MOT	Screen		0.94		0.93		0.94
C-II	MOT	Micro	0.68	0.65	0.39	0.42	0.49	0.57
	MOT	Screen		0.96		0.87		0.78
C-III	MOT	Micro	0.71	0.86	0.73	0.71	0.79	0.79
	MOT	Screen		0.88		0.66		0.88

3.3 Technician ability for estimating sperm velocities

Finally, last trial tried to evaluate the technician ability for estimating sperm velocities using the subjective assessments. In relation to subjective estimations carried out through the eyepieces of the microscope (Fig. 6), spermatozoa classified as fast, medium, or slow by the high ET showed significant differences in terms of both VCL, VSL, and VAP. However, spermatozoa classification carried out by medium and low ET did not reveal statistical differences between slow and medium spermatozoa in terms of VSL and VAP, evidencing their incapacity to evaluate properly the spermatozoa velocity.
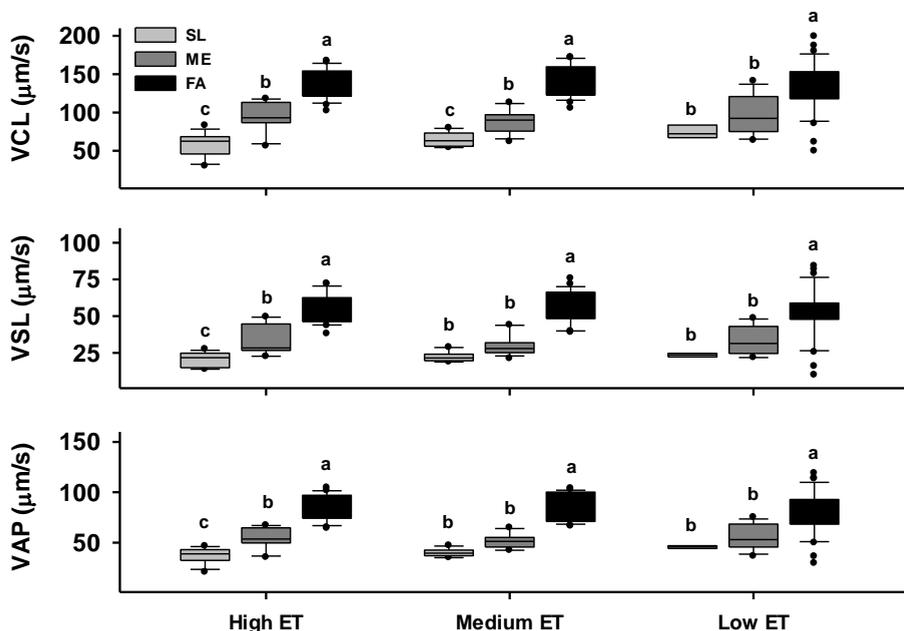
Figure 6. Average velocity values (VCL, VSL and VAP) of spermatozoa classified by different technicians as Fast (FA), Medium (ME), or Slow (SL). Velocity estimations (FA, ME, and SL) provided by High, Medium, and Low experimented technicians (ETs) were carried out through the eyepiece of the microscope. Different letters indicate statistical differences (P ≤ 0.05) between sperm velocity classes.

Concerning subjective estimations carried out through the computer monitor (Fig. 7), spermatozoa classified as fast, medium, or slow both by the high and medium ET showed significant differences in terms of VCL, VSL, and VAP, so both observers were able to do an accurate estimation of sperm velocity. However, velocities of spermatozoa classified as slow and medium by low ET did not differ statistically in neither VCL, nor VSL, nor VAP, so low ET was only able to distinguish subjectively the fast spermatozoa to the rest.
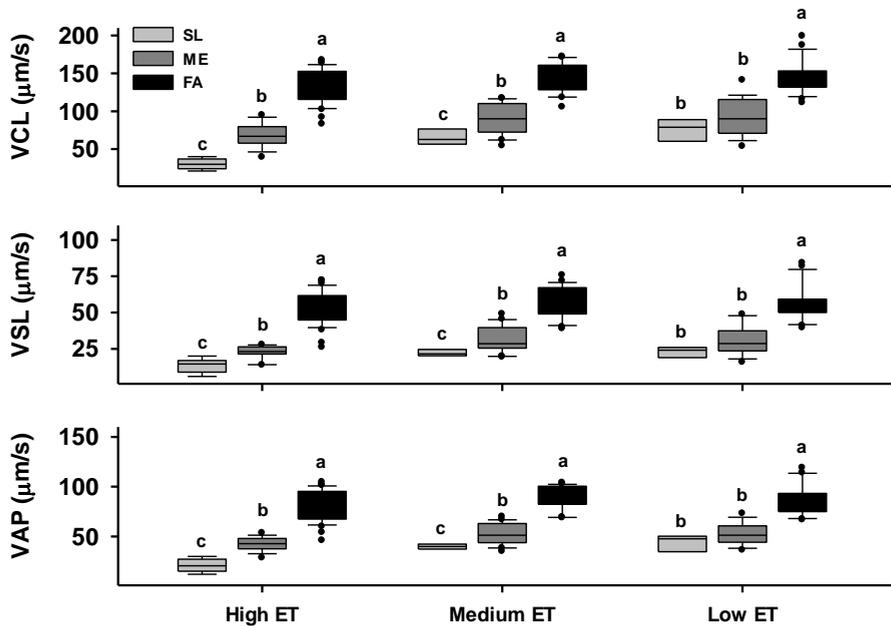
Figure 7. *Average velocity values (VCL, VSL and VAP) of spermatozoa classified by different technicians as Fast (FA), Medium (ME), or Slow (SL). Velocity estimations (FA, ME, and SL) provided by High, Medium, and Low experimented technicians (ETs) were carried out through the computer monitor (screen). Different letters indicate statistical differences (P ≤ 0.05) between sperm velocity classes.*

4. Discussion

This study show, by the first time in fish species, the importance of technique and technicians chosen for obtaining credible sperm motility assessments to be applied in fish spermatology research. Both precision and accuracy parameters were obtained in order to investigate the effect of subjective or objective methods for assessing sperm motility, at the same time that ability of different technicians (with different degrees of experience) for carrying out the different analysis.

In relation to precision, which reflects how consistent results are when measurements are repeated (even if they are far from the "real" value), the

data revealed that there were not much differences depending on the methods used (objective or subjective), and CVs were quite similar between the techniques applied. In this sense, CVs are often used for testing analytical or instrumental techniques (immunoassay tests, PCR plates, etc.), and values no bigger than 25% are usually accepted in the scientific field (McAuliffe et al. 2015). Even though there are not data from fish, CV values obtained from subjective and objective assessment techniques were similar than those reported in several mammal species. For example, in rams, CVs of sperm motility assessments ranged between 12.5 and 31.74% (Rajashri et al., 2017); on boar, CVs values ranged from 4.7 to 34.7% (Reicks, 2012); and in bull, CVs ranged between 21 to 44% (Pepper-Yowell, 2011). On the other hand, there were not much significant differences between the technical support staff (high, medium, and low experimented technicians), and statistical dispersion parameters were quite similar between them independent of the technique used and the sperm motility class analyzed. In this respect, the degree of experience in the laboratory did not become a key factor in order to achieve a high level of precision in fish sperm motility assessments.

However, in relation to accuracy, which measures the ability of technicians to carry out an accurate subjective evaluation by the difference (amplitude) between the CASA-Mot motility values and the own subjective assessment, this study yielded interesting results. When sperm motility assessments were carried out through the eyepieces of the microscope, high ET obtained subjective motility values relatively closed to the values assessed by a CASA-Mot system (with over- or under- estimations of only around 10%), However, medium and low ETs provided overestimated values up to 25 percentage points, so the data reveal that the degree of experience in the laboratory becomes a key factor in order to achieve a high degree of accuracy (even though sometimes the low ET obtained more accurate results than the medium ET).

On the other hand, when subjective motility values were obtained through the computer monitor (screen), both high and medium ETs were able to improve their assessment performance, and subjective values provided for them were closer to the CASA-Mot values. These results can be explained thanks to image quality field because while the sperm samples are analyzed

directly by the microscope, spermatozoa trajectories are difficult to distinguish in the clear field, and the overlap of trajectories can cause erroneous assessments of the samples; however, when sperm motion is assessed subjectively by the computer monitor (screen), spermatozoa appear clear over the dark field to the observer (technician), then accurate assessments can be carried out. In this sense, coefficients of correlation support this hypothesis, and both high, medium, and low ETs presented higher r values (r = 0.78–0.96) in assessments carried out by the computer monitor (screen) instead of the rude microscope evaluation (r = 0.42–0.92). Therefore, when sperm motility assessment is carried out without CASA- Mot system, it is recommended to assess the motility by the computer monitor (screen) instead of directly by the eyepieces of the microscope.

On the other hand, it is important to note that r values obtained for samples belonging to CII (r = 0.42–0.65) were much lower than those obtained for C-I and C-III classes (r = 0.71–0.92), overall for the medium and low ETs. These results show that samples with motilities between 25 and 50% have more difficulties for their accurate analyses, so subjective results can be compromised when the sperm samples are analyzed in this range of motility. Similar results have been reported in other species in which, although technicians were able to differentiate correctly the extremes of the sperm motility scale, the samples ranging between 34 and 57% were highly divergent for different technicians (Walker et al., 1982). In fact, the subjective evaluation in Walker's study was not capable of defining this boundary (limit), and fertility workups on males are incorrect 14 times out of 15 in this critical range, so the use of CASA-Mot systems seems to be an essential tool for working in fertility trials.

In relation to technician ability for estimating sperm velocities by subjective assessments, high experimented technician was able to distinguish fast, medium, and low spermatozoa, while less experimented technicians were not able to do it, evidencing their incapacity to evaluate properly the spermatozoa velocity. On this regard, sperm velocities seems to be the major component that determines fertilization success and the proportion of the paternity through the sperm competition in several fish species (Gage et al., 2004; Gasparini et al., 2010; Rudolfsen et al., 2008), so technician ability for

predicting velocity classes can be a useful tool to carry out fertilization trials in the aquaculture sector, optimizing the reproductive efficiency in the fish farms (Gallego et al., 2013b). The data obtained in this study suggest that the degree of expertise of a technician on the sperm quality analysis seems to be a key factor to predict velocities, and even though having a CASA system to make accurate assessments is the most recommended option, high experimented technicians are a requirement for investigating male fertility status as well as monitoring spermatogenesis.

To sum up, this study showed, by the first time in fish species, the importance of technique and technicians chosen for obtaining credible sperm motility assessments to be applied in fish spermatology research. Both the technique (subjective or objective) and the technician (degree of expertise) became key factors in order to reach accurate motility estimations, so the use of both qualified staff and novel CASA-Mot systems seems to be a critical request for obtaining satisfying results in species that have a motility pattern similar to that of the European eel.

In addition, because there are many different con- figurations and methods of using CASA-Mot systems, it is important to establish standard methods of enhancing the reliability, comparability, and applicability of data produced by different research groups (Boryshpolets et al., 2013; Castellini et al., 2011; Gallego et al., 2013a). All studies that use CASA must describe its methodology very clearly, particularly concerning image acquisition rate, track sampling time, number of cells sampled, type and depth of the chamber used, microscope magnification, etc. in order to make it possible to compare the results obtained by different laboratories.

Acknowledgements

CHAPTER 2

Recombinant vs purified mammal gonadotropins as maturation hormonal treatments of European eel males

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Abstract

In the past three decades the European eel *Anguilla anguilla* experienced up to 99% decline in recruitment in some parts of its distribution range, thus breeding in captivity is nowadays considered key in order to save this species. With this in mind, obtaining high quality gametes is fundamental, as is the ongoing study of new hormonal treatments in order to improve current methods. Therefore, the aim of this research study was i) to assess the effect of two hormonal treatments (OVI, a recombinant α-choriogonadotropin; and VET, a human chorionic gonadotropin purified from female urine) on the reproductive performance of European eel males and, after choosing the best hormone, ii) to compare the effects of three doses in order to cut the costs of artificial maturation.

Our results indicated that the type of hormone used (recombinant vs purified gonadotropins) significantly affected the progression of spermiation in European eel males, and that the recombinant hormone (OVI) produced better results in terms of sperm quantity and quality in most of the weeks of the treatment, remaining thus an effective treatment to induce spermiation in this species. On the other hand, in terms of the doses experiment, our results showed that from the lowest to the highest dose (0.25 to 1.5 IU/g fish) all the treatments were able to induce the whole spermiation process. However, a weekly dose of 1.5 IU/g fish of recombinant hormone (OVI) was necessary in order to provide a notable amount (volume and density) of high quality (motility and velocity) samples throughout the treatment.

Finally, the economic analysis demonstrated that the recombinant hormone (OVI, 1.5 IU/g fish) had a greater profitability than the other treatments, making it possible to obtain high-quality sperm for a lower price. In this context, and considering the fact that in the first few weeks of any hormonal treatment there is no high-quality sperm production, long-term hormonal therapies are necessary in order to lessen the cost of high-quality European eel sperm.

1. Introduction

The European eel (*Anguilla anguilla*) is an important species for European aquaculture (5000 t per year, FAO 2014), being highly appreciated both in the European and Asian markets. However, its current production still consists in the fattening of eels captured in the natural environment, due to the fact that it is not yet possible to reproduce eels in captivity. In addition, a drastic decrease has been observed in the number of wild European eels migrating from Europe and North Africa to the spawning sites in the Atlantic Ocean, leading to the species being included in the IUCN red list as critically endangered. Therefore, breeding in captivity is postulated as a key alternative in order to save this species, which will help to reduce the pressure on natural populations, it will facilitate the supply to the eel farms, and it will allow repopulation in areas where those that historically were located the eel.

Although in some fish species reproduction in captivity can be controlled exclusively by environmental factors (Rocha et al., 2008), sometimes it is impractical or even impossible to simulate the environmental conditions in which sexual maturation happens (i.e. depth, pressure, spawning migration, etc.), so the use of exogenous hormones is the only effective way of inducing reproduction (reviewed by Mylonas et al., 2010, 2017). This is the case of the eel species (*Anguilla* spp.), as they do not mature spontaneously in captivity, and the maturation of both males and females must be induced with long-term hormonal treatments (Asturiano et al., 2005; Lokman et al., 2016; Ohta et al., 1997; Sorensen and Winn, 1984).

In the case of European eel males, human chorionic gonadotropin (hCG) has been the most widely used hormone for achieving spermiation, but it has been administered to the animals in several different formats (Gallego et al., 2012). The first studies date back to the middle of the 20th century, where gonadal maturation in eel males was induced by intraperitoneal injections of urine from pregnant women (Fontaine, 1936). At the end of the century, several companies were able to isolate hCG from female urine, so the induction of spermiation of this species became a much more simple and standardized process (Dollerup and Graver, 1985; Khan et al., 1987; Pérez et

al., 2000). Studies from the beginning of the 21st century served to develop and optimize hormonal treatments based on purified hCG, optimizing the sperm production and sperm quality through weekly intraperitoneal injections of 1.5 IU/g fish (Asturiano et al., 2006). However, both the duration of the spermiation period (limited in time) and the interruption of the availability of hCG (in its purified form) in the market meant that new studies addressing the use of alternative hormones became necessary. In this context, the arrival of human recombinant gonadotropins (hCGrec, produced by recombinant DNA technology) became an effective alternative due to the similar structure of the native human hormone, and throughout the last few years they have yielded good results (Gallego et al., 2012). Nevertheless, the effectiveness of treatments based on hCGrec apparently depends on the batch of hormones used, and sometimes it is possible to find groups of animals where although gonadal maturation occurs, the sperm quality parameters (such as motilities and velocities) are not good enough for scientific or aquaculture purposes. Recently, new studies using specific (native) European eel recombinant gonadotropins were also able to induce spermiation in eel males, but the sperm volume and motility results were low for carrying out fertilization trials (Peñaranda et al., 2018). In addition, the production of these native hormones is a tedious and sophisticated process that can only be carried out by companies, thus the end cost of the hormones is prohibitive for many research groups.

Therefore, studies into alternative hormonal treatments must be ongoing in order to improve current methods to date. With this in mind the aim of this work was *i*) to assess the effect of two hormonal treatments (recombinant vs purified mammal gonadotropins) on the reproductive performance of European eel males and, after choosing the best treatment, *ii*) to compare three different hormone doses in order to cut the costs of artificial maturation in this species both for fish farms and laboratories.

2. Material and Methods

2.1 Fish maintenance

Eel males from the fish farm *Valenciana de Acuicultura, S.A*. (Puzol, Valencia; on the east coast of Spain) were moved to our facilities, in the Aquaculture Laboratory at the Universitat Politècnica de València, Spain. The fish were distributed into aquaria equipped with separate recirculation systems, thermostats/coolers, and covered to reduce the light intensity and fish stress. The eels were gradually acclimatized to sea water (salinity 37 ± 0.3 g/l) over the course of one week, and later once a week they were anaesthetized with benzocaine (60 ppm) and weighed to calculate the individual doses of the hormone, which were then administered by intraperitoneal injection. The fish were not fed throughout the experiment and were handled in accordance with European Union regulations (see Ethics statement section).

2.2 Experimental design

2.2.1 Experiment 1. Hormonal treatments: recombinant vs purified gonadotropins

Twenty adult eel males (mean body weight 107.9 ± 1.6 g) were equally and randomly distributed into two 150-L aquaria (10 males per treatment) where they underwent two hormonal treatments: OVI (a recombinant α-choriogonadotropin produced in Chinese hamster ovary cells by recombinant DNA technology and marketed as Ovitrelle; Merck S.L., Madrid) and VET (purified human chorionic gonadotropin marketed as Veterin Corion; Divasa-Farmavic S.A., Barcelona).

The VET hormone was dissolved in a saline serum (NaCl 0.9%) to obtain a concentration of 1 IU/μL serum. The OVI hormone was diluted to obtain a similar concentration. The hormones were injected weekly at a dose of 1.5 IU/g fish and were administered for 25 weeks.

2.2.2 Experiment 2: Different doses of recombinant gonadotropin

After choosing the recombinant gonadotropin (OVI) as the best hormone in terms of sperm quality and profitability, 30 adult eel males (mean body weight 102.3±3.7 g) were equally and randomly distributed into three 150-L aquaria (10 males per treatment). Each group (aquarium) received a different hormonal treatment doses (OVI1.5: 1.5 IU/g fish; OVI0.75: 0.75 IU/g fish; or OVI0.25: 0.25 IU/g fish; respectively) with the final aim of reducing production costs. The hormone was diluted 1:1 (IU/μl) in saline solution (NaCl 0.9%) and the doses were administered weekly for 12 weeks.

2.3 Sperm collection and sampling

Sperm samples were collected weekly by the application of abdominal pressure 24 h after the administration of the hormone (following the protocol described by Pérez et al. (2000)), and taking special care to avoid contamination with faeces, urine and sea water. Samples were diluted 1:9 (sperm:extender) in P1 medium (Peñaranda et al., 2010b) and kept in plastic tubes at 4 ºC until the sperm kinetic analyses, which were carried out in the 2 hours following sperm collection. Sperm volume was previously measured using graduated tubes and sperm density was determined by a CASA system (see next section).

2.4 Evaluation of sperm motility and kinetic parameters

Samples were activated by mixing 0.5 μl of P1-diluted sperm with 4.5 μl of artificial sea water (Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). All the motility analyses were performed in triplicate using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain) as described by Gallego et al. (2013a). The chamber used in all the experiments was a SpermTrack-10® (Proiser, Paterna, Spain) with a 10x negative contrast phase lens in a Nikon Eclipse (E-400) microscope.

The parameters considered in this study were total motility (MOT, %); progressive motility (pMOT, %), defined as the percentage of spermatozoa which swim forwards in 80% of a straight line; curvilinear velocity (VCL, in

µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its average spatial trajectory; and straight line velocity (VSL, µm/s), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position. Spermatozoa were considered motile if their progressive motility had a VSL over 10 µm/s.

In order to perform an in-depth analysis of the results, sperm samples were classified into three classes based on the percentage of motile spermatozoa provided by the CASA system: Class I (C-I) = 0 - 25% of motile cells; Class II (C-II) = 26 - 50% of motile cells; and Class III (C-III) = 51 - 100% of motile cells.

2.5 Economic analysis

To analyze the economic profitability of each hormonal treatment (both in experiment 1 and 2) three factors were taken into account: *i*) the price of the hormone; *ii*) the total amount of hormone used (dose) throughout the whole treatment; and *iii*) the total volume of sperm of the highest motility class (C-III) produced by each treatment. The aim was essentially to relate the investment made with the level of good quality sperm produced by each hormonal treatment.

2.6 Statistical analysis

The mean and standard error were calculated for all sperm parameters (volume, density, motility and the rest of the kinetic parameters). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. A two-way repeated measures ANOVA was used to analyze the sperm production and quality parameters. Significant differences were detected when *p-value* < 0.05. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Experiment 1. Hormonal treatments: recombinant vs purified gonadotropins

The sperm production parameters are shown in Figure 1. Most of the OVI-treated fish (90%) started to produce sperm in the 6th week of treatment, while only 60% of VET-treated fish generated sperm in this week (Figure 1A). From the 12th to the 18th week, the VET treatment generated higher percentages of spermiating males (90-100%) than the OVI treatment (70-80%) and, finally, the decreasing percentages of spermiating males were similar in the last few weeks (19th to 25th) in both treatments.

Regarding volume, there was an increasing trend from the beginning to the end (Figure 1B) in both treatments. Volume values were generally higher in OVI treated males, although statistical differences were only found in weeks 8 and 9, probably due to the high dispersion of data found in the OVI-treated males during the last few weeks. Density values were slightly higher in VET treated males from weeks 11 to 21, but significant differences were only found in weeks 13 and 15 (Figure 1C).

