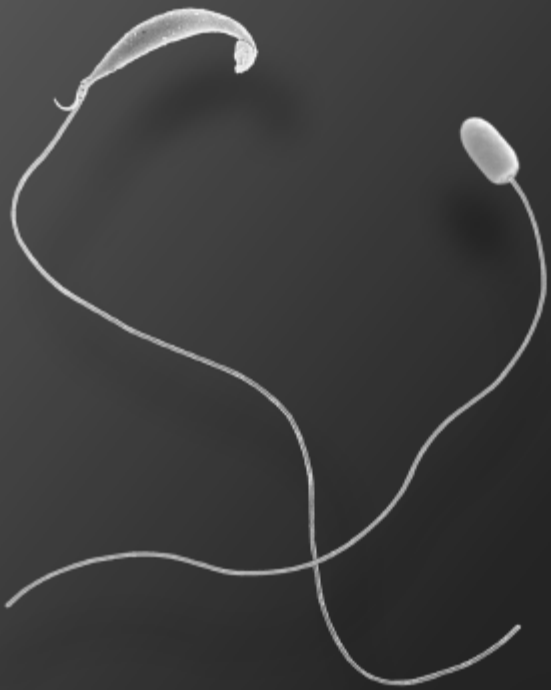
SPERM PHYSIOLOGY AND QUALITY IN TWO MARINE TELEOSTS:

Anguilla anguilla & Takifugu niphobles



Ph.D. Thesis
Víctor Gallego
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SPERM PHYSIOLOGY AND QUALITY IN TWO MARINE TELEOSTS: *Anguilla anguilla* & *Takifugu niphobles*

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Por último, he tenido la suerte de disfrutar de 3 estancias durante la realización de esta tesis, gracias a las cuales he podido conocer a gente de la que nunca podré olvidarme. En León, me quedo con dos ardillas que me hicieron sentir como un leones más!!! (y eso que Martuki es del Bierzo y Carmencilla de Valladolid)… muchas gracias por esos cortos, esas espichas y esas excursiones a la montaña!!! Supongo que cuando estéis leyendo esto las dos estaréis aquí… así que me tocara a mi sacaros a beber agua de valencia!!! Yupiiiiiii!!!! En Japón, nada hubiera sido igual sin mi novio Gareth (un apuesto y guapo gales), que estuvo de compartiendo el día a día conmigo en la estación biológica y gracias al cual los días fueron menos duros… Thanks so much for your kidness and your friendship man, I found a friend and I think I would not have survived in the station without you!!! We enjoyed a lot of great moments, overall in our travel around Japan… I will never forget it!! I miss you putiniiiiiiii!! (ahora entendéis lo de novio no??... jjjjj). Además, en Japón fui feliz gracias a tres grandes amigas que descubrí allí: Ayako, Megu y Nori-chan. Es difícil de explicar, pero cuando no tienes nada y te dan algo (incluso una sonrisa al decirte los buenos días), te sientes la persona más feliz del mundo.

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SUMMARY

The conservation status of the species studied in this thesis (European eel and pufferfish) is currently frail, thus the main goal of this research was to develop, improve and apply several techniques and protocols with the aim of increasing the knowledge about their sperm biology, improving their reproductive performance and even helping future breeding in captivity.

The reproductive performance of the males is often assessed through the sperm motion parameters analysed by the CASA system, so first we focused on how to standardize this technique in terms of procedural and biological settings. In this respect, we laid the foundations for applying a standard method to assess sperm quality in fish, making it possible for sperm studies to be compared both intra- and inter-laboratories using the proper CASA settings.

Secondly, with the aim of improving the reproductive performance of European eel males, 3 thermal regimes (two of them variable: T10 and T15; and one of them constant: T20) and 3 hormonal treatments (hCG, hCG_{rec} and PSMG) were assessed based on different sperm quality parameters. In the case of the thermal regimes, our results demonstrated that the onset and progression of spermiation are strongly influenced by water temperature, with treatment T20 showing the best results in all the sperm quality parameters. In the case of hormonal treatments, hCG_{rec} produced the best results in all the sperm quality parameters, becoming an economically profitable/viable treatment and an effective alternative to the standard hCG treatment often used to induce spermiation in eel species.