Regarding the sperm quality parameters, OVI males showed higher motilities than VET males during the first few weeks of treatment (Figures 2A and 2B), reaching maximum values of 76 and 45% of MOT and pMOT, respectively. However, VET-treated males showed a marked rise from week 12 (with 77 and 35% of MOT and pMOT, respectively), and motility parameters were similar for both hormones until the end of the treatment, with values remaining over 50% in the 25th week. The sperm velocities (Figures 2C, 2D and 2E) showed a similar pattern to the motility traits: OVI-treated males showed higher velocities (VCL, VSL and VAP) than VET-treated males during the first few weeks of the treatment, but the kinetic values were similar in both hormone treatments from the 12th week until the end of the treatment.
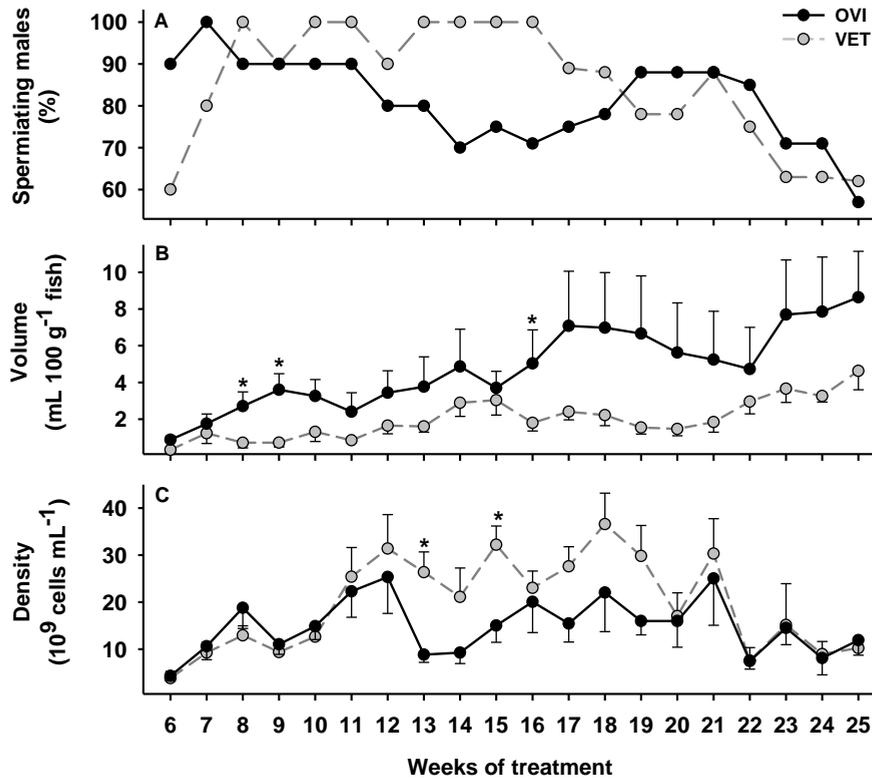
Figure 1. *Evolution of sperm production parameters throughout the hormonal treatments (OVI and VET; 1.5 IU/g fish): A) Percentage of spermating males; B) Sperm volume; and C) Sperm density. Data are expressed as mean ± SEM and asterisks indicate significant differences between treatments at each week of treatment.*

Finally, when the volume and the sperm motility classes were considered simultaneously (Table 1, experiment 1), it was observed that the OVI treatment displayed better total volume results (with volume values over 500 mL of C-III sperm) than the VET treatment, which yielded total volume values of around 200 mL of C-III sperm. In addition, in terms of the production of high quality sperm week-by-week (Figure 3), the OVI treatment showed a higher number of weeks (8th, 9th, 10th, 12th, 22th, 23th, 24th and 25th) providing higher volumes of good quality sperm (C-III) than the VET treatment.

Table 1. *Total sperm volumes (mL) recovered from the different hormonal treatments of experiment 1 (OVI and VET; 1.5 IU/g fish) and experiment 2 (OVI$_{1.5}$: 1.5 IU/g fish; OVI$_{0.75}$: 0.75 IU/g fish; and OVI$_{0.25}$: 0.25 IU/g fish) for each sperm motility class (CI-CIII).*

Sperm Class	Experiment 1		Experiment 2		
	OVI	VET	OVI$_{1.5}$	OVI$_{0.75}$	OVI$_{0.25}$
C-I	29.7	14.7	29.9	199.6	206.5
C-II	45.1	85.0	26.5	15.8	94.4
C-III	544.9	201.4	99.0	13.7	15.9

Regarding the economic analysis, the investment needed to obtain mature males was quite different in each hormonal treatment (Table 2; experiment 1). The VET treatment investment was smaller, at 0.69 €/week per male, nevertheless, although the OVI treatment required a higher investment per male (1.17 €/week per male), the total volume of class III sperm obtained from OVI-treated males was much higher than VET males (Table 1). Therefore, the final profitability of this hormone was higher in OVI treated males, where it was possible to obtain 1 mL of the highest quality sperm (C-III) for a lower price (0.44 €/mL). The other hormone (VET) produced worse economic results because it was necessary to invest 0.86 € to obtain 1 mL of good quality (C-III) sperm.
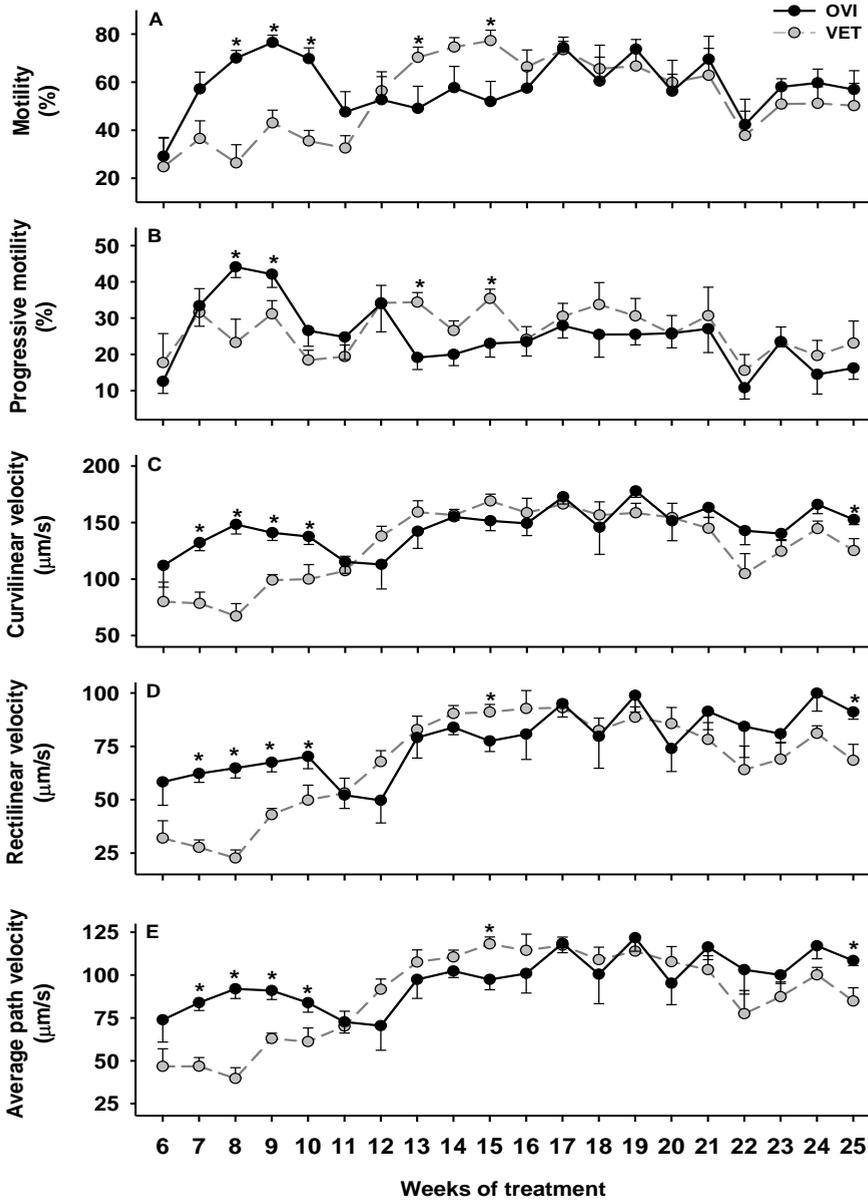
Figure 2. *Evolution of sperm quality parameters throughout the hormonal treatments (OVI and VET; 1.5 IU/g fish): A) Percentage of motile cells; B) Percentage of progressive motile cells; C) Curvilinear velocity; D) Rectilinear velocity; and E) Average path velocity. Data are expressed as mean ± SEM and different letters indicate significant differences between treatments at each week of treatment.*
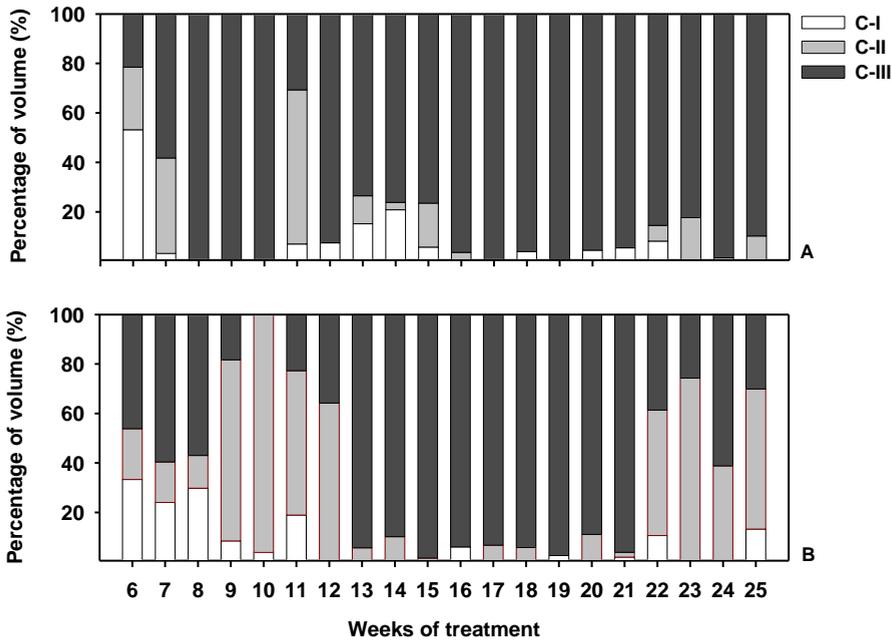
Figure 3. *Percentage of sperm volume from each motility class (I-III) in each week throughout the different hormonal treatments: A) OVI and B) VET.*
Motility classes: Class I = 0- 25%; Class II = 26-50%; and Class III >50% of motile cells.

3.2 Experiment 2: Different doses of recombinant gonadotropin

In terms of the sperm production parameters (Figure 4), all the doses of recombinant gonadotropin (OVI) were able to induce high percentages of spermiating males (around 80%) during most of the weeks of treatment. However, the $OVI_{0.25}$ group produced the lowest percentages of spermiating males during the last few weeks (around 60%).

Regarding volume, an increasing trend from the beginning to the end was seen in all three treatments (Figure 4B). The volume values were generally higher in the $OVI_{0.25}$ group, showing statistical differences in several (6, 7, 8 and 10). On the contrary, density patterns were slightly higher in males treated in the $OVI_{1.5}$ group, and significant differences were found from the 9th week to the end of the experiment (Figure 4C).

Table 2. *Profitability of hormonal treatments of experiment 1 (OVI and VET; 1.5 IU/g fish) and experiment 2 (OVI$_{1.5}$: 1.5 IU/g fish; OVI$_{0.75}$: 0.75 IU/g fish; and OVI$_{0.25}$: 0.25 IU/g fish) in relation to economic investment and production of high-quality (Class III) sperm.*

|		Experiment 1		Experiment 2		
		OVI	VET	OVI$_{1.5}$	OVI$_{0.75}$	OVI$_{0.25}$
Dose	IU/g fish	1.5	1.5	1.5	0.75	0.25
Hormone price	€/IU	0.008	0.005	0.008	0.008	0.008
[a]Dose price	€/g fish	0.012	0.007	0.012	0.006	0.002
[b]Investment/male	€/male	1.17	0.69	1.17	0.58	0.19
[c]C-III sperm price	€/mL	0.44	0.86	1.78	6.44	1.85

[a] *Dose x Hormone Price*
[b] *Investment to maturate one male (100 g approx.) per week.*
[c] *Total investment (€) / Total C-III sperm volume (mL).*

Concerning sperm quality parameters, OVI$_{1.5}$-treated males provided samples with higher values of MOT and pMOT throughout almost all the treatment (Figures 5A and 5B), reaching maximum values of 72 and 46%, respectively. Conversely, medium and low doses (OVI$_{0.75}$ and OVI$_{0.25}$) provided samples which only showed maximums of 30 and 20% of MOT, respectively. The spermatozoa velocities (Figures 5C, 5D and 5E) showed similar patterns to those of motility, and OVI$_{1.5}$-treated males showed higher velocities with significant differences (VCL, VSL and VAP) throughout most of the treatment.

Finally, when the volume and the sperm motility classes were evaluated simultaneously (Table 1, experiment 2), it was observed that the highest dose (OVI$_{1.5}$) was the only treatment able to produce acceptable volumes (near 100 mL) of good quality (C-III) sperm. On the contrary, medium and low doses (OVI$_{0.75}$ and OVI$_{0.25}$) provided large volumes of bad quality sperm (C-II especially and C-I), which represented more than 95% of total volume production for each treatment. In addition, when looking at the production of high quality sperm week-by-week, the OVI$_{1.5}$ treatment showed a higher

number of weeks (8th, 9th, 10th, 11th and 12th) providing higher volumes of C-III sperm than the other treatments (Figure 6).
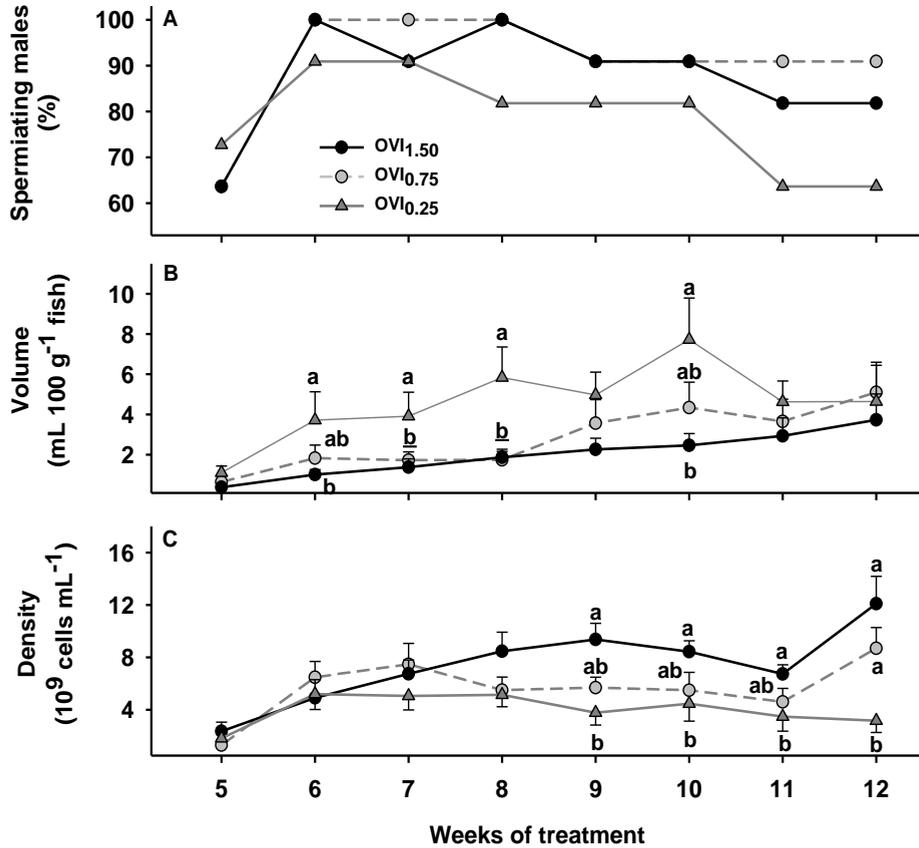
Figure 4. Evolution of sperm production parameters throughout the different hormonal doses of OVI treatment (OVI$_{1.5}$: 1.5 IU/g fish; OVI$_{0.75}$: 0.75 IU/g fish; and OVI$_{0.25}$: 0.25 IU/g fish): A) Percentage of spermiating males; B) Sperm volume; and C) Sperm density. Data are expressed as mean ± SEM and different letters indicate significant differences between doses at each week of treatment.
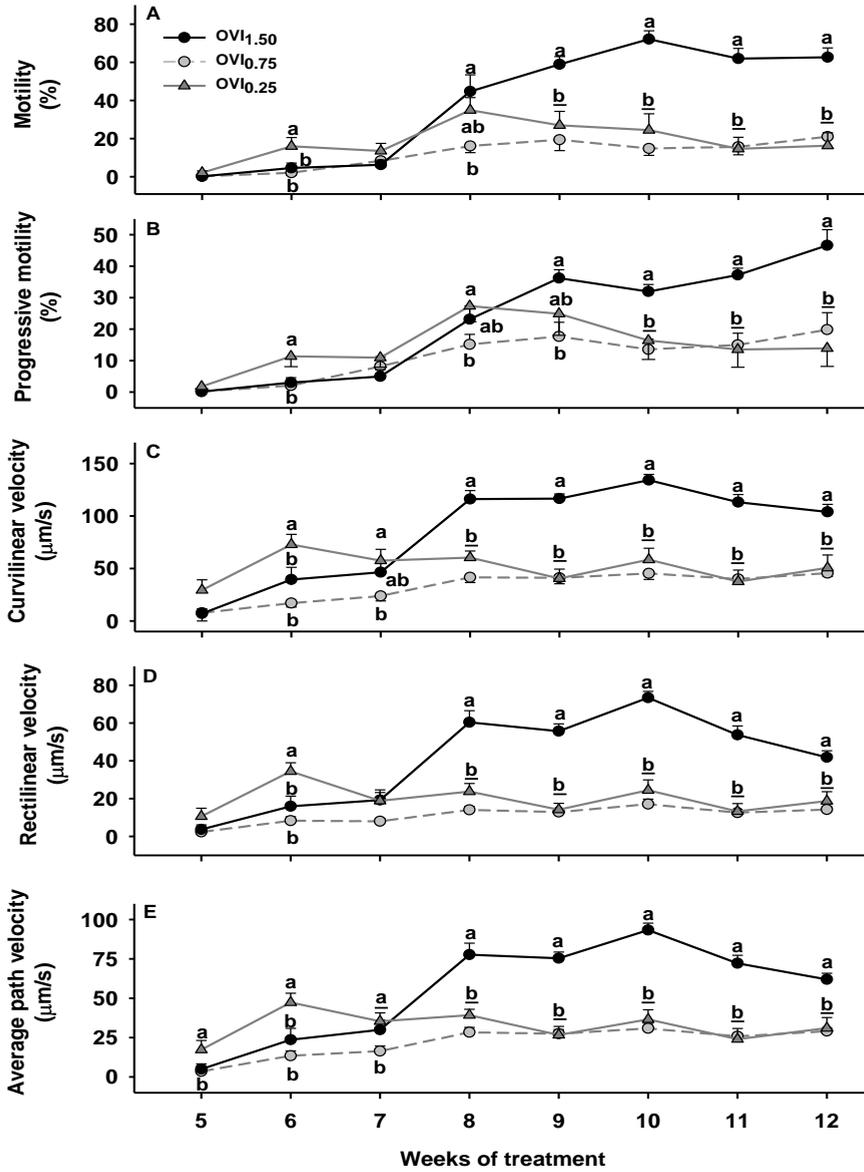
Figure 5. *Evolution of sperm production parameters throughout the different hormonal doses of OVI treatment (OVI$_{1.5}$: 1.5 IU/g fish; OVI$_{0.75}$: 0.75 IU/g fish; and OVI$_{0.25}$: 0.25 IU/g fish): A) Percentage of motile cells; B) Percentage of progressive motile cells; C) Curvilinear velocity; D) Rectilinear velocity; and E) Average path velocity. Data are expressed as mean ± SEM and different letters indicate significant differences between doses at each week of treatment.*
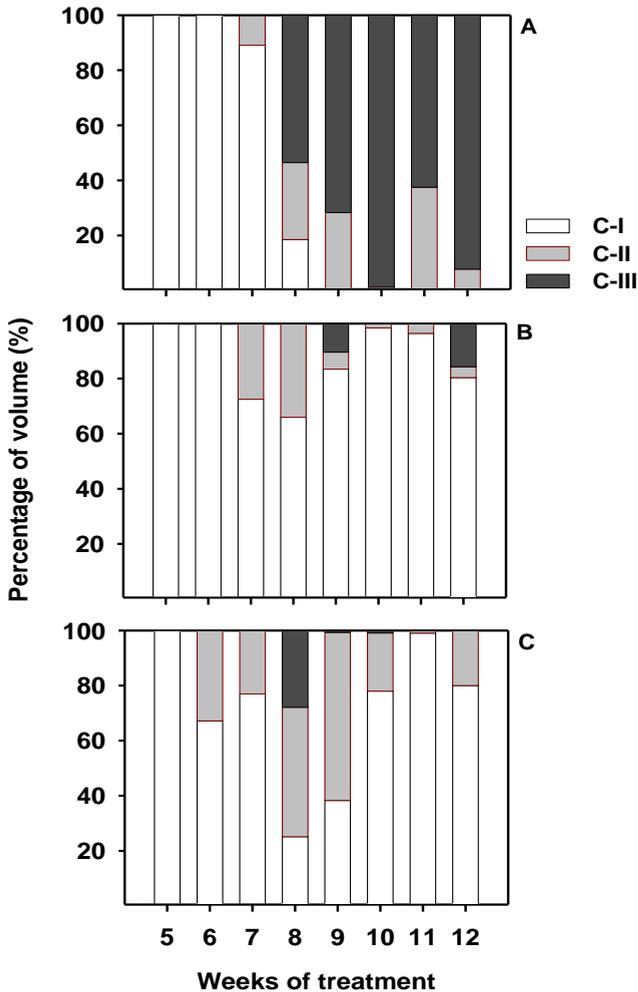
Figure 6. Percentage of sperm volume from each motility class (I-III) in each week throughout the different hormonal doses OVI treatment: A) $OVI_{1.5}$: 1.5 IU/g fish; B) $OVI_{0.75}$: 0.75 IU/g fish; and C) $OVI_{0.25}$: 0.25 IU/g fish. Motility classes: Class I = 0- 25%; Class II = 26-50%; and Class III >50% of motile cells.

Regarding the economic analysis, the investment needed to obtain mature males was quite different in each hormonal treatment (Table 2, experiment 2). The $OVI_{1.5}$ treatment required the highest investment per male per week (1.17 €), while an investment of 0.58 and 0.19 € were necessary in order to mature fish with $OVI_{0.75}$ and $OVI_{0.25}$, respectively. However, the total volume of class III sperm obtained from $OVI_{1.5}$-treated males was much higher than that produced by $OVI_{0.75}$ and $OVI_{0.25}$ males (Table 1), so the final profitability of the standard dose ($OVI_{1.5}$) was the highest, with it being possible to obtain 1 mL of the highest quality sperm (C-III) for the lowest price (1.78 €/mL).

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4. Discussion

4.1. Hormonal treatments: recombinant vs purified gonadotropins

The study of alternative hormonal treatments to improve both sperm production and quality parameters must be ongoing in order to enhance gonadal maturation in fish (Mylonas et al., 2017), specifically in species with serious reproductive problems, such as the European eel (Gallego et al., 2012; Peñaranda et al., 2018). In the present study, our results indicated that the type of hormone used (recombinant vs purified gonadotropins) significantly affects the progression of spermiation in European eel males, with the recombinant hormone (OVI) producing better results in most of the weeks.

First of all, it is important to note that sperm quantity and quality have become a key factor in controlled reproduction both for aquaculture and scientific purposes, thus reasonable volumes of high quality samples are necessary in order to fertilize the maximum number of eggs (Migaud et al., 2013; Tvedt et al., 2001). In this context, although both hormones (OVI and VET) were able to induce a high percentage of spermiating males (>70%), there was a notable difference in sperm volume and density patterns between the treatments. In terms of volume, OVI-treated males produced approximately twice (even triple) the volume than VET-treated males in all the weeks, thus the final amount of sperm resulting from the OVI hormone was much higher than that produced by VET-treated males. In this context,

Gallego et al. (2012) reported similar results in this species when using the recombinant hormone (OVI), where a purified hormone (from a different brand) used in that previous study yielded remarkable results (up to 8 mL). In addition to the volume, the density values provided by VET treated males in the present study were not high enough to compensate for the lower volumes produced by this hormone in most of the weeks, thus the recombinant hormone (OVI) was the best treatment according to both the sperm production parameters.

Moreover, in addition to sperm quantity, sperm quality is a crucial factor in fertilization trials, and several kinetic parameters (characterizing sperm

motility and velocity) are nowadays considered to be the best fish sperm quality biomarkers (Gallego and Asturiano, 2018b). In experiment 1, both hormones yielded remarkable motility and velocity values during most of the treatment, although the recombinant hormone (OVI) was able to provide high quality samples during a greater number of weeks (18/20) than the purified hormone (12/20). In this context, it is noteworthy that an essential factor in European eel breeding captivity programs is the ability to obtain high quality sperm for a large number of weeks in order to synchronize egg production by the females (Butts et al., 2014; Tomkiewicz et al., 2013), thus the recombinant hormone (OVI) was identified as the best treatment according to the sperm quality indicators.

From a physiological point of view, the different responses found in eel males regarding the different hormonal treatments could be explained by the biological activity of each hormone: while the VET hormone was a native hCG hormone, purified and isolated from human urine (Birken et al., 1996), the OVI hormone was a recombinant version of endogenous hCG produced by recombinant DNA technology (Choi and Smitz, 2015). Even though both hormones (OVI and VET) act as analogues of the luteinizing hormone (LH), Bassett et al. (2005) reported that purified-hCG preparations contained a high number of urine derived protein contaminants as well as hCG related metabolites, whereas recombinant hCG was confirmed to be essentially intact hCG (free from contaminant proteins and with very low levels of oxidised hCG). Therefore, the different nature and origins of these hormones (with different degrees and types of glycosylation) could induce gonadal maturation in different ways, generating different patterns in sperm volume or density as seen in previous studies reported by Gallego et al. (2012). In addition, recent reports showed that new recombinant hCGs are available in the market (Pregnyl, Ovidrel, etc.), and they could be probably useful for gonadal maturation in fish due to the high degree of structural and functional similarity with the reference format Ovitrelle (Leão and Esteves, 2014; Thennati et al., 2018).

On the other hand, new hormonal therapies using specific recombinant gonadotropins are being developed to induce spermiation in eel species. Although results in European eel has not been good enough for applying in

aquaculture purposes (Peñaranda et al., 2018), recombinant Japanese eel LH induced a much higher amount of high-quality sperm when compared to hCG injections in this species (Ohta et al., 2017). In this context, studies into alternative hormonal treatments must be ongoing in order to improve current methods for inducing the successful artificial maturation of endangered species, such as the European eel.

To sum up, our results demonstrated that the progression of spermiation in European eel males was notably influenced by the hormone used. Recombinant gonadotropin (OVI) showed the best results in terms of both sperm production and quality parameters, becoming an effective treatment to induce spermiation in the European eel.

4.2. Different doses of recombinant gonadotropin (OVI)

In addition to the task of pursuing new hormones in order to extend the spermiation period and enhance sperm quality, attempts to optimize hormonal therapies are also a key premise to be applied in both scientific and aquaculture sectors. In this context, and once the most efficient hormonal treatment from experiment 1 had been chosen, the effects on the induction of spermiation of several doses of the recombinant hormone (OVI) were evaluated.

Our results showed that from the lowest to the highest dose of the recombinant hormone (0.25 to 1.5 IU/g fish), all the treatments were able to induce the whole spermiation process. Previous studies reported that a single injection of hCG was enough to induce spermatogenesis and spermiation both in European and Japanese eel species (Khan et al., 1987; Miura et al., 1991b), but a continuous supply of hormone was necessary to maintain both the sperm production and the sperm quality throughout the weeks (Asturiano et al., 2005). In this context, our results agree with these previous studies, and a periodic supply of hCG (even using the lowest doses) was able to maintain the spermiation process over the weeks.

Concerning the sperm production rates (volume and density), the $OVI_{0.25}$ group surprisingly yielded the highest sperm volumes throughout the treatment, with values reaching close to 8 mL in the 10th week. However,

sperm density was lower in this group ($OVI_{0.25}$) compared to the other two groups where higher recombinant hormone doses were injected ($OVI_{0.75}$ and $OVI_{1.5}$), thus the total amount of spermatozoa (volume x density) produced weekly was similar for all treatments. This density-volume pattern has already been described in other species, and this effect seems to be controlled by the maturation inducing steroids (MIS) which regulate the final stages of sperm maturation (Asturiano et al., 2002; Schulz et al., 2010). In this context, high densities would usually be linked to small volumes and conversely, low densities would need to be compensated by high sperm volumes. In addition, the density data yielded in this study using the standard doses of recombinant hCG (1.5 IU/g fish) agree with previous values obtained by administering the recombinant hormone in this species (Gallego et al., 2012), but density data were significantly higher than those obtained using purified hormone a decade ago (Asturiano et al., 2006; Pérez et al., 2000).

On the other hand, and concerning the sperm quality parameters, notable differences were found between the treatments. In this context, only the group with the highest dose ($OVI_{1.5}$) was able to generate samples with acceptable motility values from the 8th-9th weeks until the end of the treatment, while $OVI_{0.25}$- and $OVI_{0.75}$-treated males produced bad quality sperm (<35% of motility) in all the weeks. This low response in terms of motility in the groups receiving the lowest doses could be due to a hormonal failure in the maturation process. In this sense, an insufficient weekly dose of gonadotropin could generate a deficient production of steroidogenic enzymes, which in turn would produce a low production of the steroids involved in gonadal maturation, therefore causing a poor production of good quality sperm (Jamalzadeh et al., 2014; Peñaranda et al., 2010c; Schulz and Miura, 2002). Throughout the bibliography, the most common dose applied in fish has been 1 IU/g fish, but doses are usually species-dependent, varying from 0.15 IU/g fish in pikeperch (*Sander lucioperca*) (Falahatkar and Poursaeid, 2014) to 50 IU/g fish in silver perch (*Leiopotherapon plumbeus*) (Denusta et al., 2014). Considering European eel references, previous experiments carried out a decade ago also showed that doses of 0.75 IU/g fish were unable to provide high quality samples throughout the treatment, as per this study. However, a dose of 1.5 IU/g fish of purified hCG

administered every 2 weeks provided a greater number of samples but of a similar quality (Asturiano et al., 2005), given more chances for carrying out hatchery operations related to fertilization trials.

To sum up, our results have demonstrated that in order to achieve a successful maturation process in the European eel, a minimum dose of 1.5 IU/g fish of recombinant hCG administered weekly is necessary, inducing the production of reasonable volumes of high quality sperm samples.

4.3. Economic analysis for the different hormones and doses

From a biological point of view, the best hormonal treatment should provide a large amount (volume and density) of high quality (motility and velocity) samples for as many weeks as possible. However, from an economic point of view, a reduction in the costs of hormonal therapies is essential in order to obtain affordable and more effective treatments (Mylonas et al., 2017).

In the present study, the economic performance of the treatments was assessed by taking into account both the economic investment made (type, price and dose of hormone) and the total volume of high-quality sperm generated by each treatment. In experiment 1 and concerning high-quality sperm price, the recombinant hormone (OVI) generated the best results throughout the experiment, improving the performance yielded by the purified hormone (VET). In this context, and despite the fact that the investment required for maturating males using the recombinant hormone was almost double that of the purified gonadotropin (1.17 vs 0.69 €/male per week, respectively), the large amount of high-quality sperm produced by OVI-treated males (triple that of VET-treated males) meant a greater profitability, making it possible to obtain high-quality sperm for half the price (0.44 €/mL) of the purified hormone (0.86 €/mL). These results agree with previous studies carried out by Gallego et al. (2012), where recombinant hCG also generated better economic performances (0.5 €/mL) than gonadotropins purified from pregnant women and mares (0.72 and 1.8 €/mL, respectively).

Moreover, the analysis of the economic return of the different doses used in experiment 2 yielded interesting results. Although the highest (OVI$_{1.5}$) and

the lowest dose (OVI$_{0.25}$) of recombinant hormone generated similar economic performance in terms of high-quality sperm price (1.78 and 1.85 €/mL, respectively), the total C-III volume generated by OVI$_{0.25}$ was too low (2.5 mL/week) for a sustainable application in eel aquaculture, including large-scale fertilizations. In addition, when comparing the results of the economic profitability of the same hormone (OVI) and dose (1.5 IU/g fish) from experiments 1 and 2, the results were notably different: the recombinant hormone showed a much better economic performance in experiment 1 (0.44 €/mL) than in experiment 2 (1.78 €/mL). This difference can be explained by the large-scale production concept, where the cost advantages obtained by applying a different scale of operation (in this case 25 vs 12 weeks for experiment 1 and 2, respectively) decrease the cost per unit of output (high-quality sperm). In fact, when the economic profitability of experiment 1 was calculated just for the first 12 weeks, the economic performance of OVI was lower (1.06 €/mL) than for values obtained in the same experiment for 25 weeks (0.44 €/mL). Thus, because during the first few weeks of any hormonal treatment there is no high-quality sperm production, long-term hormonal therapies are necessary in order to lessen the cost of production of high-quality European eel sperm.

Finally, and linking the production of large amount (volume and density) of high-quality (motility and velocity) sperm samples to the essential hatchery tasks such as *in vitro* fertilization trials (IVF), Butts et al. (2014) showed that the sperm to egg ratio became a critical step towards establishing successful *in vitro* fertilization protocols. In this context, and taking into account the optimum value of sperm:egg ratio reported in this species (1:25,000), a large amount of eggs (approx. 10 million per week) could be fertilized using a batch of 10 males induced with recombinant hCG (1.5 IU/g fish).

5. Conclusions

In conclusion, this study shows that the type of hormone used significantly affected the progression of spermiation in European eel males, and the recombinant hormone (Ovitrelle at 1.5 IU/g fish) produced the best results in terms of sperm quantity (volume and density) and quality (motility and

velocity). In addition, the economic analysis demonstrated that the recombinant hormone had a greater profitability than the other treatments, hence becoming an effective method to induce the spermiation process in this species with the aim to provide high quality samples during a great number of weeks

Acknowledgements

CHAPTER 3

Comparison of European eel sperm cryopreservation protocols with standardization as a target

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Abstract

The critical situation of the European eel (*Anguilla anguilla*) has urged the development of sperm cryopreservation protocols for reproduction in captivity and cryobanking. In the last years, two research groups have developed their own protocols in Spain and Hungary with positive results, but difficult to compare.

Here, a series of experiments were conducted to test the quality of thawed sperm after using both protocols, determining which of them produce the best results and aiming for standardization. The quality of thawed sperm was assessed by studying the motility and kinetic values of thawed sperm from both cryopreservation protocols using a computer-assisted sperm analysis (CASA-Mot) system. In addition, a viability analysis was performed using flow cytometry to test if the cryoprotectants or the freezing-thawing process led to a reduction in spermatozoa survival. Furthermore, since during cryopreservation the sperm was treated with methylated cryoprotectants (DMSO or methanol) that may induce epigenetic changes in the sperm DNA (cytosine methylation) and could affect the offspring, we conducted a luminometric methylation assay (LUMA) to study the DNA methylation levels induced by both protocols.

In this work, all the above-mentioned parameters were analyzed in fresh and frozen-thawed sperm samples. Our results showed that thawed sperm samples from both protocols presented lower sperm motility and velocity, and lower percentage of live cells than those shown in fresh sperm samples. Furthermore, sperm samples from the methanol based protocol showed significantly higher motility, velocity and percentage of live spermatozoa than the same sperm samples treated with the DMSO based protocol. In addition, the DMSO based protocol induced a hypomethylation of sperm DNA compared to fresh samples whereas the methanol based protocol did not alter sperm DNA methylation level. Our results indicate that the methanol based protocol is a more suitable protocol that preserves better the motility and genetic qualities of the European eel sperm.

1. Introduction

During the last years, a drastic decrease has been observed in the number of European eels (*Anguilla anguilla*) returning from Europe and North Africa to the spawning sites in the Atlantic Ocean (Dekker, 2000; Jacoby and Gollock, 2014). Several impacts such as water pollution, overfishing or habitat fragmentation, have led the European eel to be included on the IUCN red list as critically endangered (Jacoby and Gollock, 2014). Consequently, the development of techniques and protocols for reproduction in captivity are necessary to reverse this situation.

The maturation of the European eel in captivity is only achieved by costly and long hormonal treatments (Asturiano et al., 2006; Gallego et al., 2012; Pérez et al., 2000), and still the production of gametes in both sexes can be unsynchronized (Asturiano et al., 2017). During the last years, several researchers have worked in the development of new maturation protocols such as alternative hormonal treatments with recombinant hormones (Peñaranda et al., 2018) or androgen implants (Di Biase et al., 2017; Mordenti et al., 2018), but the timing of final maturation in females is still highly variable and difficult to control (Mylonas et al., 2017). Therefore, the development of cryopreservation protocols for European eel sperm has been considered important for reproduction management, by guaranteeing the availability of both types of gametes when female spawns (Asturiano et al., 2017), besides its application for cryobanking and future broodstock management.

Cryopreservation of European eel sperm has been faced by different groups since early 2000s. Mainly two groups of research established successfully their own cryopreservation protocols in Spain (Asturiano et al., 2003; 2004; Peñaranda et al., 2009) and Hungary (Müller et al., 2004; Szabó et al., 2005). These protocols differ greatly in many aspects such as the composition of the extenders, the cryoprotectants used, the volume of the straws or the cooling rates within others, evidencing the need for standardization (Asturiano et al., 2017; Rosenthal et al., 2010).

The success of a sperm cryopreservation protocol is commonly assessed using parameters such as sperm viability and motility, fertilizing capacity and

the quality of the offspring (Cabrita et al., 2010). However, in the case of the eel both protocols have yielded high post-thaw sperm viability (58 to 63%) and motility values ranging between 18 and 38% (Asturiano et al., 2017). Furthermore, the fertilizing capacity of the Spanish protocol (from now on referred as DMSO protocol) was successfully tested by producing European eel larvae after fertilization with thawed sperm (Asturiano et al., 2016), and following the Hungarian protocol (from now on referred as methanol protocol), hybrid larvae were successfully produced using thawed sperm from European eel and eggs from Japanese eel (*Anguilla japonica*) (Müller et al., 2012; 2018). In this last study, Müller and collaborators showed that the malformation rate of larvae was higher when using cryopreserved sperm than in the control groups using fresh sperm, suggesting that the cryopreservation methodology needs further refinement.

Additionally, a growing concern is that the epigenetic effects of cryopreservation on the sperm DNA might be altered by the freezing, cryobanking and thawing process (Labbé et al., 2017; Pérez-Cerezales et al., 2010). The use of methylated cryoprotectants such as methanol or dimethyl sulfoxide (DMSO) has been proven to produce reactive oxygen species (ROS) that can induce cytosine methylation in fish sperm (De Mello et al., 2017; Kawai et al., 2010). Methylation of cytosine residues in DNA is considered to be one of the major epigenetic mechanisms stabilizing gene silencing (Schaefer et al., 2007). Furthermore, cytosine methylation can be altered by cryopreservation, inducing hypo- and hypermethylation profiles in sperm DNA (Labbé et al., 2017). Therefore, the study of epigenetic effects of cryopreservation may be a good indicator of the success of a cryopreservation protocol, since damaged DNA or abnormal DNA regulation have been observed to have a negative effect on the generated embryos (Herráez et al., 2017).

The main objective of this work was to compare the main protocols previously developed for European eel sperm cryopreservation, aiming for standardization. The comparison was made considering sperm quality after thawing, when sperm motilities, sperm velocities, and sperm viability were analyzed. Furthermore, epigenetic effects of sperm cryopreservation were

studied by analyzing whether DNA methylation patterns were affected by the different cryopreservation protocols.

2. Materials and methods

2.1 Ethics statement

The protocol was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2015/VSC/PEA/00064).

2.2 Fish handling

For this experiment, 28 immature male European eels from the fish farm Valenciana de Acuicultura S.A. (Puzol, Valencia) were brought to our facilities in the Universitat Politècnica de València. Fish were distributed in two 200 L aquaria with recirculation systems, and thermostats and coolers to maintain water temperature at 20 ºC. They were gradually acclimated to seawater (salinity 37 ± 0.2 g/L) increasing the salinity 10 ppt each 2 days for 8 days, and 2 days more of resting at 37 ppt. The aquaria were covered to maintain a constant shadow and reduce fish stress.

After 10 days of acclimation, male fish anesthetized with benzocaine (60 ppm) were weekly treated with injections of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Madrid, Spain, 1.5 IU/g fish) to induce maturation and spermiation (Gallego et al., 2012; Pérez et al., 2000). From the sixth week of hormonal treatment, sperm samples were collected weekly, 24h after the hormone injections.

For sperm collection, fish were anesthetized with benzocaine. Thereafter, the genital area was carefully cleaned with distilled water and thoroughly dried with paper to avoid contamination with feces, urine or seawater to avoid accidental sperm activation. Then, sperm was collected by applying a ventral massage from the pectoral fins to the genital opening and collected in graduated Falcon tubes using a vacuum pump.

Sperm samples were collected after 11-14 weeks of hormonal treatment. The samples were diluted 1:9 (sperm:extender) in P1 medium (in mM: NaCl 125, NaHCO$_3$ 20, MgCl$_2$ 2.5, CaCl$_2$ 1, KCl 30; pH adjusted to 8.5, described by Peñaranda *et al.*, 2010a) , kept in plastic tubes at 4 ºC and evaluated for motility.

2.3 Evaluation of sperm motility

In a maximum of 2 h after the sperm extraction, sperm samples were evaluated in triplicates following the method described by Gallego et al. (2013a). Briefly, each sperm sample was activated by mixing 0.5 µL of P1-diluted sperm sample with 4.5 µL of artificial seawater (in mM: NaCl 354.7, MgCl$_2$ 52.4, CaCl$_2$ 9.9, Na$_2$SO$_4$ 28.2, KCl 9.4, in distilled water) with 2% (w:v) bovine serum albumin (BSA), pH adjusted to 8.2 and osmolality of 1100 mOsm/kg. The activation was performed in an ISAS Spermtrack 10 counting chamber (Proiser R+D, S.L., Spain) on a microscope in negative phase with a 10X magnification (Nikon Eclipse 80i) connected to a computer with an ISAS 782M camera (Proiser R+D, S.L., Spain), recording 60 frames per second (fps). All samples were analyzed 15 s after activation, using the CASA module ISAS v1 software (Proiser R+D, S.L., Spain). Several kinetic parameters such as percentage of motile spermatozoa (MOT, %), progressive motility (pMOT, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and average path velocity (VAP, µm/s), as well as percentage of slow (average path velocity (VAP) = 10-50 µm/s), medium (VAP = 50-100 µm/s) and fast (VAP >100 µm/s) spermatozoa were recorded for further analysis (Gallego and Asturiano 2018a for details). Samples with motility values higher than 65% were selected for cryopreservation.