A preliminary physiological study regarding the changes to the main ions involved in the fish sperm activation process was carried out. Our results showed that intracellular concentrations of Ca^{2+} and K^{+} increased upon eel sperm activation, while pH gradually decreased over time, thus it is likely that all of them play an important role in the initiation of sperm motility in the European eel, as with other marine and freshwater teleosts.

In the second part of this thesis, which focuses on the pufferfish, an in-depth study into the sperm of this species was carried out for future application in aquacultural matters. A short-term storage method for pufferfish sperm was developed, enabling us to preserve the sperm quality parameters for a relatively long time period (7 days) compared to fresh sperm samples. Moreover, the effects of both the osmolality and the ion composition of the activation media on the sperm motion parameters were evaluated, concluding that both factors play an essential role in the initiation of sperm motility of pufferfish sperm and probably, in marine fish sperm.

Finally, *in vitro* fertilization trials were developed to assertain how the quantity and quality of male gametes affects the fertilization and hatching rates. We demonstrated that sperm/egg ratio and sperm quality are strongly related factors, suggesting that both should be taken into account as unique interrelated elements. In addition, coefficients of correlation among all the spermatozoa motion parameters provided by a CASA system and fertilization and hatching rates were estimated for the first time in a marine species. Spermatozoa velocities showed the highest coefficients (≥0.80), suggesting that the kinetics of the spermatozoa are a key factor in the fertilization process.

RESUMEN

Las especies objeto de estudio de esta Tesis Doctoral (anguila europea y pez globo) se encuentran en un delicado estado de conservación, por lo que el objetivo principal de este trabajo fue el desarrollo, la mejora y la aplicación, de varias técnicas y protocolos para aumentar el conocimiento sobre la fisiología de su esperma, mejorando distintos aspectos reproductivos y ayudando a una futura cría en cautividad.

El rendimiento reproductivo de los machos suele evaluarse mediante la medida de parámetros de motilidad espermática usando sistemas CASA, por lo que el primer objetivo fue estandarizar esta técnica, tanto en parámetros técnicos como biológicos. De esta manera, se han establecido las bases para poder evaluar de una manera estandarizada y precisa los parámetros espermáticos, haciendo posible la comparación de los análisis tanto inter- como intra-laboratorios.

En segundo lugar, y con el objetivo de mejorar el proceso de inducción a la maduración de los machos de anguila europea, 3 regímenes térmicos (dos de ellos variables: T10 y T15, y uno de ellos constante: T20) y 3 tratamientos hormonales (hCG, hCGrec y PSMG) fueron evaluados en base a diferentes parámetros de calidad espermática. La aparición y progresión de la espermiación resultó fuertemente afectada por la temperatura del agua, y el tratamiento T20 generó los mejores resultados en todos los parámetros de calidad espermáticos. En el caso de los tratamientos hormonales, hCG_{rec} generó los mejores resultados, pasando a ser una alternativa rentable y eficaz al tratamiento estándar de hCG utilizado habitualmente para la maduración de machos de anguila.

A continuación, y completando el estudio en machos de anguila europea, se llevó a cabo un estudio fisiológico sobre los cambios que se producen en las concentraciones intracelulares de los principales iones implicados en la activación del esperma. Los resultados mostraron que, tal y como ocurre en otras especies marinas, las concentraciones intracelulares de Ca^{2+} y K^{+} aumentaron tras la activación, mientras que el pH mostró una disminución

gradual, sugiriendo un papel importante de estos iones durante el proceso de activación de los espermatozoides.

Durante la segunda parte de esta Tesis, centrada en el pez globo, se llevó a cabo un amplio estudio sobre diferentes aspectos del esperma de esta especie que permitió, en primer lugar, el desarrollo de un método capaz de preservar el esperma de esta especie durante un periodo relativamente largo de tiempo (7 días), sin diferencias significativas respecto a los parámetros de calidad de las muestras en fresco. Por otra parte, se evaluaron los efectos que la osmolaridad y la composición iónica del medio de activación tienen sobre los parámetros espermáticos, concluyendo que ambos factores juegan un papel esencial en la iniciación de la motilidad del esperma en pez globo y, probablemente, en el esperma de teleósteos marinos.