2.4 Experimental design

A total number of 18 sperm samples were selected for cryopreservation. Each sample was first evaluated for motility and then frozen and thawed following both protocols. In addition, before freezing, each sample was evaluated for motility approximately 10 minutes after diluted with the freezing media corresponding to each protocol. Then, four straws (IMV

Technologies, l'Aigle, France) of 250 µL for the DMSO protocol and four straws of 500 µL for the methanol protocol were frozen. Therefrom, three straws per protocol were thawed and immediately analyzed with CASA-Mot for sperm quality. Moreover, 50 µL of fresh and thawed sperm from each sample were used for the viability analysis using the flow cytometer (see down). The left straw per protocol was maintained frozen in liquid nitrogen and was sent to INRA's lab in Rennes (France) for sperm epigenetic analysis, by studying the DNA methylation level. In addition, 100 µL of fresh sperm from each sample were frozen as well by directly throwing the tube with the sperm into the liquid nitrogen and then storing it at -80 ºC for DNA methylation analysis of the sperm control. We demonstrated previously that such snap freezing allows that the DNA methylation level of the fresh sperm is preserved (unpublished data).

2.5 Cryopreservation protocols

Every selected sample was frozen and thawed following both protocols. For the DMSO protocol, a freezing medium was prepared in advance by mixing a modified P1 extender solution (in mM: NaCl 50, $NaHCO_3$ 100, $MgCl_2$ 2.5, $CaCl_2$ 1, KCl 30; described by Peñaranda *et al.*, 2009; and named M5 in that paper), 25% (v/v) of fetal bovine serum (FBS) and 10% (v/v) of DMSO. The freezing medium was adjusted to a pH of 6.5, an osmolality of 330 mOsm/kg and maintained at 4 ºC. Thereafter, a dilution 1:2 of sperm: freezing medium, was prepared and immediately packed in 250 µL straws, sealed with modeling clay and frozen for 5 min in liquid nitrogen vapor 1 cm above the surface using a floating structure. Following, the straws were thrown into the liquid nitrogen where the sperm was preserved as long as needed. The thawing consisted in a water bath at 30 ºC for 8 s.

For the methanol protocol, modified Tanaka´s extender (in mM: NaCl 137, $NaHCO_3$ 76.2) was prepared in advance and maintained at 4 ºC. Then, a dilution consisting in sperm:Tanaka's extender:methanol (1:8:1) was prepared and packed in 500 µL straws, and frozen for 3 min in liquid nitrogen vapor 3 cm above the liquid nitrogen before throwing the straws into the

liquid nitrogen. For thawing, the straws were immersed in a water bath at 40 ºC for 13 s.

2.6 Thawed sperm evaluation

The quality of thawed sperm samples was assessed by analyzing several sperm motility parameters with CASA-Mot, sperm viability (membrane integrity) with a flow cytometer and epigenetic effects with an analysis of sperm DNA methylation pattern.

The motility analysis was performed using CASA-Mot as explained above. In addition, a viability analysis was conducted with flow cytometry using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) containing the membrane-permeating dye SYBR 14, that stains the nuclei of membrane-intact cells fluorescent green and the non-permeating propidium iodide (PI), that counterstains the nuclei of cells with a damaged membrane fluorescent red. Here, 0.5 µL of SYBR 14 (100 µM) and 2 µL of PI (2.4 mM) were added to 50 µL of fresh or thawed sperm samples and incubated at room temperature in the dark for 10 min. Thereafter, samples were diluted in 500 µL of extender solution (P1 medium for the Spanish protocol or Tanaka´s medium for the Hungarian protocol) and were analyzed with a flow cytometer (Beckman Coulter FC500). The analyses were performed using the voltages: SS= 199, FS= 199, FL1= 377 and FL2= 372; for a maximum number of 5,000 events or 15 s at low flow.

Finally, a study of DNA methylation level was conducted in fresh and thawed sperm. Sperm DNA was extracted using the phenol/chloroform method: about 20×10^6 spermatozoa in 10 µL Hank´s balanced salt solution (HBSS) 300 were digested overnight at 42 °C under agitation in 1mL of TNES buffer (125 mM NaCl, 10 mM EDTA, 17 mM SDS, 4 M urea, 10 mM Tris-HCl, pH 8) with 75 µg of proteinase K (Sigma Aldrich, P6556). One mL phenol-chloroform-isoamyl alcohol (25:24:1) was added and vigorously mixed. After centrifugation for 15 min at 8,000 g at 4 °C, the upper phase (800 µL) was mixed with 200 µL NaCl 5 M and 2 mL of cold (-20 °C) 100% ethanol. After centrifugation, the dried DNA pellet was mixed with 100 µg/mL RNase in water (Promega, A7973) and incubated 1 h at 37 °C. Whole DNA methylation

level was estimated using LUMA (luminometric methylation assay) (Karimi et al., 2006). Genomic DNA from each sperm (0.5-1 μg) was digested 4 h at 37 °C with 7.5 units of either HpaII and EcoRI (NEB R3101) or MspI and EcoRI in a total volume of 30 μL in duplicate. For pyrosequencing of the digested samples, 20 μL of digested DNA were mixed with 20 μL of annealing buffer (Qiagen, 979009) and samples were placed in a Qiagen PyroMark Q96 ID. The instrument was programmed to add dNTPs in the following order: A, C+G, T, C+G, water, A, T. Peak heights (PH) were analyzed using the PyroMark Q96 software. A and T peaks refers to the amount of DNA cleaved by EcoRI (DNA content controls) whereas C + G peaks show the amount of DNA cleaved by MspI and HpaII. The percentage of methylation was calculated as 100 x (1- (PH HpaII/PH MspI)). The PH HpaII/PH MspI ratio was calculated by doing (PH HpaII/PH EcoRI) / (PH MspI/PH EcoRI).

2.7 Statistical analysis

Sperm viability and motility parameters were subjected to analysis of variance (General Linear Model, GLM). As fixed effect was chosen fresh or thawed sperm from both protocols, i.e. "fresh sperm", "thawed DMSO" and "thawed methanol". For all models, an examination of the residual plots verified that no systematic patterns occurred in the errors. Model results of p-values<0.05 were considered significant.

For the statistical analysis of DNA levels, a non-parametric test (paired Wilcoxon test) was performed. Differences were considered as significant if p<0.05.

All analyses were conducted in the R-environment (R_Development_Core_Team, 2010)

3. Results

Results from this comparison experiment showed that all samples, independently of the protocol used, decreased their percentage of motile cells and cell velocity after cryopreservation (Figure 1). In addition, the motility results from thawed samples of sperm cryopreserved with the

methanol protocol showed higher motility (32.4 ± 1.8%) than those from the DMSO protocol (10.8 ± 0.9%) (Figure 1). All the sperm kinetic parameters analyzed showed the same pattern, with higher motility and faster velocities in samples preserved with the methanol protocol than those preserved with the DMSO one (Figure 1).
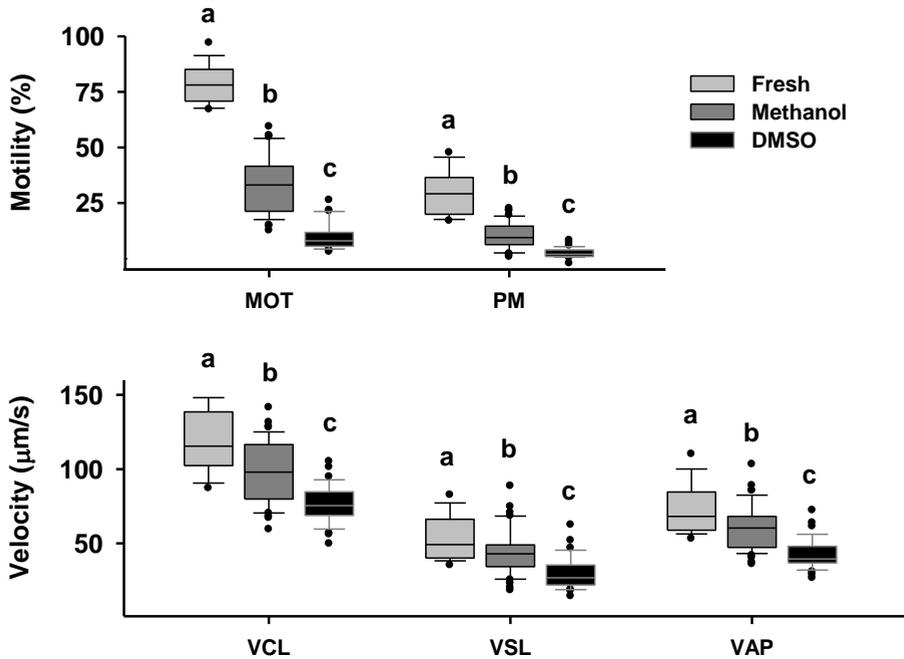
*Figure 1. Sperm kinetic results from fresh sperm, thawed sperm from methanol cryopreservation protocol (Hungarian protocol) and thawed sperm from DMSO protocol (Spanish protocol). The motility analyses show MOT (motility) and PM (progressive motility). The velocity results presented here are VCL (curvilinear velocity), VSL (straight-line velocity) and VAP (average path velocity). Boxplots with different letters are significantly different (p < 0.05; n = 16-18).*

Furthermore, the proportion of fast cells (faster than 100 µm/s) was also significantly reduced after cryopreservation (Figure 2). Nevertheless, thawed samples of sperm cryopreserved with the methanol protocol presented a higher percentage (47.9 ± 1.5%) of fast cells than using the DMSO one (29.6 ± 2.1%). Note that the sperm was instantly activated when diluted in the

freezing medium of the DMSO protocol before freezing, clearly affecting the motility after thawing (Figure 3), whereas samples diluted in the freezing medium containing methanol were not activated (no differences with fresh samples) and did not affect the sperm motility prior to freezing (Figure 3).
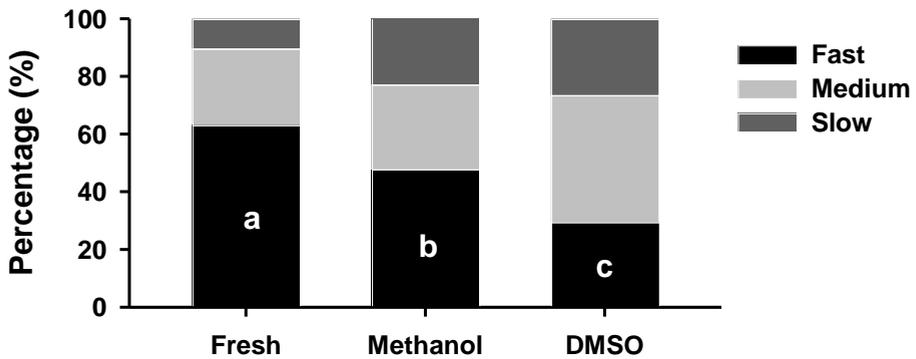
Figure 2. Comparison of the percentage of different velocity groups [slow (VAP = 10-50 µm/s), medium (VAP = 50-100 µm/s) and fast (VAP > 100 µm/s)] of thawed sperm samples from the DMSO and methanol protocols, and from fresh sperm. Different letters indicate significant differences between percentages of fast cells (p < 0.05; n = 16-18).

Cell viability results (Figure 4) showed that there were more live spermatozoa in thawed sperm samples from the methanol protocol than from the DMSO one, and although survival in both cases was high (>75%), it was still lower than viability measured in fresh sperm samples.
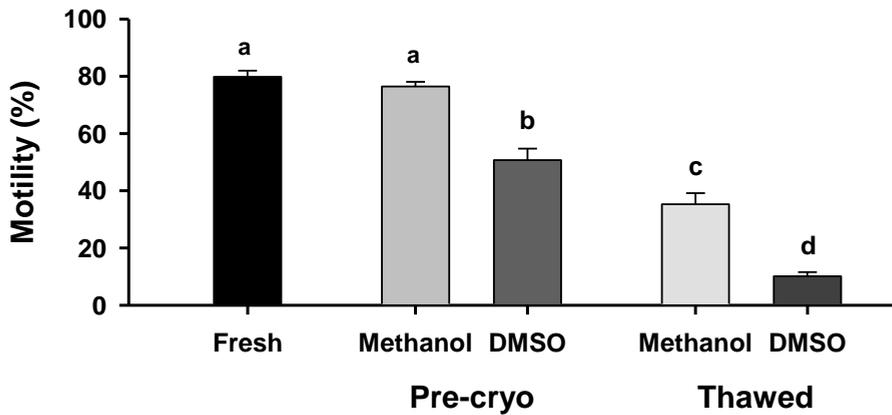
Figure 3. *Effect of freezing medium dilution on sperm motility. Percentage of motile cells after activation with artificial sea water. "Fresh" column shows motility from fresh samples. "Pre-cryo" columns shows the sperm motility of sea water-activated samples after being diluted with freezing medium containing DMSO or methanol before cryopreservation, and "Thawed" columns shows the sperm motility of thawed and sea water-activated samples from the DMSO or methanol protocol. Values are means ± SEM of sperm from 16 samples. Means with different letters are significantly different (p < 0.05).*

The analysis of cysteine methylation in fresh and thawed sperm (Figure 5) showed that sperm samples treated with the DMSO protocol had lower DNA methylation than fresh samples and samples treated with the methanol protocol, whereas these two showed no differences between each other.
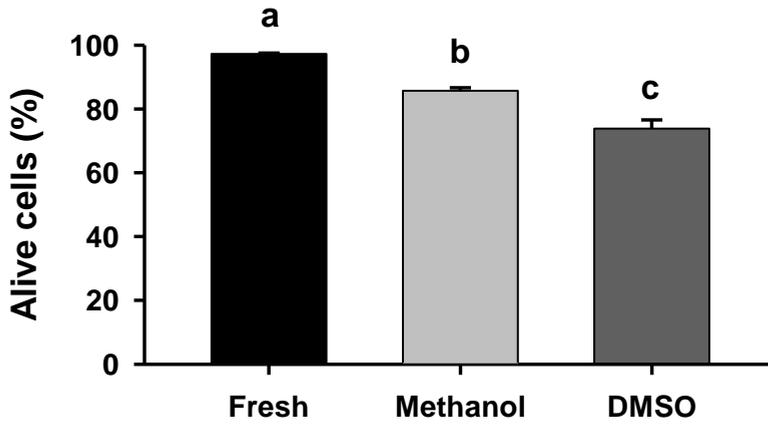
Figure 4. *Comparative viability data from flow cytometry of fresh sperm and thawed sperm from methanol and DMSO cryopreservation protocols. Values represent means ± SEM (n = 12). Different letters indicate significant differences (p < 0.05) between means.*

Figure 5 *Global DNA methylation of eel sperm. Average percentage ± SD (n=9) of 5-methylcytosine on fresh and thawed samples. Different letters indicate significant differences (p<0.05).*

4. Discussion

In this work, we described and compared the two main protocols available for European eel sperm cryopreservation. Our results indicated that in every case, the sperm motility of thawed sperm was lower than in fresh sperm. The reduction in post-thawing sperm quality compared to fresh sperm is consistent with the available bibliography, although there is a great variation between fish species (Asturiano et al., 2017; Horváth et al., 2015). For instance, Dziewulska et al. (2011) used several cryoprotectants (DMSO and methanol as in the present study) to freeze fresh sperm samples of Atlantic salmon (*Salmo salar*) with a motility of 70-95%. The study showed that the sperm motility after thawing was significantly lower than in fresh samples, with post-thawing motility values in the best protocol of 8.2%, using DMSO as cryoprotectant. Oppositely, a different study with cryopreserved sperm from brown trout (*Salmo trutta*) using methanol as cryoprotectant, obtained motilities of thawed sperm higher than 60%, which represented a reduction of only 20% of motility compared to fresh samples (Horváth et al., 2015).

In the present study, the data of sperm quality from thawed samples showed that cryopreservation using the methanol protocol caused higher motility values than the DMSO protocol. Although the values obtained with the methanol protocol were consistent with the bibliography (Müller et al., 2004; Szabó et al., 2005), the motility results from the DMSO protocol were lower than previously reported (Asturiano et al., 2003; 2004; Peñaranda et al., 2009). Although the samples were frozen immediately after the addition of the freezing media containing DMSO to the sperm, it has been proved that the presence of DMSO in the freezing media activates the European eel sperm (Peñaranda et al., 2009), and lead to a reduced post-thawed sperm motility. Even though the DMSO protocol was improved to reduce activation by increasing the concentration of $NaHCO_3$, decreasing the pH of the media (Peñaranda et al., 2009; Vílchez et al., 2017), fast manipulation was still required. In this study, we show that the sperm was activated after diluting in the freezing media (before freezing) containing DMSO. This pre-freezing activation naturally affects the final sperm motility of thawed sperm samples.

Although DMSO is the most used cryoprotectant for fish sperm (Gallego and Asturiano, 2018a; Martínez-Páramo et al., 2017), methanol has also been widely used in freshwater species such as sturgeons, salmonids, tench or Eurasian perch within others (reviewed by Asturiano *et al.,* 2017). Furthermore, it has been recently used in cryopreservation protocols for Japanese eel sperm (Koh et al., 2017; Müller et al., 2017; 2018). As cryoprotectant, methanol has been reported to penetrate more rapidly the cells and being less toxic than DMSO (Horváth et al., 2015). In addition, methanol is osmotically inert and therefore does not activate sperm by osmotic shock (De Baulny et al., 1997; Horváth et al., 2005). In our study, the methanol was apparently less toxic than the DMSO, because thawed samples from the methanol protocol presented higher survival than samples from the DMSO protocol. Furthermore, we confirmed that since methanol is osmotically inert, it did not activate the sperm, oppositely to the DMSO that activated the sperm due to the increase of osmolality. This difference could partially explain the higher motility and velocity of thawed samples treated with the methanol protocol. Furthermore, both protocols differ in other aspects such as extender composition, dilution rate, volume and freezing rate that could also affect the thawed sperm motility.

Sperm from the DMSO protocol presented a loss of methylation compared to fresh sperm, whereas sperm from the methanol protocol remained similar to the fresh control. Changes in cytosine methylation levels after cryopreservation have been little explored in fish. Primarily, the concern arose for the use of methylating cryoprotectants that in the presence of ROS may led to cytosine methylation (Kawai et al., 2010). Indeed, Riesco and Robles (2013) observed in zebrafish that some promoter regions were hypermethylated after genital ridge cryopreservation in DMSO. However, in tambaqui (*Colossoma macropomum*) sperm, cryopreservation with either DMSO or methanol induced in both cases a sperm DNA hypomethylation (De Mello et al., 2017), contrarily to what could have been expected from the model study from Kawai et al. (2010). It is therefore not clear if the cryoprotectant molecule is the main parameter affecting DNA methylation. It was reported that cryopreservation-induced changes in DNA methylation could be species dependent (Labbé et al., 2017), and that cryopreservation

with methods which are not optimal for a given species would induce more epigenetic effect (Labbé et al., 2014). In our case, the fact that the methanol protocol did not change the overall DNA methylation level would indicate that the epigenetic risk is reduced with this method.

5. Conclusions

In conclusion, this study show that the methanol cryopreservation protocol, is nowadays the most suitable protocol for European eel sperm cryopreservation, giving the best sperm motility, sperm velocity and cell survival values. Furthermore, the methylation level of sperm DNA from thawed samples with this method are the same as in fresh sperm, indicating that there are not drastic epigenetic changes when sperm is cryopreserved in this way.

Acknowledgements

CHAPTER 4

European eel sperm storage: optimization of short-term protocols and cryopreservation of large volumes

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Abstract

Maturation in captivity of European eel (*Anguilla anguilla*) requires long and costly hormonal treatments that often lead to asynchronic maturation between sexes. Therefore, optimization of sperm short-term storage methods and cryopreservation protocols can be a key factor for successful artificial fertilization. Two experiments were carried out to optimize the existing protocols.

For the short-term storage experiment, sperm was diluted in P1 extender and then stored at different dilution ratios (1:9 and 1:49). The best outcome was then tested at different temperatures (4 and 20 ºC) and in constant agitation or still. In the cryopreservation experiments, large sperm volumes (cryotubes of 2 and 5 ml), different cooling rates (freezing tubes 1 or 3 cm above liquid nitrogen during 15 and 20 min), and different extender compositions (methanol 10% was used as cryoprotectant, and complemented with FBS 20%, BSA 5% or egg yolk 5%) were tested. Sperm kinetic parameters were analyzed with a CASA-Mot system both in fresh and short- or long-term stored samples.

In the short-term storage trial, sperm quality did not show significant differences in the first 24 h after sperm collection between the different storage conditions tested. For longer time, 1:49 dilution ratio showed significantly better results than 1:9, and low temperature (4 ºC) was better for sperm preservation after 3 days.

Cryopreserved sperm samples showed good motility results when they were frozen in cryotubes of 2 and 5 ml, with no significant differences compared to samples cryopreserved in lower volumes (straws of 0.5 mL). Furthermore, the combination of methanol (10%) and egg yolk (5%) as freezing medium, induced significant higher post-thawing motility values (over 50%) than the control (methanol 10%), whereas the addition of FBS (20%) and BSA (5%) led to a significant reduction of the sperm motility. The establishment of these storage and cryopreservation protocols will be important for the improvement of European eel artificial reproduction programs.

1. Introduction

European eel (*Anguilla anguilla*) is a catadromous fish with a complex life cycle that includes several metamorphoses. In the last 35 years, the number of European eels arriving to the European coasts, have been dramatically reduced in over 90% (van den Thillart et al., 2009), and strategies for artificial reproduction have become a priority to recover the species. Furthermore, the European eel is very appreciated as food delicatessen with great economical value, reinforcing the need for a program for reproduction in captivity to release the fishing pressure on natural individuals.

Maturation in captivity of European eel requires hormonal treatments that last for several weeks in males and even months in females (Butts et al., 2014; Gallego et al., 2012; Mylonas et al., 2017), and frequently there is a maturation asynchrony between genders. Furthermore, in females, the period of time after ovulation that the eggs are viable for fertilization is very short (Butts et al., 2014). Therefore, short-term preservation of fresh sperm diluted in extender medium, or cryopreservation in liquid nitrogen is necessary to facilitate artificial fertilization in European eel (Asturiano et al., 2016).

The optimal sperm storage conditions are normally at low temperatures to avoid bacterial growth, and diluted in extender solution, that mimics the composition of the physiological seminal plasma, to maintain the spermatozoa capacities for longer time (Asturiano et al., 2016; Bobe and Labbé, 2009). However, the time that the sperm maintains motility and fertilization capacity varies widely between species, and the optimal temperature, dilution ratio and other physiochemical storing conditions are species specific (Bobe and Labbé, 2009).

Several research groups have studied the effect of different storing conditions on European eel sperm quality at temperatures above freezing (Peñaranda et al., 2010a,b), with good sperm motility results for over three days, and even one week or more under specific air-limited conditions (Peñaranda et al., 2010a). Nevertheless, in these studies, the assessment of sperm motility was conducted subjectively, which make these results difficult to compare both intra- and inter-laboratories (Gallego et al., 2018).

For preservation of sperm during a longer period, cryopreservation is the best option, and protocols developed in fish species can keep the sperm quality up to several years (Fabbrocini et al., 2015). Moreover, sperm cryopreservation presents many other applications in broodstock management, including the transport of gametes from different centers, or preservation of selected genetic lines (Asturiano et al., 2017; Cabrita et al., 2010; Martínez-Páramo et al., 2017). Although sperm cryopreservation present several benefits, these techniques face different issues such as the membrane damage produced by the freezing and thawing process (Labbé et al., 2013). The use of cryoprotectants can partially protect the sperm cells from damaging and are absolutely necessary for successful cryopreservation (Cloud and Patton, 2009). Furthermore, the use of membrane protectants such as sugars, bovine serum albumin (BSA), or egg yolk, have been used to improve the preservation of sperm membrane integrity (Cabrita et al., 2010; Martínez-Páramo et al., 2017). However, the sperm characteristics vary greatly between fish species and therefore the development and improvement of cryopreservation protocols should be adapted to the characteristics of each one (Asturiano et al., 2017).

In European eel, several researchers have developed different sperm cryopreservation protocols (Asturiano et al., 2003; 2004; Herranz-Jusdado et al., 2019a; Müller et al., 2004; Peñaranda et al., 2009; Szabó et al., 2005). Moreover, cryopreserved European eel sperm have been successfully used in fertilization trials (Asturiano et al., 2016) and in hybridization trials with Japanese eel (*Anguilla japonica*) eggs (Müller et al., 2012; 2018), although an increased rate of larval deformities were observed when fertilizing with cryopreserved sperm (Müller et al., 2018), evidencing the need of refinement of the protocols. Recently, Herranz-Jusdado et al. (2019a) have compared the available European eel cryopreservation protocols with the aim of choose the most efficient one and standardize its use, but this protocol still consists of small volumes of 0.5 mL, which is impractical for large-scale fertilization programs needed in e.g. hatcheries. Furthermore, the use of additives may improve the protection of the spermatozoa membrane, increasing the viability of cryopreserved sperm and optimizing the motility results of post-thawed sperm.

With the objective of improving the storage conditions and cryopreservation of large European eel sperm volumes, two experiments were designed. The first experiment was performed to test different sperm short-term storage conditions. Here, we tested whether the dilution ratio of eel sperm (1:9 or 1:49) in extender solution or the temperature (4 or 20 ºC) had any effect on the sperm preservation time. Further, to reduce degradation, we tested if constant stirring had a positive effect on the stored sperm. The second experiment was performed to design a new cryopreservation protocol for larger volumes, using 2 and 5 mL vials. Furthermore, we tested whether the use of additives that previously have been successfully used in other fish species, such as fetal bovine serum (FBS), BSA or egg yolk, could improve the motility of cryopreserved European eel sperm.

2. Materials and methods

2.1 Animal rights

This study was carried out in strict accordance with the recommendations given in 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 protocols were approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and the final permission was given by the local government (Generalitat Valenciana, Permit Number: 2015/VSC/PEA/00064). The fish were not fed throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Fish handling and sperm collection

Male European eels (n = 30) from Valenciana de Acuicultura, S.A. fish farm (Puzol, Spain) were moved to the aquaculture laboratory at the Universitat Politècnica de València (Spain). Fish were distributed in two 150-L aquaria (15 males per aquarium) and gradually acclimatized to seawater (salinity = 37 ± 0.4 g/L) during a week. The eels were kept at a constant temperature of 20 °C and the aquaria were covered to reduce the light intensity minimizing

fish stress. After 10 days of acclimation, the eels were anesthetized weekly with 60 ppm of benzocaine (Thermo Fisher, Kandel, Germany) for injecting 1.5 IU g−1 fish of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Merck S.L., Madrid) to induce maturation.

After ten weeks of hormonal treatment, sperm samples were weekly collected by abdominal pressure 24 h after the administration of the hormone (Gallego et al., 2012; Pérez et al., 2000). Sperm samples were immediately diluted 1:9 (sperm:extender) in P1 medium (in mM: NaCl 125, NaHCO3 20, MgCl2 2.5, CaCl2 1, KCl 30; and pH adjusted to 8.5, described by Peñaranda et al. (Peñaranda et al., 2010b) and kept in 15 mL centrifuge tubes at 4 °C until sperm kinetic analyses with Computer Assisted Sperm Analyzer (CASA-Mot).

2.3 Evaluation of motility

Within the 2 h following the sperm extraction, sperm samples were evaluated with CASA-Mot system following the method described by Gallego et al. (2013a). Briefly, 1 mL of each sperm sample (1:9 diluted in P1) was transferred to a 1.5 mL plastic tube. Then, each sample was activated by mixing 0.5 µL of P1-diluted sperm sample in 4.5 µL of artificial seawater (in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4, in distilled water) with 2% (w:v) bovine serum albumin (BSA) (Sigma Aldrich Química SA, Madrid, Spain), pH adjusted to 8.2 and osmolality of 1100 mOsm/kg. The activation was performed in a counting chamber ISAS Spermtrack 10 (Proiser R+D, S.L., Spain) on a microscope in negative phase with a 10X magnification (Nikon Eclipse 80i) connected to a computer with an ISAS 782M camera (Proiser R+D, S.L., Spain), recording 60 frames per second (fps) during 1 s. All samples were analyzed 10 s after activation, using the CASA module ISAS v1 software (Proiser R+D, S.L., Spain). Several kinetic parameters were studied: percentage of motile spermatozoa (MOT), progressive motility (pMOT), defined as the percentage of spermatozoa swimming forward, curvilinear velocity (VCL) defined as the average velocity of a spermatozoa in a curvilinear trajectory and straight-line velocity (VSL), defined as the average velocity of a spermatozoa along a straight line. In addition, percentage of

slow (average path velocity (VAP) = 10-50 μm/s), medium (VAP = 50-100 μm/s) and fast (VAP >100 μm/s) spermatozoa were recorded (see Gallego and Asturiano (2018b) for details). Samples with motility values higher than 65% were selected for the experiments.

2.4 Sperm viability

A viability analysis was conducted for the cryopreservation experiment, in every fresh and thawed sample with flow cytometry using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) containing SYBR 14, that stains in green the nuclei of living cells, and propidium iodide (PI) that stains in red the nuclei of dead cells. For each sample, 0.5 μL of SYBR 14 (final concentration 100 nM) and 2 μL of PI (final concentration 12 μM) were added to 50 μL of fresh or thawed sperm samples and incubated at room temperature and darkness for 10 min. Thereafter, each sample was diluted in 500 μL of P1 extender and was analyzed with the flow cytometer (Beckman Coulter FC500). All analyses were performed using the voltages: SS= 199, FS= 199, FL1= 377 and FL2= 372, and for a maximum number of 5000 events or 15 s at low flow.

2.5 Experimental design

The study was divided into two independent experiments. The experiment 1 aimed to find the best short-term storing conditions for European eel sperm, and the experiment 2 aimed to adapt the latest European eel sperm cryopreservation protocol to larger volumes, and test whether the use of additives may improve the quality of cryopreserved samples.

2.5.1 Chilled storage

The chilled storage experiment was divided in two parts. First, it was tested which dilution ratio preserved better sperm quality through time. For so, 11 sperm samples with motilities higher than 65%, were diluted 1:9 or 1:49 in P1 extender solution with a final volume of 1 mL and stored at 4 ºC in 1.5 mL Eppendorf tubes. Each sperm sample was then analyzed for sperm kinetics with CASA-Mot at 2, 24, 48, 72 h after the sperm collection.

In a second part of the experiment, using the dilution that preserved the best motility longer time, it was tested whether temperature or movement while storing would affect the sperm quality through time. Hence, sperm samples from 12 different males, with motility over 65% were diluted 1:49 in P1 extender solution (v = 1 mL) and stored in 1.5 mL Eppendorf tubes at 4 or 20 ºC and still or in constant stirring. The stirring consisted in placing the samples over a shaking device at 80 rpm. Then samples were again analyzed with CASA-Mot at 2, 24, 48, 72 h and 7 days after the sperm collection.

2.5.2 Cryopreservation

The cryopreservation experiment was also divided in two parts. First, it was tested if the latest eel sperm cryopreservation protocol could be used with larger containers (2 and 5 mL). For so, sperm samples from 14 males, with motilities over 65% were selected for the experiment and each sample was frozen in a straw of 0.5 mL (standard container) (IMV Technologies, I'Aigle, France), cryotube (Deltalab SL, Barcelona, Spain) of 2 mL and cryotube of 5 mL. The straws were frozen following the protocol described by Herranz-Jusdado et al. (2018b) , but for the cryotubes different cooling conditions were tested. The different freezing conditions consisted in placing the cryotubes on a floating structure 1 or 3 cm over the liquid nitrogen (LN) for 15 or 20 min.

For the second part of the experiment, the best outcome for 5 mL cryotubes was further tested if by adding BSA, FBS (Sigma Aldrich Química SA, Madrid, Spain) or egg yolk, had a positive effect in the preservation of the sperm. Each sample from 10 different individuals, was analyzed for sperm kinetics with CASA-Mot as described above, before freezing and after thawing. In addition, the samples were analyzed with the flow cytometer for cell viability as explained above.

2.6 Cryopreservation protocols

First, a dilution with sperm:P1-extender:methanol at a proportion 1:8:1 was prepared for each sample and packed in duplicate for each volume and cooling condition. The diluted samples were then incubated for one hour at 4 ºC to ensure a stable penetration of the cryoprotectant into the cells. Further, the 0.5 mL straws were cooled for 3 min, 3 cm over the LN, and then threw them into the LN. The 2 mL tubes were cooled by placing them during 15 or 20 min at 1 or 3 cm above the LN, and then they were thrown into the LN. For the 5 mL tubes, preliminary studies showed that 15 min were not sufficient time for cooling enough the sperm, therefore all 5 mL tube samples were placed for 20 min, 1 or 3 cm over the LN before throwing them into the LN. For thawing, frozen sperm samples were submerged in water at 40 ºC for 13 s (0.5 mL straws), 70 ºC for 75 s (2 mL crytubes) or 70 ºC for 105 s (5 mL cryotubes). All samples were analyzed immediately after thawing with CASA-Mot for sperm motility and with flow cytometry for cell viability.

For the second part of the experiment, the same cryopreservation protocol was used. Each sperm sample was divided in four treatments containing 5% of egg yolk, 20% FBS, 5% BSA or no additives, as control. The proportions of the mixture containing sperm:(P1+additive):methanol were 1:8:1, and it was prepared by diluting the additive in P1 first, then added the methanol and finally the sperm (Table 1). Note that the egg yolk was extracted directly from a commercial hen egg. Then, samples from each treatment were packed in 5 mL tubes (two tubes per treatment), incubated for 1 h at 4 ºC and then frozen for 20 min, 1 cm above the LN surface. Thereafter, the samples were thrown into the LN and stored in a LN tank. Frozen samples were thawed by warming them in water at 70 ºC during 105 s, and stored at 4 ºC for 24 h. The samples were then analyzed with CASA-Mot, and an additional analysis was performed 24 h after thawing. The samples were also tested for cell viability with the flow cytometer approximately one hour after thawing.

Table 1. Volume proportion of the different components of the cryopreservation mixture. All volumes are represented as mL of a total volume of 10 mL. The additives tested were fetal bovine serum (FBS), bovine serum albumin (BSA) and egg yolk from hen. The order of mixture was first P1 extender and additive, followed by methanol and finally the sperm. The mixture was incubated for 1 h before freezing.

Additives	Sperm	P1-extender	Methanol	Additive
Control	1	8	1	-
FBS	1	6	1	2
BSA	1	7.5	1	0.5
Egg yolk	1	7.5	1	0.5

2.7 Statistical analysis

Analysis of sperm viability, motility and velocity parameters were subjected to analysis of variance (General Linear Model, GLM). For the short-term experiment, the considered fixed effects were first the dilution rates, and then the temperature of incubation and stirring or still at each time point (1, 24, 48, 72 h and 7 days). The cryopreservation experiment included each of the different cooling conditions and tube size as fixed effects, and for the second part of the cryopreservation experiment, the different treatments "MeOH", "FBS", "BSA" and "egg yolk", were the chosen fixed effects.

For all models, an examination of the residual plots verified that no systematic patterns occurred in the errors. Model results of p-values < 0.05 were considered significant. All analyses were conducted in the R-environment (R_Development_Core_Team, 2010).

3. Results

3.1 Chilled storage

Sperm quality of samples diluted 1:9 and 1:49 in P1 was tested at 5 different time-points (1, 24, 48, 72 h and 7 days) (Fig. 1). The results showed no reduction in MOT in the first 24 h independently on the dilution ratio, and after 48 h, sperm samples of both dilutions showed a significant decrease in MOT, but the MOT values were significantly higher in samples diluted 1:49 than in sperm samples diluted 1:9. The pMOT results showed a reduction

already at 24 h independently on the dilution ratio, but the pMOT results after 48 h showed that 1:49 preserved better this parameter. In the analysis of the velocities (VCL and VSL) very little differences were found, but sperm samples diluted 1:49 preserved the velocity for 48 h (the VCL) and 72 h (the VSL), whereas samples diluted 1:9 showed a reduction of VCL and VSL after 48 h.
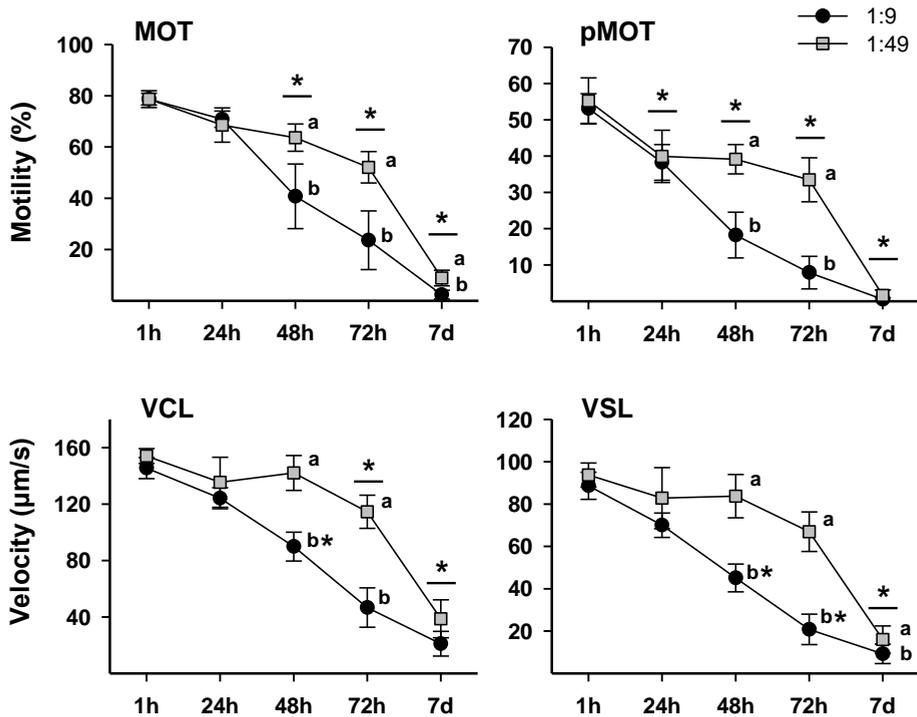
*Figure 1. Sperm kinetic results of sperm stored diluted 1:9 or 1:49 into P1 extender at 1, 24, 48, 72 h and 7 days after collection. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Results are given as means ± SEM (n = 11). Different letters indicate significant differences (p<0.05) between different dilution ratios within each time point. * indicates significant differences (p<0.05) with the control (t = 1 h).*

Since samples diluted 1:49 in P1 showed better sperm quality results, this dilution was tested then for different temperatures (4 or 20 ºC) and stirring or still storing (Fig. 2). The results showed few differences in the first 24 hours. Only still storage at 20 ºC showed a significant reduction compared to control (1h stored) samples, but no significant differences were found between different storing conditions. After 48 h, all samples showed a reduction in MOT and pMOT independently of the storing condition compared to control and only samples stored at 20 ºC and still showed lower MOT than samples stored under the other conditions. However, the sperm velocities (VCL and VSL) were maintained unchanged in all storing conditions. At 72 h, samples stored at 4 ºC independently if they were stored still or stirring, showed significant higher MOT and pMOT than samples at 20 ºC. Similar patterns were found in the velocities (VCL and VSL) but only samples stored at 20 ºC and still were slower. Finally, after 7 days all samples showed a strong reduction in motility (0 - 12%) and velocity (0 - 38 µm/s).