Por último, se realizaron distintos ensayos de fertilización *in vitro* con el objetivo de conocer el efecto que la cantidad y la calidad del esperma tienen en las tasas de fertilización y eclosión en esta especie. El ratio huevo:esperma y la calidad del esperma resultaron factores estrechamente relacionados, por lo que ambos deberían ser tenidos en cuenta como un único elemento multi-factorial en ensayos de fertilización. Además, los coeficientes de correlación entre todos los parámetros espermáticos obtenidos mediante un sistema CASA y las tasas de fertilización y eclosión fueron estimados por primera vez en una especie marina. Los parámetros de velocidad presentaron los coeficientes más elevados (≥0,80), sugiriendo que la cinética de los espermatozoides es un factor clave en el proceso de fertilización.

RESUM

Les espècies objecte d´estudi d´aquesta Tesi Doctoral (anguila europea i peix globus) gaudeixen actualment d´un fràgil estat de conservació, doncs l´objectiu principal d´aquest treball fou el desenvolupament, la millora i l´aplicació de vàries tècniques i protocols per incrementar el coneixement sobre la fisiologia del seu esperma, millorant diferents aspectes reproductius i col·laborant per a una futura cria en captivitat.

El rendiment reproductiu dels mascles és habitualment avaluat mitjançant paràmetres de motilitat espermàtica a través de un sistema CASA, doncs el primer objectiu va ser estandarditzar aquesta tècnica, tant en aspectes tècnics como biològics. D'aquesta manera, s´han establit les bases per a poder avaluar d'una manera estandarditzada i precisa els paràmetres espermàtics en esperma de teleostis marins, fent possible la comparació de resultats tant inter- como intra-laboratoris.

En segon lloc, i amb l´ objectiu de millorar la inducció de la maduració en mascles d´anguila europea: 3 règims tèrmics (dos d´ells variables: T10 i T15, i un d´ells constant T20) i 3 tractaments hormonals (hCG, hCGrec i PSMG) van ser avaluats a través de diferents paràmetres de qualitat espermàtica. L´inici i la progressió de l'espermiació va ser afectada per la temperatura de l´aigua, on el tractament T20 va generar els millors resultats en tots el paràmetres de qualitat de l´esperma. En relació amb els tractaments hormonals, hCG_{rec} va generar els millors resultats, passant a ser una alternativa rentable i eficaç al tractament estàndard de hCG utilitzat habitualment per a la maduració de mascles en aquesta espècie.

Completant l´estudi en mascles d´anguila europea, es va fer un estudi en relació als canvis en les concentracions intercel·lulars dels principals ions implicats en la activació de l´esperma. Els resultats van mostrar que, al igual que succeeix en altres espècies marines, les concentracions intracel·lulars de Ca^{2+} i K^{+} van augmentar després de la activació, mentre que el pH va presentar una disminució gradual, suggerint una funció important d'aquests ions durant el procés d´activació dels espermatozoides.

Durant la segona part d'aquesta tesi, centrada en el peix globus, es va fer un ampli estudi sobre diferents aspectes de l´esperma d'aquesta espècie que va permetre, en primer lloc, el desenvolupament d'un mètode capaç de conservar l'esperma durant un període de temps relativament llarg (7 dies), sense diferències significatives respecte als paràmetres de qualitat de les mostres control. D´altra banda, es van avaluar els efectes que la osmolaritat i la composició iònica del medi d'activació tenen sobre els paràmetres espermàtics, concloent que tots dos juguen un paper essencial en la iniciació de la motilitat de l´esperma en el peix globus i, provablement, en l´esperma de teleostis marins.

Finalment, es van realitzar diferents assaigs de fertilització *in vitro* amb l'objectiu de conèixer l´efecte que la quantitat i la qualitat de l´esperma tenen en les taxes de fertilització i eclosió en peix globus. El rati ou:esperma i la qualitat de l'esperma van ser factors estretament relacionats, doncs tot dos deurien de ser considerats com un únic element multi-factorial en assaigs de fertilització. A més, els coeficients de correlació entre tots els paràmetres espermàtics obtinguts a través de un sistema CASA i les taxes de fertilització i eclosió van ser estimats per primera vegada en una espècie marina. Els paràmetres relacionats amb la velocitat presentaren els coeficients més elevats (≥0,80), suggerint que la cinètica de l´espermatozoide és un factor clau en el procés de fertilització.