3.2 Cryopreservation

Results from the cryopreservation experiment showed that all samples reduced their sperm kinetic parameters (MOT, pMOT, VCL and VSL) after cryopreservation independently on the cooling conditions or tube/straw size (Fig. 3). Between the different cooling conditions and tube sizes were very few differences. Only samples from tubes of 2 mL cooled for 15 min at 3 cm over LN and tubes of 5 mL cooled 20 min 3 cm over LN showed a decrease in MOT. The other cooling conditions did not show significant differences between each other or with the straw control. The results of pMOT showed that only thawed samples from 5 mL cryotubes, cooled 20 min 3 cm above the LN showed significant lower pMOT compared to thawed samples from straws, and sperm from 2 mL tubes cooled for 20 min at 3 cm over the LN had higher pMOT than the sperm from the control straws. Finally, the velocity results showed very little variation between different sizes or cooling rates. Only thawed samples from 5 mL cryotubes cooled 3 cm above LN for 20 min showed slower sperm velocities (VCL and VSL) compared to samples from straws or from other cooling rates and tube sizes.
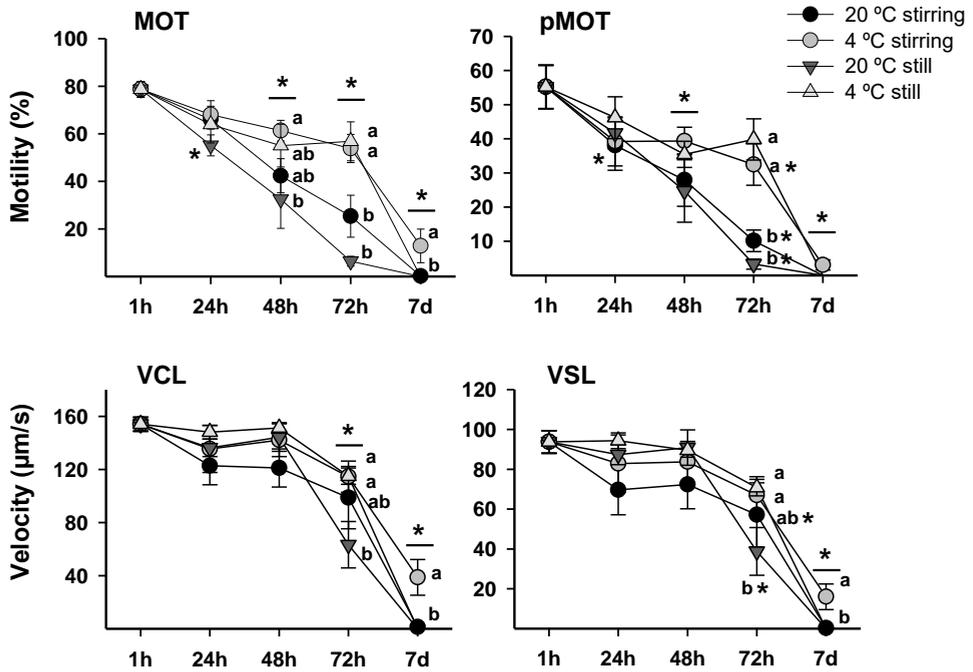
Figure 2. *Sperm kinetic results of sperm stored at different conditions (temperature and still or stirring) at 1, 24, 48, 72 h and 7 days after collection. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Results are given as means ± SEM (n = 12). Different letters indicate significant differences (p<0.05) between different storing conditions within each time point. * indicates significant differences (p<0.05) between a storing condition compared with the control (t = 1 h).*

In addition, the spermatozoa survival after cryopreservation was studied (Fig. 4). The results indicated that all samples showed a reduction in cell survival after cryopreservation, and few differences were found compared to samples from straws. Samples from 2 mL tubes cooled 1 cm above LN independently of the cooling time showed a small reduction in cell survival. The same was found when samples from 5 mL tubes were cooled for 20 min, 3 cm over the LN.
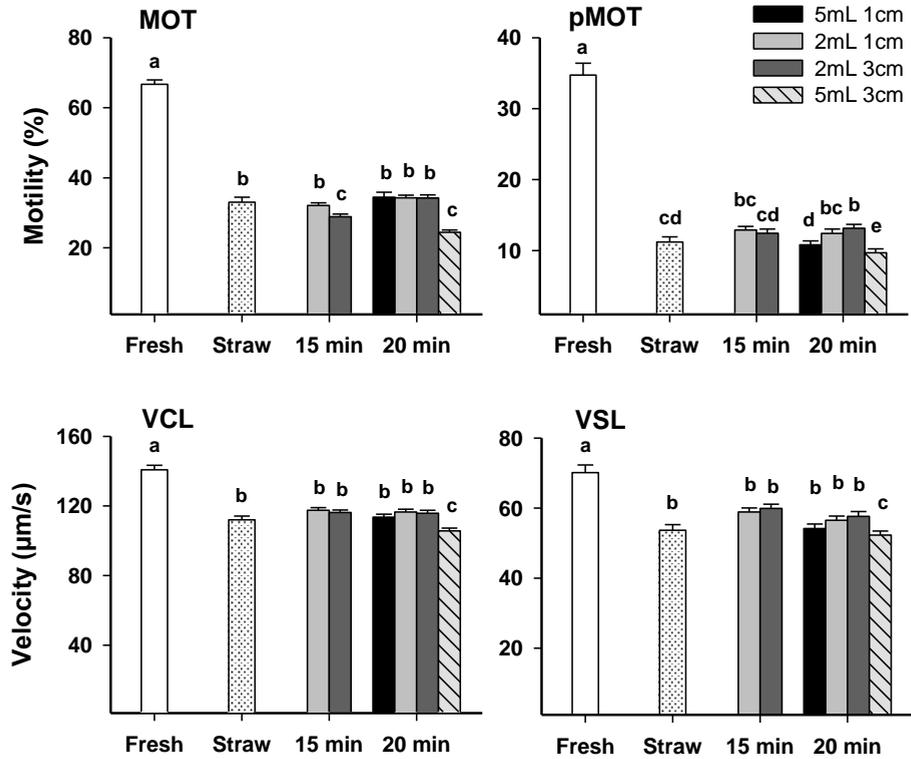
Figure 3. *Sperm kinetic results from fresh and thawed sperm samples treated with different cryopreservation conditions (straw/tube size, cooling height and cooling time). Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Values represent means ± SEM (n = 8-14). Different letters indicate significant differences (p < 0.05).*

The second part of the cryopreservation experiment tested the effect of additives in the quality of thawed sperm using samples in 5 mL tubes cooled 20 min, 1 cm above LN. All thawed sperm samples independently of the additives used, showed a reduction in the motilities and velocities (as occurred in the first part of the experiment) compared to fresh samples (Fig. 5). However, sperm samples treated with egg yolk showed higher motility than samples treated with other additives and control (without additives). Furthermore, samples with egg yolk showed MOT higher than 50%, which is the highest value obtained so far in European eel. Further, the addition of

BSA and FBS induced a reduction in pMOT compared to samples without additives and with egg yolk. The analysis of velocities indicated that the addition of egg yolk resulted in thawed sperm with faster spermatozoa. Furthermore, the percentage of fast cells were also higher in thawed samples with egg yolk compared to samples with other additives or without them (Fig. 6). Moreover, the analysis of cell viability showed a reduction on spermatozoa survival of all samples after cryopreservation, without significant differences in spermatozoa viability when using additives compared to thawed samples without additives (Fig. 7).
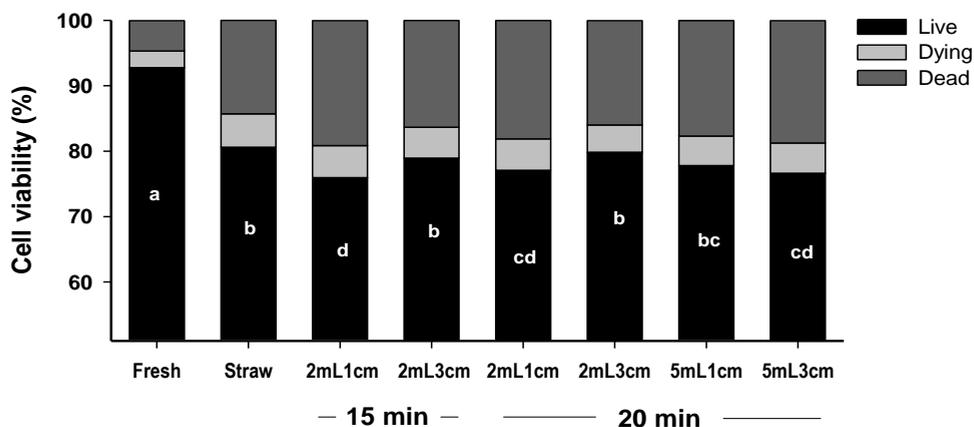
Figure 4. *Sperm viability data from flow cytometry analysis of fresh and thawed sperm samples from the different cryopreservation conditions (straw/tube size, cooling height and cooling time). Data (n = 8-14) are expressed as percentage of live, dying and dead cells. Different letters indicate significant differences (p < 0.05) in the percentage of live cells between different cryopreservation conditions.*
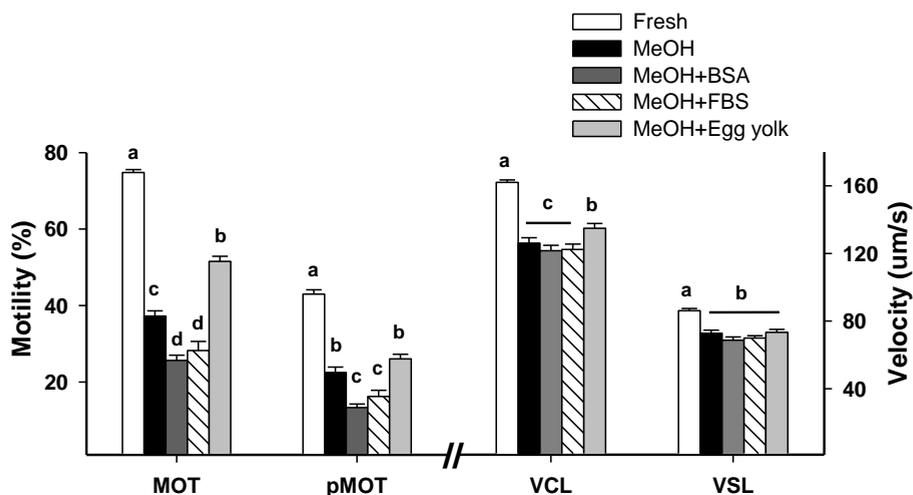
Figure 5. *Sperm kinetic results from fresh and thawed sperm samples from different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant and without additives. All samples were cryopreserved in 5 mL cryotubes. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Values are shown as means ± SEM (n = 9). Different letters indicate significant differences (p < 0.05) between means.*

The samples with additives were maintained at 4 ºC for 24 h and then analyzed for MOT (Fig. 8). The results showed that samples with egg yolk showed similar MOT values than samples without additives, whereas samples with FBS or BSA as additives showed lower sperm MOT. However, sperm samples maintained their MOT unchanged for 24 h independently on the additive used (Fig. 8).
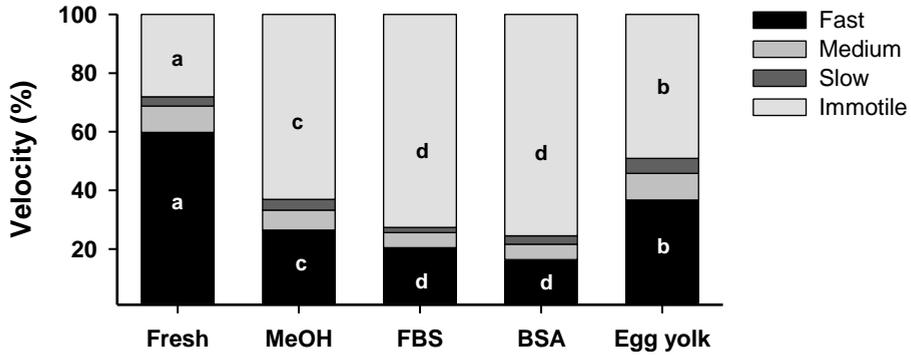
Figure 6. Comparison of the percentage of different velocity groups [fast (VAP = 100 μm/s), medium (VAP = 50-100 μm/s), slow (VAP = 10-50 μm/s) and immotile] of sperm samples from fresh sperm and from thawed sperm cryopreserved using methanol (MeOH), MeOH and FBS, MeOH and BSA, and MeOH and egg yolk. Different letters indicate significant differences between percentages of immotile and fast cells (p < 0.05; n = 9).
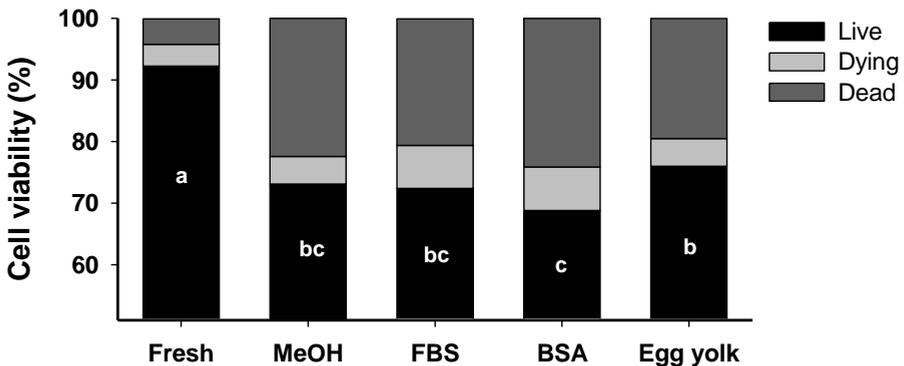
Figure 7. Viability data from flow cytometry of fresh and thawed sperm from the different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant without any other additive. Data (n = 9) are expressed as percentage of live, dying and dead cells. Different letters indicate significant differences (p < 0.05) between the mean percentages of live cells.
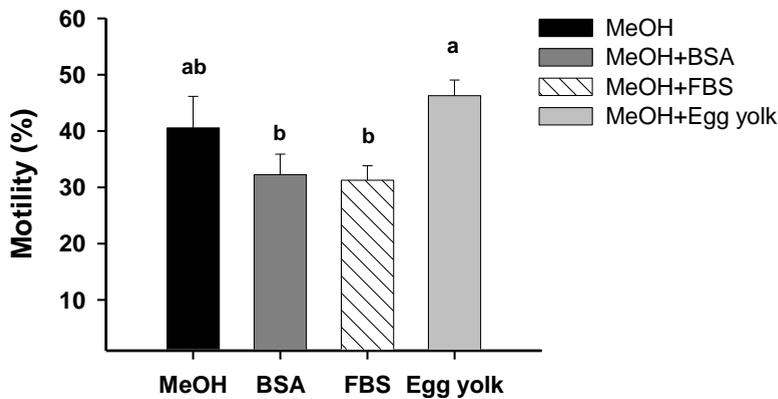
Figure 8. Comparison between sperm motility results from sperm immediately and 24 h after thawing from different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant and without additives. Values are presented as means ± SEM (n = 9). Different letters indicate significant differences (p < 0.05) between means.

4. Discussion

The use of extender diluents for short-term preservation of sperm is widely used in fish reproduction, due to its low cost and efficiency (Bobe and Labbé, 2009; Pérez-Cerezales et al., 2009; Trigo et al., 2015). The extender used in this study, P1 extender, has been previously refined and optimized for its use as diluent for European eel sperm (Asturiano et al., 2003; 2010b; Peñaranda et al., 2010a), and it aims to maintain the sperm inactive by mimicking the physicochemical characteristics of the seminal plasma, where in natural conditions the sperm is immotile (Lahnsteiner et al., 1997; Ohta and Izawa, 1996).

In European eel, sperm dilutions raging from 1:10 to 1:100 have been previously tested under different conditions (Peñaranda et al., 2010a,b). In these studies, 1:50 showed the best outcome, but the motility analysis were conducted subjectively, and therefore difficult to compare to objective

studies (Gallego et al., 2018). In the present study, two sperm dilution ratios in P1 extender were tested, 1:9 and 1:49. We found that in the first 24 h, both dilution ratios successfully preserved sperm quality, but after two days, dilutions of 1:49 provided better results maintaining high sperm motility for over 3 days. These positive effects of higher sperm dilution ratios have been proposed to be related to a reduction in the effect of urine contamination, a better preservation of pH or a reduction in bacterial growth due to a lower spermatozoa concentration (Bobe and Labbé, 2009). Yet, these results support the previous findings showing that samples diluted 1:50 preserved better sperm motility through several days, but in the first 24 h, samples from both dilutions maintained the motility without differences with fresh samples. Therefore, for use in the first 24 h, 1:9 sperm dilution may be more practical for fertilization trials, since the concentration of spermatozoa in the semen would be higher, but after that time-period, 1:49 should be the used dilution ratio for preserving better sperm quality.

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However, since 1:49 dilutions preserved better sperm quality for longer time, this dilution was used to test whether temperature and still or stirring storing had an effect on sperm quality preservation. In this context, previous studies showed that semen storage at low temperature decreases spermatozoa metabolism (Cosson et al., 1985) and therefore maintained its quality. Nonetheless, higher storage temperatures can be more practical in certain situations such as long distance transportation, since then maintaining low temperatures require specific cooling equipment.

Furthermore, studies with salmonids sperm reported lower mortality when sperm was shaken during storage (Parodi et al., 2017), and this way of storing is common in sperm short-term storage protocols of various salmonid species (Trigo et al., 2015; Ubilla et al., 2015). In the present study, no strong effect of stirring was found on sperm preservation. However, low temperatures (4 ºC) preserved higher sperm motility after 2 days of storage, and significant improvements from stirring the samples were found only after a week. These results are in agreement with several previous studies, that indicate that chill storage of sperm preserved better spermatozoa motility through time due to a reduction in spermatozoa metabolism and a lower bacterial growth in the sperm at low temperatures (Bobe and Labbé, 2009; Cosson et al., 1985). Yet, this study shows that in the first 24 h of storing, European eel sperm maintained its motility independently of the temperature.

The storing time analyzed here was up to 7 days after collection. The preservation of sperm motility through time is species specific and varies greatly. For instance, sperm samples from Atlantic halibut (*Hippoglossus hippoglossus*) preserved at optimal conditions, remained motile after 79 days of storage (Babiak et al., 2006), whereas in common carp (*Cyprinus carpio*), sperm motility was maintained for a maximum of 84 h (Ravinder et al., 1997). In European eel, previous studies showed that under air-limited conditions, sperm could maintain some motility for as much as 14 days (Peñaranda et al., 2010a). That protocol required the used of polycarbonate bags that were closed under vacuum conditions. In the present study, the aim included finding the best short-term storing conditions that resulted in a practical and

easy handling of the samples, having in mind its potential use in large-scale reproduction programs at the hatcheries.

In the second experiment of this work, it was tested whether the latest sperm cryopreservation protocol for European eel (Herranz-Jusdado et al., 2018), that uses small straws of 0.5 mL, could be applied for larger volumes without losing sperm quality for aquaculture purposes. Even though sperm cryopreservation protocols are typically developed to solve gamete synchronization problems, the establishment of this protocols may have additional practical uses such as transfer of sperm between hatcheries (Żarski et al., 2017), and using larger volumes would be a great advantage for this purpose.

Cryopreservation of fish sperm in large volumes has already been tested in different fish species. For instance, Cabrita et al. (2001) conducted a series of experiments using rainbow trout (*Oncorhynchus mykiss*) sperm using different straw sizes for cryopreservation. The results showed similar sperm motility results from cryopreserved samples independently of the straw size, i.e. 0.5, 1.8 and 5 ml. Moreover, in a recent study, Nomura et al. (2018) successfully cryopreserved Japanese eel sperm in 2.5 and 5 mL straws, and they obtained similar fertility, hatching and survival rates using cryopreserved sperm than from fresh sperm.

In the present study, we show that it is possible to use 2 or 5 mL cryotubes for cryopreservation of European eel sperm. Compared to the cryopreservation protocol used for 0.5 mL straws, similar thawed sperm quality was obtained in larger volumes just by adjusting the cooling conditions. This represents a great advantage for fish reproduction management, since the number of spermatozoa required to fertilize an egg is relatively high (Butts et al., 2012; 2014), and therefore a large number of spermatozoa is preferred for fertilization programs. However, in this work we did not tested the fertilization outcome of this protocols, yet the cryopreservation success have been evaluated by studying the sperm survival, motility and other kinetic parameters analyzed with CASA-Mot. These parameters are widely use in fish reproduction studies, and have been proposed as good biomarkers for sperm quality showing a strong correlation

with fertilization success in several fish species (reviewed by Gallego and Asturiano, 2018b).

The second part of this experiment aimed to improve the cryopreservation protocol by using different additives: FBS, BSA or egg yolk. These additives have been widely used in sperm cryopreservation protocols of different fish species (Cabrita et al., 2010; Labbé et al., 2013; Magnotti et al., 2018b). FBS and BSA are commonly used due to their osmotic shock buffer effect, antioxidant effect and because they provide mechanical protection to the cell membrane during the freezing and thawing processes (Cabrita et al., 2005; Lewis et al., 1997; Peñaranda et al., 2009), whereas egg yolk stabilizes the sperm membrane and reduces injuries provoked by the thermal shock (Bozkurt et al., 2014; Gallego et al., 2017). Furthermore, the LDL fraction of egg yolk has been reported to protect against DNA damage that may occur through the freezing-thawing process (Hu et al., 2008; Pérez-Cerezales et al., 2010).

In this study, we showed that the addition of egg yolk had a positive effect in the post-thawed sperm motility, showing sperm cells survival values close to 80% and motilities of over 50%. The sperm motility after cryopreservation is species specific in fish and varies greatly (Asturiano et al., 2017). For instance, cryopreservation of paddlefish (*Polyodon spathula*) sperm can reach thawed sperm motility values of 85% (Horváth et al., 2006), whereas experiments with striped bass (*Morone saxatilis*) showed thawed sperm motility lower than 10% (Frankel et al., 2013). In European eel, using the latest protocol, it was obtained sperm motility values of approximately 30% (Herranz-Jusdado et al., 2019a), which is consistent with the motility values obtained in the present work in samples without additives. However, the addition of egg yolk resulted out in the highest thawed sperm motility reported in European eel. Moreover, our results indicated that the thawed sperm quality was preserved for 24 h after thawing stored at 4 ºC. This represents a great practical advantage, since the sperm could be thawed at the home institution and when required, transported to the hatchery just under refrigeration within the next 24 h, and still preserving good quality.

The benefits shown in this work from the addition of egg yolk have been proposed to depend on its chemical composition. Previous work have studied the differences in phospholipids, proteins and cholesterol content between different avian egg types and their effect as cryoprotectant in fish sperm (Bozkurt et al., 2014), but only small differences were found between the different avian egg yolks and none of the components alone could explain the sperm post-thaw variation. Although hen´s egg yolk has been previously used in fish sperm cryopreservation (Babiak et al., 2012), further research to study the effect of the different components of egg yolk as cryoprotectant are recommended, not only to understand better how egg yolk protects the sperm through the cryopreservation process, but also to standardize the protocols. Note that the egg yolk used in this study was obtained from standard commercial hen eggs, which may have variations in their composition.

5. Conclusions

Here we have described a simple method for short-term preservation of European eel sperm for a maximum of 7 days, which is long enough to compensate the gamete asynchronic release that often occurs in European eels. Furthermore, we have optimized the sperm cryopreservation protocol for European eel by increasing the volume of sperm cryopreserved without losing thawed sperm quality. Moreover, we demonstrated that by including egg yolk as additive, the sperm quality post thawing was improved reaching motility values higher than 50%. These findings represent a good advance in the development of future large scale reproduction programs for European eel.

Acknowledgements

CHAPTER 5

Eel sperm cryopreservation: an overview

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Abstract

The eels are teleost fishes from the order Anguilliformes that includes several species with high commercial value. Due to the high interest for aquaculture production of some eel species and for the need to restore eel species that are endangered, several research groups have directed their research toward developing protocols to cryopreserve the spermatozoa of Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*). In this review, we provide an overview on the different protocols that have been developed so far. The first developed protocols used DMSO as cryoprotectant in both species with good success, obtaining sperm motilies of over 45% in Japanese eel and over 35% in European eel. Moreover, sperm cryopreserved using DMSO was successfully used in fertilization trials, although with low fertilization rates. However, recent studies show that DMSO produce epigenetic changes in eel sperm and therefore, the last developed protocols used methanol as cryoprotectant instead. Cryopreservation protocols using methanol as cryoprotectant, showed improved motility values in both Japanese and European eel. In addition, the latest protocols have been adapted to cryopreserve larger volumes of sperm of up to 5 mL, which is useful for larger scale fertilization trials.