GENERAL INTRODUCTION

1. Reproductive biology of goal species

A wide diversity of reproductive strategies and fertilization modes can be found in marine fish (Serrao and Havenhand, 2009). Most of them share a common fertilization strategy, broadcast spawning, in which gametes both from males and females are released into the seawater. However, despite this common reproductive link, fish species have evolved in response to several sets of selective pressures, so they often differ in terms of their key reproductive traits. In this respect, having in-depth knowledge of each life-history seems to be essential in order to understand the sperm biology of each species.

The conservation status of the goal species of this thesis is currently frail. The European eel (*Anguilla anguilla*) is classified as a Critically Endangered Species according to the International Union for Conservation of Nature (IUCN) Red List while, in the case of the pufferfish (*Takifugu niphobles*), the current data about its population are deficient and several factors such as habitat destruction or climatic change could contribute to making it anendangered species. Therefore, the chapters in this thesis have a common link: the development and improvement of different techniques and protocols with the aim of increasing the knowledge about the sperm biology of both species, thereby improving the reproductive performance and even helping future breeding in captivity.

1.1 European eel

The European eel (*Anguilla anguilla*) is a marine fish belonging to a primitive group called elopomorpha which appeared in the early evolution of teleosts. Nowadays, the genus *Anguilla* is composed of 15 species (Watanabe, 2003), which are widely distributed around the world inhabiting tropical, subtropical and temperate areas.

The European eel species displays an interesting catadromous life history strategy, and the mystery has been always linked to its biological cycle. In this respect, the first surmise was reported by Aristotles around 350 BC,

who postulated that “the eels come from what we call the entrails of the earth”. A few centuries later, some discoveries of eel larvae at different wild locations allowed new hypothesis to be made and finally, thanks to the marine expeditions of the early 20th century conducted by Johannes Schmidt, the central mystery of the eel breeding location was elucidated. Schmidt recorded data of over 10.000 European eel larvae over a period of 25 years, and through a spatio-temporal study of the larvae distribution he discovered the breeding location of this specie: the Sargasso Sea (Schmidt, 1923). The hypothesis that all European eel migrate to the Sargasso Sea for reproduction is called the panmixia theory, and until recently has been broadly accepted. However, based on field observations, morphological traits and molecular studies there are some indications that Schmidt’s claim of the complete homogeneity of the European eel population and a unique spawning location may be an overstatement (van Ginneken and Maes, 2005).

Nowadays it is known that mature adults leave the continental waters depending on the lunar phase and atmospheric conditions (Tesch, 2003). They swim southward between 5000-6000 km using the Atlantic currents and arrive at the Sargasso Sea 6-7 months later, where they spawn and die. Eel larvae, called leptocephali, start the 8-9 month return journey to the eastern Atlantic coast transported along the Gulf Stream and actively swimming (Lecomte-Finiger, 1994). Near the coast, the leptocephali metamorphose into glass eels, swim upstream rivers and grow to partial maturity. Once in their final habitat, eels can live a long time, depending on their sex: 5-8 years for the males and 8-20 years for the females. Finally, pubertal eels return to the sea for the reproductive migration (Figure 1).

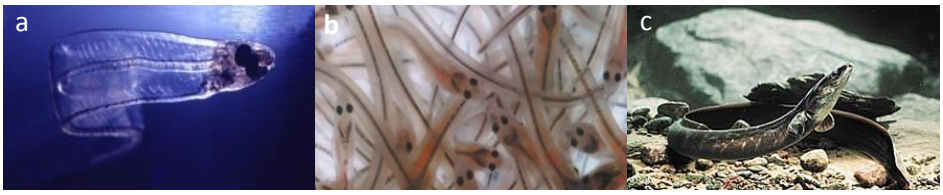

Figure 1. Pictures of European eel metamorphosis: a) leptocephali; b) glass eel; and c) adult eel.

1.2 Pufferfish

The pufferfish (*Takifugu niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean. This species belongs to the tetraodontine genus *Takifugu*, composed of 24 species known as *fugu* species. All of them have a common dangerous peculiarity: they contain a lethal tetrodotoxin, stored basically in the liver and ovaries, which is able to paralyze the muscles until eventual death from asphyxiation. However, despite this clear disadvantage, the *fugu* species has currently become a delicious delicacy in Japan.

Regarding its reproductive biology, the pufferfish displays a unique and sophisticated reproductive strategy. Before spawning, hundreds and even thousands of pufferfish gather together at the beach around the new moon during the spawning season, which occurs between June and July (Yamahira, 1996). Spawning begins 2 hours before sunset, and each spawning is conducted by a group of 10-60 males and includes only 1 female (Nozaki et al., 1976; Figure 2).

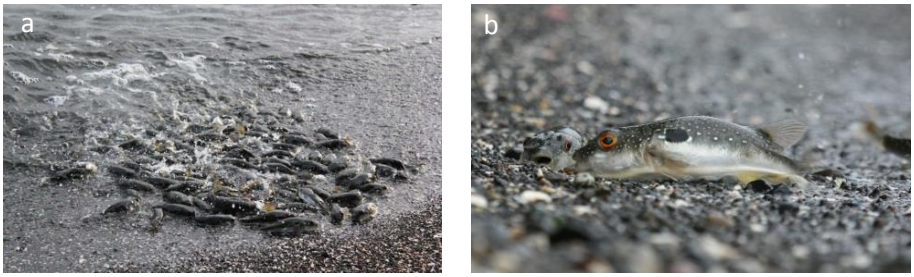

Figure 2. Pictures of spawning behaviour of pufferfish: a) males and females leaving from the seawater; b) female releasing the eggs.

During the spawning period, pufferfish leave the water at times using an incoming wave and at that moment both sexes release the gametes simultaneously. The fertilization process takes just a few seconds. However, this group spawning is repeated several times within a spawning day. Finally, fertilized eggs are dragged into the sea by a wave, and they begin their development.

2. Sperm physiology in fish

Fish are the most diverse and numerous group of vertebrates (Helfman et al., 2009). However, our knowledge of fish spermatogenesis is limited to a few species used in basic research and/or in aquaculture biotechnology such us guppy, medaka, salmon, eel, etc. (Schulz et al., 2010).

In basic terms, spermatozoa are the result of a maturation process, called spermatogenesis, in which several haploid cells are produced with the aim of transmitting paternal genetic information to the next generation. During spermatogenesis, the non-functional gametes pass through the spermiduct, turn into mature spermatozoa, and finally they become cells specifically designed to reach and fertilize the oocyte. Spermatogenesis involves both morphological and physiological changes, where sex steroids such as estrogens, androgens, and progestagens regulate the complete progression thereof (Peñaranda et al., 2010a). The duration of this process can take from a few weeks to several months, and is usually shorter in fish than in mammals (Cheng and Mruk, 2013). In addition, spermatogenesis is also influenced by several external factors such as temperature, and the development of this process under optimum species-specific conditions allows the production of high quality gametes. Therefore, understanding the reproductive strategy and reproductive cycle in relation to environmental conditions seems essential for the development of proper protocols to control maturation and spawning in fish species.

2.1 Factors determining sperm activation

During the whole spermatogenesis process, fish spermatozoa remain in a safe environment, surrounded by Sertoli and Leydig cells and later floating in their own fluid, the seminal plasma. At this stage, the sperm cells are quiescent but already prepared for accomplishing their fertilizing task (Fauvel et al., 2010). However, sperm activation will be an essential requirement in the pursuit of female gametes, and spermatozoa exploit their swimming ability in order to encounter the oocyte as fast as possible (Cosson et al., 2008a).

Sperm activation is a diverse and complex process in the animal kingdom (Ward and Kopf, 1993). In mammals, spermatozoa are partially activated in their passage through the epididymis, where some molecules are secreted causing the partial initiation of sperm motility. Finally, mammalian spermatozoa will acquire their maximum motility values when they approach the oocyte, interacting with some ions and proteins (Baldi et al., 1996). However, due to the fact that aquatic species have a radically different reproductive strategy, the sperm displays a completely different activation mechanism, and the gametes are usually activated when they are released into the aquatic environment (Morisawa and Suzuki, 1980).