The present study introduces the state of the art and future perspectives of the eel sperm cryopreservation to be applied in aquaculture and biological conservation programs.

1. Introduction

Freshwater eels of the genus *Anguilla* include 19 species (Watanabe, 2003), all of which display a complex catadromous life cycle, with oceanic migrations ranging from few hundreds to thousands of kilometers depending on the species (Arai, 2014). Recent studies have indicated that the genus *Anguilla* is originated in the deep ocean of tropical areas and freshwater eels radiated out from the tropics to colonize the temperate regions (Inoue et al., 2010; Minegishi et al., 2005).

Since the early 80's, the population of temperate eels has continuously declined. Particularly, the populations of European eel (*A. anguilla*) and Japanese eel (*A. japonica*) became reduced by 90% in the last 30 years, however the decline of the American eel (*A. rostrata*) population is less dramatic (ICES, 2011). In the case of tropical eels, the actual situation is uncertain, since data of fisheries are unavailable. The causes behind the decline of the temperate eel populations are most likely due to a combination of global climate change, habitat degradation, pollution, parasite infection and overfishing. These eels are common in the traditional diets of many countries, especially in Europe and Asia. The country where eels are consume the most is Japan. There, eels are smoked and processed into a dish named "kabayaki". This delicatessen is made using Japanese eel, but imported eels from the United States (American eel) and Europe (European eel) were also used due to the low availability of Japanese eels. However, since 2010, due to the decline of European eel catches, the EU imposed export restrictions, making illegal to sell European-caught eel to markets outside the EU (ICES, 2015). In Europe, eels are consumed smoked principally in northern European countries or consumed at elver stage in places like northern Spain. In the United States, although eels were consumed by the pilgrims from Europe, the nowadays catches are mostly used for export of elvers to Europe or Asia (Schweid, 2002). However, all three temperate eel species have been included in the Red List of the International Union for Conservation of Nature (IUCN) as threatened due to population decline, with *A. japonica* and *A. rostrata* categorized as "Endangered" (Jacoby and Gollock, 2016; Jacoby et al., 2016), and *A. anguilla*

included as "Critically Endangered" (Jacoby and Gollock, 2014), which is the highest category before extinction rating.

The conservation status of the eel species justifies the needs for taking actions such as development of reproduction in captivity and control of fisheries based on life cycle. The complexity of their life cycle include a metamorphosis. First, the larvae hatched as leptocephalus, which has a laterally compressed body and looks like a leaf with a small head. These first larvae are transported by the oceanic currents to the continental coasts, where they metamorphose into glass eels. At this stage, they display the anguilliform shape but they are thin, small and unpigmented. Thereafter, the glass eels migrate into coastal waters and turn into pigmented elver eels, that later migrate into continental waters and become yellow eels. At this stage, eels undergo a sedentary and feeding phase in freshwater prior to enter the silver eel stage (called silvering). Silvering is a puberty related event, which marks the beginning of sexual maturation, migration and the reproductive phase. Silver eels are still sexually immature when they start their reproductive migration, with sexual maturation occurring during the migration period towards the reproduction site in the ocean. However, in captivity, dopaminergic inhibitions in addition to a deficient stimulation of gonadotropin-releasing hormone (GnRH) block the eel sexual maturation as long as the reproductive oceanic migration is not performed (Dufour et al., 1988; Dufour et al., 2003; van Ginneken and Maes, 2005). Therefore, eels are blocked in a pre-pubertal stage and do not mature spontaneously in captivity. To induce an artificial full maturation in eels, costly hormonal treatments are required that last for several weeks in males and even months in females (Lokman and Young, 2000; Ohta et al., 1996; Oliveira and Hable, 2010; Pedersen, 2003). Moreover, there is frequently a maturation asynchrony between sexes. In females, the period of time after ovulation during which oocytes are viable for fertilization is very short (Butts et al., 2014; Nomura et al., 2013). Therefore, preservation of sperm would be essential to facilitate successful artificial fertilization.

Interestingly, eel spermatozoon present an unusual structure (Figure 1). It possesses a crescent-shaped nucleus with a flagellum consisting of a 9+0 pattern, whereas the typical axonemal structure of the flagellum is 9+2.

Moreover, it has a pseudoflagelum and a single large spherical mitochondrion on the anterior surface at the superior end of the nucleus (Gibbons et al., 1985; Okamura et al., 2000).
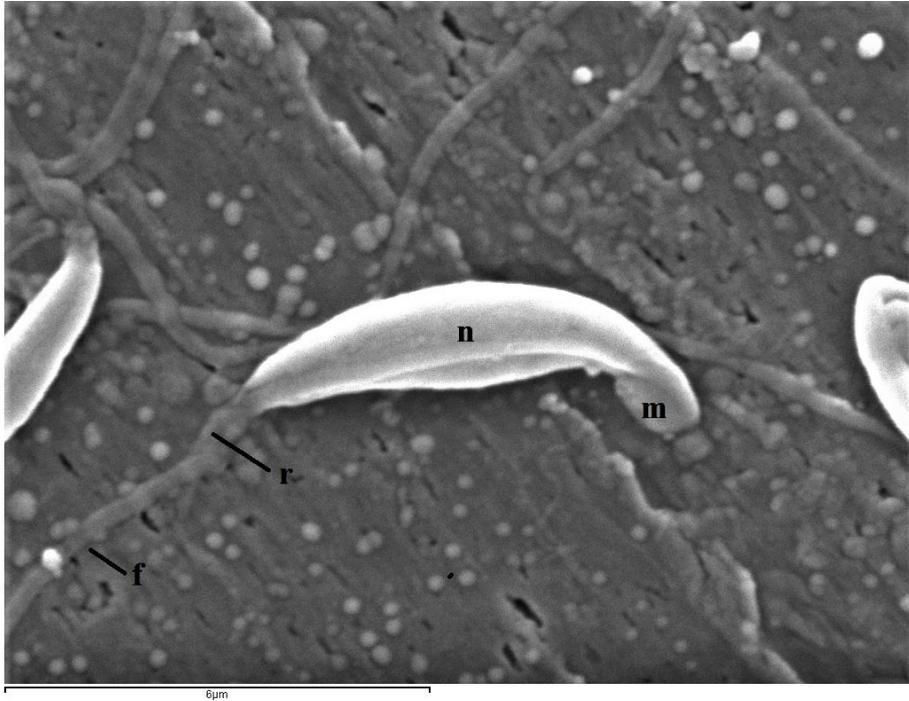
Figure 1. *Electron microscope picture of eel spermatozoa (European eel). m, mitochondria; n, nucleus; r, rootlet (pseudoflagellum); f, flagellum.*

Cryopreservation is the conservation of biological material in liquid nitrogen (LN) at very low temperatures (-196 ºC) that may potentially preserve its viability indefinitely (Bakhach, 2009). In addition to long-term conservation of sperm, cryopreservation of sperm presents several additional advantages; for instance providing research scholars with biological materials to perform comparative experiments, to promote exchange of genetic material for use in breeding and genetic studies (Asturiano et al., 2017; Bobe and Labbé, 2009).

Cryopreservation of eel sperm was first achieved by Tanaka et al (Tanaka et al., 2002a) in the early 2000´s for Japanese eel. Many advances have been

achieved since, including the development of cryopreservation protocols for European eel. In this review, we performed an overview of the historical development of different sperm cryopreservation protocols of two freshwater eels including the European and the Japanese eels.

2. Eels artificial maturation

Good gamete quality is crucial for successful sperm cryopreservation (Bobe and Labbé, 2009). Eels (*Anguilla* spp.) do not mature spontaneously in captivity, so to obtain good quality sperm in the lab, male eels need to be treated with long-term hormonal treatments, i.e. gonadotropins, to induce maturation (Asturiano et al., 2005; Gallego et al., 2012; Ohta et al., 1997). These treatments produce a boost in the plasma levels of 11-ketotestosterone (11-KT), which is the effective androgen in most fish species, including eels (Miura et al., 1991a). Leydig cells are considered as the major source of androgens, while androgen receptors are mainly expressed in Sertoli cells and in interstitial cells. However, androgen receptors are also expressed in Leydig cells, where androgens modulate the expression of steroidogenic genes (Miura et al., 2006), suggesting that androgens develop biological activity via testicular somatic cells (Schulz et al., 2010). Sertoli cells produce different growth factors during spermatogenesis, and their expression or repression seems to regulate spermatogonial mitosis and germ cell differentiation (Schulz et al., 2010). Consequently, the hormonal treatment with gonadotropins promotes spermatogenesis and spermiation.

The traditional hormonal treatment with gonadotropins to induce maturation in Japanese eel and European eel males typically consists of weekly injections of human chorionic gonadotropins (hCG) (Ohta et al., 1997; Pérez et al., 2000) and has been used as the preferred method to obtain high quality sperm for cryopreservation trials in the eels (Asturiano et al., 2004; 2016; Garzón et al., 2008; Herranz-Jusdado et al., 2019a; Marco-Jiménez et al., 2006; Müller et al., 2004; 2012; Peñaranda et al., 2009; Szabó et al., 2005; Tanaka et al., 2002a). However, application of heterologous hormonal treatments with hCG have been observed to produce low rates of fertilization and hatching due to low gamete quality (Tanaka et al., 2002a), and a new line

of studies focuses on the development of homologous gonadotropic hormones to induce eel maturation.

In European eel, Peñaranda et al. (2018) used homologous recombinant LH and FSH, which were obtained by transfection of mammalian cells of Chinese hamster ovary. They treated immature European eels with weekly injections of recombinant LH and FSH and successfully induced full spermatogenesis and spermiation *in vivo*. Nonetheless, there were high variations in sperm quality among treated males (Peñaranda et al., 2018), and thus sperm obtained from this protocol has not yet been used for any cryopreservation trial. In parallel, in the Japanese eel, Kazeto et al. (2014) succeeded in producing homologous recombinant gonadotropins of Japanese eel synthetized as well from cell lines of Chinese hamster ovary. Soon after, Ohta et al. (2017) developed a protocol for Japanese eel maturation consisting of weekly injections of recombinant LH at a dose of 500 µg/kg fish, that induced a high volume of spermiation and fast stimulation of spermatogenesis. This maturation method has been successfully used in cryopreservation and fertilization trials with positive results (Nomura et al., 2018).

3. Cryopreservation protocols

3.1 Japanese eel sperm cryopreservation

A cryopreservation protocol for Japanese eel sperm was first developed by Tanaka et al. (2002a). In this work, the researchers first designed a cryopreservation diluent or extender, to prevent cryoinjury of the spermatozoa and to avoid the spermatozoa activation. This is crucial, since when spermatozoa start their motility, the stored ATP required for the movement of the flagellum will last for only a few minutes (Ingermann, 2008). The cryoprotectant used was dimethyl sulphoxide (DMSO) at 10% v/v, which is the most common compound used as cryoprotectant in sperm of marine fish species (Gallego and Asturiano, 2018a), and the extender diluent included NaCl, $NaHCO_3$ and soya lecithin (Table 1). The use of DMSO caused a hypertonicity in the medium that activated sperm motility (Horváth et al., 2005), but due to the inclusion of $NaHCO_3$ in the extender, the spermatozoa motility was prevented and the protective capacity of the freezing medium

was improved (Tanaka et al., 2002b). Furthermore, the cryopreservation protocol used 2 mL cryovials that were cooled in LN vapor for 5 min, 2 mm above the LN surface, before immersion and storage, and the thawing consisted in immersion in a water bath at 40 ºC for 70 s.

Using this protocol, Tanaka et al. Tanaka et al. (2002a) obtained good post-thaw sperm motility values (37-46%), and therefore, they used it for fertilization trials. In these trials, they successfully fertilized Japanese eel oocytes using cryopreserved sperm, however the hatchability of the fertilized eggs was lower than eggs fertilized with fresh sperm.

For long time, this was the only published Japanese eel sperm cryopreservation protocol, until Müller et al. (2017) published a new cryopreservation protocol in which an artificial seminal plasma (ASP) and methanol were used as extender and cryoprotectant, respectively. The composition of ASP was based on the Ohta et al.'s study (1997), and was prepared with (in mM) 149.3 NaCl, 15.2 KCl, 1.3 $CaCl_2$, 1.6 $MgCl_2$ and 20 $NaHCO_3$, buffered with 20 mM TAPS-NaOH at pH 8.1, and possess iso-ionic osmolality to the seminal plasma of artificially matured Japanese eel. In contrast to DMSO, methanol is osmotically inert avoiding the spermatozoa motility activation that has been caused by DMSO. Moreover, the protocol used 0.5 mL straws and the freezing was conducted in LN vapor for 3 min, 3 cm over the LN surface, before immersion and storage, and the thawing consisted of immersion for 13 s in water at 40 ºC. Although Müller et al. (2017) successfully cryopreserved Japanese eel sperm, the embryos hatched using cryopreserved samples showed lower survival and higher malformation rate than those of fresh sperm, indicating that the protocol was still sub-optimal.

In parallel, Koh et al. (2017) conducted a series of experiments focused on the use of K30 ASP (Ohta et al., 2001) as extender in an alternative cryopreservation protocol for Japanese eel sperm. The K30 ASP consisted of (in mM) 134.3 NaCl, 30 KCl, 20 $NaHCO_3$, 1.6 $MgCl_2$, 1.3 $CaCl_2$, and buffered at pH 8.1 with 20 mM TAPS-NaOH. Moreover, they tested different cryoprotectants in various concentrations, addition of fetal bovine serum (FBS), different storage temperatures before cooling, sperm dilution ratios

and cooling rates. The cryoprotectants tested were methanol, DMSO, N-N,dimethyl formamide (DMF), N-N,dimethyl-acetamide (DMA) and a combination of methanol and DMA. Their results showed that the optimal protocol with the tested parameters consisted of 10 or 15% methanol as cryoprotectant, with 22.5% FBS and 67.5% K30 ASP as extender solutions, obtaining results close to 60% of comparative post thaw motility (CPM), which is a parameter calculated from [sperm motility (%) after cryopreservation × sperm motility (%) before preservation^{-1}] × 100. Furthermore, the cooling rates used were 6.3 – 28.6 ºC/min, corresponding to cooling at 10-16 cm above the LN surface when 0.25 mL straws were used, and the temperature at which the samples were immersed in LN was -40 to -70 ºC. Interestingly, the use of DMSO as cryoprotectant was incompatible with the use of K30 ASP as extender for Japanese eel sperm.

Following the work of Koh et al. (2017), Nomura et al. (2018) established a large-scale cryopreservation protocol for Japanese eel sperm to be used for fertilization programs. They used 5 mL straws and adapted the cooling rate to that volume, and the cryoprotectants and extenders were as described in the latest protocol (Koh et al., 2017). Further, the fertilization trials did not show any difference in egg hatching or survival rates between cryopreserved sperm and fresh sperm. Moreover, the morphology of larvae produced from cryopreserved sperm was similar to that of larvae from fresh sperm, and the larvae were further grown into normal glass eels, representing a great refinement of the Japanese eel sperm cryopreservation protocol.

3.2 European eel sperm cryopreservation

Cryopreservation of the European eel sperm was developed shortly after the first cryopreservation protocols for Japanese eel. Two independent research groups established their own cryopreservation protocols in Spain and Hungary. These protocols differed in most aspects, from the rearing conditions of the eels to the type of cryoprotectants applied into their experiments (Table 2).

Table 1. Extender composition, cryoprotectant concentration and pH used for sperm cryopreservation in Japanese eel and European eel.

Species	Extender	Extender composition (in mM)	Cryoprotectant	pH	Volume (mL)	Thawing (t/Tª)	Motility (%)	References
Japanese eel	Tanaka	137 NaCl, 76.2 $NaHCO_3$, 24 Soya lecithin.	DMSO (10%)	8.2	1	70 s/40 ºC	46.6	Tanaka et al. 2002
	ASP	149.3 NaCl, 15.2 KCl, 1.3 $CaCl_2$, 1.6 $MgCl_2$, 20 $NaHCO_3$, 20 TAPS-NaOH.	MeOH (10%)	8.1	0.5	13 s/40 ºC	-	Müller et al. 2017
	K30 ASP	134.3 NaCl, 30 KCl, 1.3 $CaCl_2$, 20 $NaHCO_3$, 1.6 $MgCl_2$, 20 TAPS- NaOH, 22.5% FBS.	MeOH (10-15%)	8.1	0.25, 2.5 & 5	10 s/20 ºC	59.7 CPM 54.8 CPM	Koh et al. 2017 Nomura et al. 2018
European eel	TNK	137 NaCl, 76.2 $NaHCO_3$, 20 TAPS.	DMSO (10%)	8.1	0.25	45 s/20 ºC	32.2	Asturiano et al. 2003
	P1	125 NaCl, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 1.4% L-α-phosphatidylcholine.	DMSO (10%)	8.5	0.25	45-60 s/20 ºC	36.6	Asturiano et al. 2004 Asturiano et al. 2007
	Kurokura	61.6 NaCl, 134.1 KCl, 1.98 $CaCl_2$, 0.84 $MgCl_2$, 2.4 $NaHCO_3$.	MeOH (10%)	8.0	0.25	5 s/40 ºC	36	Müller et al. 2004
	P1+FBS	125 NaCl, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	8.5	0.25	60 s/20 ºC		Herranz-Jusdado et al. 2019ª Marco-Jiménez et al. 2006
	Tanaka	137 NaCl, 76.2 $NaHCO_3$.	MeOH (10%)	8.2	0.25 0.5	5 s/40 ºC 13 s/40 ºC	40-47 31	Szabó et al. 2005 Müller at al. 2012, 2018
	Modified P1	125 NaCl, 75 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	8.5	0.25	60 s/20 ºC	22.2	Garzón et al. 2008
	Modified P1 (M5)	50 NaCl, 100 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	6.5	0.25	15 s/20 ºC 10 s/40 ºC	38	Peñaranda et al. 2009 Asturiano et al. 2016
	P1+egg yolk	125 NaCl, 20 NaHCO3, 30 KCl, 2.5 MgCl2, 1 CaCl2, 5% egg yolk	MeOH (10%)	8.5	2 & 5	75 s/70 ºC 105 s/70 ºC	51.6	Herranz-Jusdado et al. 2019b

Table 2. *Comparison of the main technical aspects of European eel sperm cryopreservation protocols developed by the Spanish and Hungarian research groups previous to standardization by Herranz-Jusdado et al. 2019a*

Protocols	Spanish	Hungarian
Fish origin	Farmed fish	Farmed fish
Rearing water	Seawater	Freshwater
Hormonal treatment	hCG recombinant	Natural hCG
Extender solution	P1	Tanaka
Dilution ratio	1:2	1:9
Cryoprotectants	10% DMSO & 25% FBS	10% Methanol
Straws (in mL)	0.25	0.5

The group from Spain developed a primary protocol (Asturiano et al., 2003; 2004) mimicking the protocol previously developed for Japanese eel (Tanaka et al., 2002a) using DMSO as cryoprotectant (Table 1). Different extenders were tested, including two developed for Japanese eel (Tanaka´s and K30) and two developed for the European eel (P1 and P2) designed to be iso-ionic to the seminal plasma of European eel (Pérez et al., 2003). The Tanaka medium, developed for Japanese eel had (in mM) 137 NaCl, 76.2 NaHCO$_3$ and 20 TAPS at pH 8.2, and the K30 medium with (in mM) 134.5 NaCl, 20 NaHCO$_3$, 30 KCl, 1.6 MgCl$_2$, 1.3 CaCl$_2$, and at pH 8.1. The P1 medium developed for European eel, was composed by (in mM) 125 NaCl, 20 NaHCO$_3$, 30 KCl, 2.5 MgCl$_2$, 1 CaCl$_2$, and the pH was adjusted to 8.5, and the P2 medium was prepared with (in mM) 70 NaCl, 75 NaHCO$_3$, 30 KCl, 2.5 MgCl$_2$, 1 CaCl$_2$, and pH 8.5. All media were supplemented with 10% DMSO as cryoprotectant and different sperm dilution ratios were also examined. The freezing process was carried out in 0.25 mL straws placed for 10 min, 5 cm above LN surface before plunging them into LN, and the thawing was conducted by submerging the straws in a water bath at 20 ºC during 45 s. In this first approach, Asturiano el al. (2003; 2004) reported that sperm samples diluted 1:5 in Tanaka extender or P1 extender with 10% DMSO showed the highest spermatozoa motility post thawing.

In parallel, the group from Hungary (Müller et al., 2004) developed a cryopreservation protocol using a modified Kurokura solution as extender (in mM: 61.6 NaCl, 134.1 KCl, 1.98 CaCl$_2$, 0.84 MgCl$_2$, 2.4 NaHCO$_3$) and 10%

methanol as cryoprotectant (Table 1). The dilution rate used was 1 sperm: 8 extender: 1 methanol using 0.25 mL straws, and cooling them 4 cm over the LN for 3 min before plunging them into the LN. A water bath at 40 ºC was used to thaw cryopreserved sperm for 5 s. Under application of this protocol, Müller et al. (2004) obtained similar results to those of the Spanish protocol, although protocols show differences. Following this study, Szabó el al. (2005) conducted a series of experiments to test different extenders and cryoprotectants (DMSO and methanol), aiming at improvement of the protocol described by Müller et al. (2004). They observed that application of DMSO (10%) with Tanaka extender, and application of methanol (10%) with Tanaka extender resulted in the highest success compared to other treatments. The samples cryopreserved using the protocol with methanol could be further diluted 1:9 in Tanaka's medium to reduce the toxicity of the cryoprotectant, which may be important for further short-term storage of frozen-thawed sperm. This was not a possibility when using DMSO as cryoprotectant, probably explained by the change in osmolality of sperm following dilution (Horváth et al., 2005).