With regards to teleost fish, when the sperm remain quiescent in the seminal plasma, there are three different activation sperm pathways (Figure 3, Morisawa et al., 2008). Regarding seawater and freshwater species, the sperm becomes motile when in contact with hyper- or hypo-osmotic solutions, respectively. Thus, osmolality seems to be the key factor which triggers sperm activation in these species. However, regarding salmonids, it has been suggested that high concentrations of K^+ in the seminal plasma suppresses sperm motility in the male reproductive tract so, in this case, the release of spermatozoa in a K^+ deficient environment would be the trigger for sperm motility.

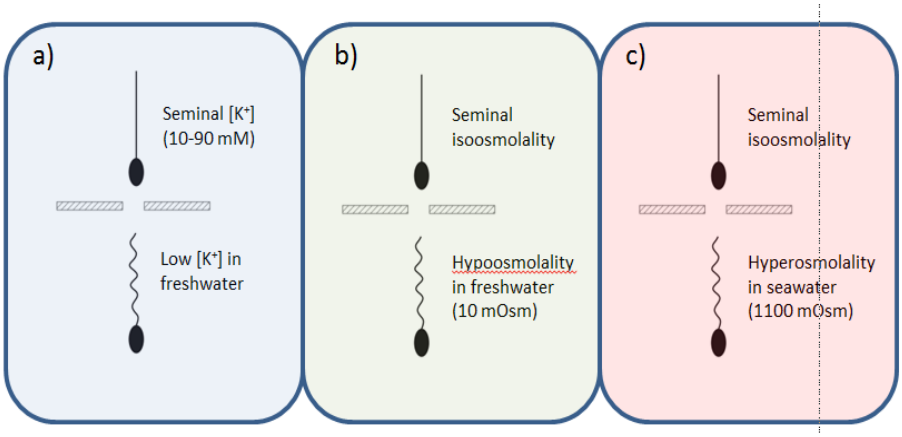

Figure 3. Schematic illustration showing how spermatozoa of salmonids (a), freshwater fish (b) and marine fish (c) initiate the motility.

Finally, the activation process in some marine invertebrates deserves a special mention. It has been reported that the activation of sperm motility in some ascidians or sea urchins is directly induced by signals released from the egg or egg coating and not by the release of the gametes into the environment (Yoshida et al., 1993). In this respect, for sea urchin or ascidian spermatozoa, finding an egg to fertilize in a vast ocean might seem like looking for a needle in a haystack, and some external fertilizers such as those cited above have devised an effective strategy in order to overcome this problem: the eggs are able to release chemical elements that guide the sperm towards them (Kaupp et al., 2006). During this process, the spermatozoa are able to detect them and orient their swimming direction up the concentration gradient towards the oocyte, thus fulfilling the task for which they were designed, to fertilize the oocyte (Miller, 1985).

2.2 Role of ions during the sperm activation

Although it is widely accepted that hyperosmotic shock is the main factor triggering sperm motility in marine fish, the ion composition of the activation medium is considered the second most important factor (Alavi and Cosson, 2006). From a general overview, the osmotic shock faced by the spermatozoa when it is released into the marine environment leads to a rapid flux of ions and water between the intra- and extracellular medium (Takai and Morisawa, 1995). During this activation process, both *i*) the ion composition of the activation media (extracellular factor) and *ii*) intracellular factors such as ATP availability, pH, intracellular ions, etc., can affect the spermatozoa motion performance (Cosson et al., 2008b).

Currently, there are conflicting studies about the effect of the ion composition of the activation medium on the initiation of sperm motility. For example, while in species such as pufferfish, halibut, European sea bass or cod, sperm activation can be induced by hyperosmotic non-electrolyte solutions (e.g. glucose); in other species such as tilapia or Pacific herring extracellular Ca^{2+} seems to be essential for the initiation of sperm motility. In addition, Detweiler and Thomas (1998) have shown that although the sperm of Atlantic croaker was able to move in a medium without Ca^{2+},

spermatozoa kinetic parameters were negatively affected by the depletion of this ion. Thus, it seems clear that there is a wide interspecific variability in relation to the presence/absence of extracellular ions and the effect thereof on the sperm motion parameters, so more studies focusing on this topic are necessary in order to design optimum species-specific activation media.

On the other hand, scarce studies regarding the intracellular ion changes after sperm activation of marine fish have been published (Oda and Morisawa, 1993; Takai and Morisawa, 1995). Nowadays, the widely accepted model in marine species suggests that a hyperosmotic shock causes a spermatozoa membrane depolarization, which in turn results in an increase in Ca^{2+} and K^{+} inside the cell, which is the key factor triggering sperm motility (Figure 4).

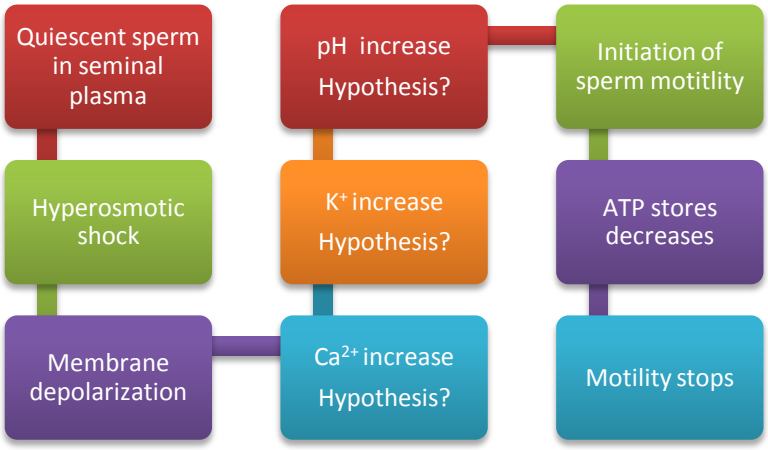

Figure 4. Activation process and signal transduction in marine spermatozoa: scheme of the interacting processes occurring during the motility period.

However, neither the origin of these ions (from the extracellular medium or intracellular stores), nor their specific effects on motility and the kinetic parameters measured by CASA systems have been investigated. In this respect, new studies involving detailed protocols, channel blockers, etc. must be carried out in order to find out the specific mechanisms which occur during motility activation in marine fish sperm.

3. Sperm quality in fish

From an aquacultural point of view, breeding in captivity of some marine species is a key factor in reaching reproductive success, and the main limitations are the quantity and quality of male and female gametes. However, until recently gamete quality has not received much attention, and only a few studies have been published about the effect of specific factors on gamete quality (see review Bobe and Labbé, 2000). Traditionally, hatcheries have focused more on the quality of eggs and larvae rather than that of sperm, even though sperm quality also affects the performance of larvae production (Trippel and Nielson, 1992). In fact, sperm in commercial hatcheries is often inadequate both in terms of quantity and quality and often optimal fertilisation rates are not reached through *in vitro* fertilization trials (Rurangwa et al., 2004). Therefore, it appears that the assessment of sperm quality would be a useful tool that could be applied in a wide range of reproduction fields, and several factors such as artificial fertilization, chilled storage, cryopreservation or even the effect of environmental pollutants on fish reproductive success could be improved by sperm quality evaluation (Cabrita et al., 2005a; Fabroccini et al., 2010).

First of all, it seems essential to define and understand what sperm quality means. From a biological standpoint, sperm quality could be defined as the ability ofthe spermatozoa to reach and fertilize the oocyte (Fauvel et al., 2010). In this respect, any quantifiable parameter directly correlated with the fertilization capacity could potentially be used as a sperm quality indicator. Sperm is the final result of a maturation process in which several haploid cells are produced with the aim of transmitting paternal genetic information to the next generation. It is important to highlight that throughout this process, several factors can affect the sperm quality and thus, fertilization success. The most important factors are displayed in Table 1, all of different origins, from the reproductive age of the breeder to the hormonal treatment (if required) applied to achieve artificial maturation. Therefore, in order to achieve good quality sperm it is essential to take all these factors into account, because slight variations in any of them could negatively affect the sperm quality and thus, decrease fertilization success.