Similarly, the Spanish group conducted a study where they tested the effect of DMSO, methanol and other cryoprotectants, with different dilution ratios and freezing medium supplementation with FBS, on European eel spermatozoa motility, viability and on spermatozoa head size (Garzón et al., 2008; Marco-Jiménez et al., 2006). Here, they found that viability for frozen-thawed eel spermatozoa with DMSO and methanol was similar, but the spermatozoa heads when cryopreserved in methanol medium were smaller than with DMSO. Furthermore, the researchers found a positive effect when the freezing medium was supplemented with FBS (25%). In a different study, a similar effect as spermatozoa membrane protector was found when adding L-α-phosphatidylcholine (Asturiano et al., 2007). However, this compound also increased the osmolality and density of the media, being therefore impractical to use.

Although valuable results were obtained following the protocol using DMSO in terms of percentage of spermatozoa motility, viability and spermatozoa head size, the use of this cryoprotectant still increased the medium osmolality resulting in inducing spermatozoa motility activation and

premature ATP consumption. To avoid this drawback, Peñaranda et al. (2009) tested different combinations of pH and $NaHCO_3$ concentrations. The use of $NaHCO_3$ was previously included in the Japanese eel sperm cryopreservation protocols, developed by Tanaka et al. (2002a,b) because of its inhibitory role on spermatozoa motility. Based on this feature, Peñaranda et al. (2009) developed an improved medium based on the P1 medium, but containing 100 mM $NaHCO_3$ and pH 6.5 that partially prevented the activation effect of DMSO. Furthermore, the researchers refined the protocol and used a 1:2 (sperm:freezing medium) dilution, and the 0.25 mL straws were cooled 1.6 cm above LN surface for 5 min before being immersed into LN. With this protocol, they obtained post-thaw spermatozoa motility values close to 40%, which is well sufficient for fertilization trials.

Following this last protocol, Asturiano et al. (2016) successfully used cryopreserved sperm in fertilization trials, to produce viable offspring. Despite the low percentage of fertilized eggs, lower than that observed with fresh sperm, embryos developed and a few larvae from cryopreserved sperm were obtained at 55 h after fertilization. Similarly, Müller et al. (2012; 2018) successfully used cryopreserved European eel sperm in fertilization trials, but in this case, they used Japanese eel eggs and successfully obtained hybrid larvae of *A. japonica x A. anguilla.* The sperm used in this fertilization trial was cryopreserved following a protocol based on those described by Müller et al. (2004) and Szabó et al. (2005), using a modified Tanaka solution (Tanaka et al., 2002a) as extender and methanol 10% as cryoprotectant (Table 1), obtaining progressive motility results in the thawed sperm samples of 12.3 ± 10.87%. Although the hatching rate was low, they demonstrated that the cryopreservation protocol worked successfully on the European eel sperm.

Despite the fact that cryopreservation protocols developed by the groups from Spain and Hungary were proved to succeed in fertilization trials, they differed in many aspects (Table 2) and a need of standardization of the protocol was evident. With this aim, both groups conducted together a joined study (Herranz-Jusdado et al., 2019a), where both protocols were tested using the same sperm samples. In this study, in addition to analysis of viability and motility in frozen-thawed samples, epigenetic effects of cryopreservation on spermatozoa DNA was also tested. Several studies

suggested that the drastic changes occurring during freezing and thawing may affect the DNA of cryopreserved spermatozoa (Labbé et al., 2017; Pérez-Cerezales et al., 2010). Furthermore, the use of methylated cryoprotectants is known to induce the production of reactive oxygen species (ROS) that can cause several damages, such as cytosine methylation in fish spermatozoa DNA (Kawai et al., 2010), which is one of the principal epigenetic mechanisms (Bird, 2002), and have been suggested to be a good indicator for sperm quality (Herráez et al., 2017), affecting consequently the success of a cryopreservation protocol. In this comparative study, Herranz-Jusdado et al. (2019a) showed that the protocol using methanol, initially developed by the Hungarian group (Müller et al., 2012; Szabó et al., 2005), was better in terms of higher spermatozoa viability and motility than the protocol with DMSO developed by the Spanish group (Asturiano et al., 2016; Peñaranda et al., 2009). Furthermore, the protocol with DMSO induced a hypo-methylation of them spermatozoa DNA, whereas no changes in DNA methylation were observed when sperm was cryopreserved with the protocol with methanol.

The most recent work on the European eel sperm cryopreservation aimed at the improvement of the protocol by using sperm membrane protection additives and to adapt the protocol to larger volumes (Herranz-Jusdado et al., 2019b) as done with Japanese eels (Nomura et al., 2018). In this latest work, using the protocol described by Herranz-Jusdado et al. (2019a), the researchers successfully scaled up the volume of sperm cryopreserved using 2 and 5 mL cryotubes, by adapting the cooling rate. Furthermore, adding egg yolk to the extender solution, they improved the frozen-thawed sperm quality, reaching motility values over 50%, which are the highest motility ever reported in cryopreserved European eel sperm. These improvements in the protocol represent a great advance for future large-scale reproduction programs in European eel.

4. Vitrification

Vitrification is a cryopreservation technique that leads to a glass like-solidification while preventing intracellular and extracellular ice crystallization, that has been proposed as an alternative to traditional

cryopreservation (Tavukcuoglu et al., 2012). Although exists several methods for vitrification (Katkov et al., 2006), its application with fish sperm is typically based on the combined use of high concentrations of cryoprotectants and fast cooling rates (Magnotti et al., 2018a). The use of vitrification of fish spermatozoa is a relatively new application, however, it has been already tested on sperm of several fish species (Xin et al., 2017). The success of sperm vitrification depends on several factors, including initial sperm quality, type and concentration of cryoprotectants, equilibration time and cooling and warming rates (Tsai et al., 2015). Normally, the concentration of cryoprotectants used must be very high to prevent the formation of ice crystals during the fast cooling process, but can be toxic to the cells. Therefore, finding a proper cryoprotectant and its concentration is critical to develop new sperm vitrification protocols (Magnotti et al., 2018a).

Recently, a new vitrification protocol has been developed for European eel sperm (Kása et al., 2017). The protocol consisted in a sperm:diluent ratio of 1:1, with 40% cryoprotectant (20% methanol and 20% propylene glycol), and 10% FBS using Cryotops of 2 μL as cooling device. The percentage of spermatozoa motility obtained from this vitrification protocol was low compared to conventional sperm cryopreservation. However, this was the first protocol described for European eel, proving the feasibility of this technique with European eel sperm.

5. Conclusion and future remarks

Since first was developed an eel sperm cryopreservation protocol in the early 2000´s, lots have been changed and improved. The latest protocols for sperm cryopreservation of European and Japanese eel use methanol as cryoprotectant and they have been adapted to large volumes. In the case of the protocol for Japanese eel sperm, successful fertilization have been achieved and with similar survival rates as with fresh sperm. Moreover, the morphology of the larvae produced with cryopreserved sperm was similar as larvae produced from fresh sperm. In the case of the protocol for European eel sperm, the latest protocol has not been tested for fertilization trials yet, but the motility of frozen-thawed sperm obtained was over 50%, which is the

highest ever obtained in this species and future studies should aim to test whether is suitable for large-scale fertilizations. Moreover, future work should aim to investigate the effect of large periods of cryogenic storage (over 2 years) on eel sperm.

Acknowledgements

GENERAL DISCUSSION

1. Alternative methods for European eel artificial maturation

The natural population of European eel has drastically decreased in the last decades. To reverse this tendency, it is necessary immediate correcting measures, being the control of artificial reproduction crucial to reduce the pressure on natural populations and to fulfill the demands of eel farms (Feunteun, 2002). To develop breeding captivity programs, the first step is to obtain high quality gametes, which is crucial to reach high fertilization and hatching rates (Bozkurt and Secer, 2006).

In European eel, the hormonal treatments required to obtained `high quality gametes, and in particular good quality sperm in males, have been developed since the middle 20th century (Fontaine, 1936), and later improved and standardized by the use of weekly injections of hCG (Asturiano et al., 2005; Gallego et al., 2012; Pérez et al., 2000). This method using weekly injections of hCG, has also shown successful spermiation results in other eel species such as Japanese eel, American eel, shortfinned eel and longfinned eel (Lokman and Young, 2000; Ohta et al., 1997; Sorensen and Winn, 1984). In this thesis, alternative hormonal methods for European eel maturation have been tested aiming to improve several male reproductive traits such as the onset and the duration of the spermiation period and the quantity and quality of sperm (see Chapter 2). Here, we tested the effect of two different hormonal treatments, purified hCG and hCGrec. The results obtained indicated that the recombinant hormones produced better sperm in terms of quality and quantity than purified hormones, and the optimal dose was 1.5 IU/g fish, since lower doses produced lower quality sperm.

These results are consistent with previous work of Gallego et al. (2012) that compared a protocol using hCGrec with a protocol using a different brand of purified hCG than the one tested in this thesis. The different hormonal treatments tested possess different glycosylation profiles that affect the bioactivity of the gonadotropins (Hearn and Gomme, 2000; Ulloa-Aguirre et al., 1999). Furthermore, the advantages of using recombinant gonadotropins include that in their production, the protein resulted is more pure and safe, and its production is not dependent on urine collection and hormone

extraction, avoiding contamination during the purification (Thennati et al., 2018).

Although the protocol have been improved and standardized here (see Chapter 2), there are still aspects that may be optimized. One aspect of the protocol that is susceptible of being improved is the type of recombinant hormones. The hormones used here are heterologous, and although they have successfully been used to induce eel maturation obtaining good quality sperm, their use also have been linked to low rates of fertilization and hatching (Palstra and van den Thillart, 2009). Therefore, recent studies in teleost reproduction have been focused on the development of homologous recombinant gonadotropins (Chauvigné et al., 2012; Hayakawa et al., 2008; Molés et al., 2011; So et al., 2005; Zmora et al., 2007).

Following this line of studies, Kazeto et al. (2014) succeeded in producing homologous recombinant gonadotropins of Japanese eel synthetized from cell lines of Chinese hamster ovary. Soon after, Ohta et al. (2017) developed a protocol for Japanese eel maturation consisting of weekly injections of recombinant LH at a dose of 500 µg/kg fish, that induced a high volume of spermiation and fast stimulation of spermatogenesis. This maturation method has been successfully used in fertilization trials with positive results (Nomura et al., 2018). In European eel, Peñaranda et al. (2018) also tested homologous recombinant LH and FSH, which were again obtained by transfection of mammalian cells of Chinese hamster ovary. They treated immature European eels with weekly injections of recombinant LH and FSH and successfully induced full spermatogenesis and spermiation *in vivo*. Nonetheless, there were high variations in sperm quality among treated males, perhaps due to the low doses of recombinant gonadotropins used compared to those used by Ohta et al. (2017) with Japanese eel. Moreover, the price of the treatment was too high to make the method sustainable. Therefore, further work using European eel recombinant hormones are required to improve the present hormonal treatments.

Another aspect of the maturation protocol susceptible of improving is the hormonal administration method. The method used here requires weekly injections that involve repetitive handling of the animals, which demand

substantial labor, time, and monitoring cost. Moreover, the intense manipulation causes stress and increases mortality of the fish. Therefore, the use of alternative hormonal delivery methods may improve the actual protocol. For this purpose, there are several available hormonal delivery methods such as solid implantable pellets of cholesterol (Weil and Crim, 1983) or Ethylene-Vinyl Acetate (EVAc) (Mylonas et al., 2007) or in the form of biodegradable microspheres (Mylonas et al., 1995). Each method has shown specific advantages, and all have been proved to effectively deliver the hormones in different fish species. Different implant treatments have already been tested in eel species. For instance, Lokman et al. (2015) showed that pretreatment of shortfinned eels with androgen implants in females improved greatly the maturation protocol, resulting in less handling, less hormone and less time. Moreover, Kagawa et al. (2009; 2013) used an osmotic pump as an effective method for inducing vitellogenesis in female Japanese eels. The success obtained using implants or osmotic pumps in eel species, infer that these may be good alternative delivery methods for the hormonal treatment of European eel.

2. Sperm quality analysis: Future remarks

The success of artificial reproduction of fish species including the validation of cryopreservation protocols, depend greatly on the accurate evaluation of the sperm quality, which is the best way to define the fertility potential of males (Gallego and Asturiano, 2018a; Kime et al., 2001; Rurangwa et al., 2004). There are several characteristics of semen that contribute to define the sperm quality, however, sperm motility is considered the best biomarker and shows high correlation with fertilization and hatching ratios in some fish species (Gallego and Asturiano, 2018b). In Chapter 1 of this thesis, it has been described the wide variation existing when the analysis of sperm motility is conducted subjectively, and how the experience of the observer affects the sperm motility estimation. The results in Chapter 1 suggest that using CASA-Mot systems to assess sperm quality in fish is the only reliable method to compare results between research groups and it is crucial to assess accurate sperm quality evaluation. CASA-Mot analyses supply data of several sperm

parameters in addition to sperm motility, that improve the method to assess sperm quality (Gallego and Asturiano, 2018b).

Although the use of CASA-Mot to assess sperm quality has been widely accepted as an accurate, reliable and objective method to assess sperm quality, it is important to describe thoroughly the settings used, in both hardware and software, for the analysis. This is important for several reasons, but mainly to be able to replicate experiments and to compare results between different experiments (Verstegen et al., 2002), since different settings may directly influence the results from CASA-Mot. In this matter, Caldeira et al. (2019) recently reviewed the optimal settings for analyzing European eel sperm with CASA-Mot. In their study, they showed that using low recording frame rates when analyzing European eel sperm, led to underestimation of real kinetic trait values. Particularly the VCL was highly affected when using the optimal frame rate (200 fps) compared to low frame rate (60 fps) recordings.

Alike the effect of the frame rates, the temperature also seems to play a key role in the motility of eel sperm, affecting their kinetic traits. It has been observed in several fish species that temperature affects speed and duration of sperm movement (Bobe and Labbé, 2009). The record of the temperature of analysis when using CASA-Mot should be included in every experiment. In this thesis, all sperm analyses with CASA-Mot were performed using sperm samples and activation medium (seawater) at 4 ºC, however, the microscope is placed at room temperature and immediately warms the samples, being the real temperature at which the sperm was analyzed uncertain. Future work should study how temperature affects sperm motility and velocities, and which is the optimal temperature of analysis for European eel sperm.

Additionally to motility and kinetic parameters analyzed with CASA-Mot, there are other parameters that offer additional information to accurately assess sperm quality in fish for instance spermatozoa viability, mitochondrial activity, DNA integrity and membrane fluidity. These parameters have been typically studied using live/dead staining method, counting live and dead spermatozoa (differentiated by colors) under the microscope (McNiven et al., 1992) or using a more advanced method such as the flow cytometry

(Cabrita et al., 2005; Gillan et al., 2005). These parameters are particularly relevant in cryopreservation studies since the plasma membranes of spermatozoa are very sensitive to cryoinjury from ice crystals formed during the freezing-thawing cycle that may affect the spermatozoa structures. In this thesis, these analyses have been included in the chapters that addresses sperm cryopreservation (Chapters 3 and 4), providing information on the success of the different cryopreservation protocols.

Additionally to analyses with flow cytometry, effects on sperm DNA have been studied further in Chapter 3. The effects of the extreme low temperatures and chemicals (cryoprotectants) required in the cryopreservation process may provoke epigenetic changes in the spermatozoa DNA (Labbé et al., 2017). Here we showed that cryopreservation of European eel sperm using DMSO as cryoprotectant, lead to hypo-methylation of DNA. Since phenotype is resulting from the genotype associated with epigenetic information, and the spermatozoa carries both the genetic and the epigenetic information that will be transferred to the offspring. Hence, epigenetic alteration should be avoided in any reproduction program (Labbé et al., 2017).

However, there are still several questions regarding the epigenetic effects resulted from cryopreservation technology. In this thesis, the analysis consisted in a Luminometric methylation assay (LUMA) that analyzed sperm global methylation levels, but other analysis showing genome areas more sensitive to epigenetic changes, or individual genes affected by the methylation would contribute to understand better the epigenetic effects. Moreover, further studies tracking the consequences on the offspring should be considered.

3. Prospects for European eel cryopreservation

The main objective of this thesis was the development and improvement of different techniques and protocols for European eel sperm cryopreservation. This has been covered in two chapters and reviewed in one more of this thesis where first the standardization (Chapter 3) and then improvement (Chapter 4) have been addressed. Further, Chapter 5 has reviewed within a

historical perspective, the evolution and development of the different cryopreservation protocols. In chapter 3, we standardize the cryopreservation protocol from the pre-existing protocols developed by the Spanish and the Hungarian groups. The Hungarian protocol (Müller et al., 2012; Szabó et al., 2005), that used methanol as cryoprotectant, was better in terms of spermatozoa viability and motility than the protocol with DMSO developed by the Spanish group (Asturiano et al., 2016; Peñaranda et al., 2009). In chapter 4, we successfully scaled up the volume of sperm cryopreserved using 2 and 5 mL cryotubes, by adapting the cooling rate. Furthermore, adding egg yolk to the extender solution, the frozen-thawed sperm quality was improved, reaching motility values over 50%, which are the highest motility ever reported in cryopreserved European eel sperm.

Although this last protocol has shown the best outcome quality to date, still it is susceptible of improvement. Firstly, this protocol uses egg yolk as an additive, and although its role as sperm protectant during freezing and thawing is still poorly understood, it is hypothesized that egg yolk supplies lipids and proteins to the extender medium that act as spermatozoa membrane stabilizer, becoming more resistant to cryoinjuries (Aboagla and Terada, 2004). The use of egg yolk from commercial hen eggs has the problem that the composition vary depending on the strain and size of the eggs (Ahn et al., 1997), and therefore it is difficult to standardize its use. Thus, future work using the low-density lipoprotein (LDL) fraction of egg yolk should be evaluated since positive results have been described in other fish species (Pérez-Cerezales et al., 2010) and it possible to control the accurate proportion of LDL used.

Another interesting additive that may be tested in a future in cryopreservation of European eel sperm, are the anti-freeze proteins (AFP). These proteins that are naturally found in arctic fish species, binds to water molecules preventing ice crystal formation, and interact with sperm membranes protecting them of cryoinjuries. There are three types of AFP (I-III) and a glycoprotein (AFGP) that have been tested as cryopreservation additives in fish sperm, eggs and embryos of fish and mammals with different results (Kim et al., 2017; Robles et al., 2019). In fish, Beirão et al. (2012) showed that using AFPs, mainly AFPIII, in the extender solution improved the

cryopreservation protocol for gilthead seabream sperm, decreasing the loss of sperm quality and helping maintain the lipid composition of the plasma membrane. In a more recent study, Shaliutina-Kolešová et al. (2019) in their work with common carp sperm cryopreservation obtained similar results where AFPs (I and III) showed a dose dependent protective effect to frozen-thawed sperm.

Alternatively to sperm cryopreservation, the cryopreservation of eel oocytes may be an interesting option to preserve genetic material and future artificial reproduction of European eel. Cryopreservation of fish oocytes is less developed than cryopreservation of fish sperm, partially due to the intrinsic difficulties for freezing oocytes, i.e. larger cell volume, lower permeability to cryoprotectants, high chilling sensitivity or the presence of chorion. However, several studies have been successfully carried out mainly on model species such as zebrafish (*Danio rerio*) (Godoy et al., 2013), but also on marine species such as gilthead seabream (*Sparus aurata*) (Zhang et al., 2007) and on some South American freshwater species (Streit Jr. et al., 2014). In recent years, the cryopreservation of oocytes have been mainly focus on the development of slow freezing cryopreservation and vitrification of early oocyte developmental stages such as stage I and stage II, since at these stages have been obtained the best results. However, these oocytes will need to undergo *in vitro* maturation, ovulation, and fertilization, after cryogenic storage. Therefore, the optimization of the protocols for cryopreservation and for *in vitro* maturation of fish ovarian follicles, represent the future challenges for further development of these techniques (Martínez-Páramo et al., 2017).

Besides these alternative methods, the logical next work on European eel sperm cryopreservation should aim to test whether the latest protocol described in this thesis is suitable for large-scale fertilization trials. Moreover, future work also should aim to investigate the effect of large periods of cryogenic storage (over 2 years) on eel sperm, since the use of cryopreserved sperm for cryobanking should maintain sperm quality intact through a long period.

CONCLUSIONS

i.   The subjective evaluation of sperm quality was proved to be dependent on the technicians' degree of expertise. Therefore, the use of qualified technicians and CASA-Mot systems is critical for obtaining reliable results of European eel sperm quality.

ii.   The spermiation process in European eel males was strongly affected both by hormonal dose and type, and an effective and economically profitable treatment was established, improving the reproducion performance of eel males.

iii. A simple short-term sperm storage method for European eel sperm was developed, by adjusting the dilution rate in extender solution and the storage temperature, preserving the sperm for up to 7 days.

iv.   The cryopreservation protocol of European eel sperm was standardized after compareing two protocols resulting more effective the protocol using methanol as cryoprotectant. Moreover, the use of DMSO as cryoprotectant was proved to induce negative epigenetic effects in cryopreserved eel sperm.

v.   The previously standardized cryopreservation protocol was adapted to larger volumes (up to 5 mL) without losing frozen-thawed sperm quality compared to smaller volumes.

vi.   The European eel sperm cryopreservation protocol for large volumes was improved by adding egg yolk obtaining the best quality frozen-thawed sperm ever reported for European eel.

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