Table 1. Main factors affecting the reproductive success using fish sperm (modified from Fauvel et al., 2010).

Parental effect	Activation medium	Human interaction
Temperature	Temperature	Hormonal induction
Photoperiod	Ion composition	Preservation
Domestication	Osmolality	Chilled storage
Reproductive age	pH	Cryopreservation
Nutrition		Xenobiotics
Selection		

3.1 Basic parameters for assessing sperm quality

Over the years a relatively high number of sperm parameters have been used to assess sperm quality. These sperm biomarkers have so far been documented in scientific papers, and several traits of the fish/sperm itself, such as osmolality, pH and chemical composition of the seminal plasma (Asturiano et al., 2005; Ciereszko, 2008); enzymatic activity (Burness et al., 2005); ATP concentration (Suquet el al., 2010); spermatocrit and sperm density (Harald et al., 2001; Sørensen et al., 2013); sperm motility (Ottesen et al., 2009); or sperm morphology and ultrastructure (Marco-Jiménez et al., 2006; Asturiano et al., 2007; Sadiqul and Akhter, 2011) have been linked to the ability of sperm to fertilize the ova. Some of these parameters can be scored relatively easily and are commonly used because no expensive equipment is needed and they are quick and easy to assess. On the other hand, other biomarkers need sophisticated laboratory analyses or expensive equipment, thus at themoment the application of these is not directly available to fish farms.

On the other hand, despite it having been reported that it is the set of sperm biomarkers as a whole that contribute to determining sperm quality and that each one, by itself, is not a single predictive parameter, sperm motility is currently considered the most useful tool to assess sperm quality

in fish (Kime et al., 2001; Fauvel et al., 2010; Boryspholets et al., 2013). However, the most used technique to assess sperm motion has been subjective evaluation, and some problems have emerged from this method. In this respect, the subjective assessment depends on an experienced observer, and several aspects such as sperm density, sperm velocity, drift, etc. can cause over- or underestimation readings. Therefore, the low reproducibility of this motility analysis through subjective evaluation, which can result in variations of 30 to 60% from the same sample, often makes it difficult to interpret and compare the results between labs (Verstegen et al., 2002; Rosenthal et al., 2010). In this sense, the gradual appearance of computerized techniques has provided us with an objective and rapid method for obtaining a correct and accurate evaluation of sperm motion features (Rurangwa et al., 2004, Cabrita et al., 2011).

3.2 New tools to determine sperm quality

The appearance of Computer Assisted Sperm Analysis (CASA) systems has made it possible to estimate a higher number of sperm motion parameters using an objective, sensitive and accurate technique. These systems are the evolution of multiple photomicrography exposure and video-micrography techniques for spermatozoa track, using a computer equipped with imaging software (Rurangwa et al., 2004). This technique was first introduced in the 80´s in mammalian sperm and only in the last few years have modern CASA systems been adapted for fish spermatozoa studies (Van Look et al., 2000; Wilson-Leedy and Ingermann, 2007). The differences in the biology of fish and mammalian spermatozoa may explain the delay in the release of adequate tools for the measurement of sperm motility in fish. To date, these systems have been used and validated in a wide range of animal groups such as marine invertebrates (Gallego et al., 2013), birds (Kleven et al., 2009), marine mammals (Montano et al., 2012), reptiles (Tourmente et al., 2011) or even insects (Al-lawati et al., 2009).

A long time ago and by subjective assessment, the experimented observer was only able to estimate two sperm motion traits: the percentage of motile sperm cells and the total duration of spermatozoa movement. Then,

faced with the difficult task of estimating correct and accurate sperm motility values, researchers used an arbitrary scale of criteria usually comprising of five categories (Pérez et al., 2000; Asturiano et al., 2005; Peñaranda et al., 2010b). Nowadays, CASA systems are able to accurately quantify a high number of sperm motility parameters, including some that could be not detected by visual inspection. Although there are several companies which market the product (Projectes i Serveis R+D S.L.; Microptic S.L.; Hamilton Thorne, etc.), the parameters provided by the system are almost identical. It is noteworthy that most of the parameters provided by CASA systems have been positively correlated with fertilization rates, so these computer systems have proven themselves to be useful tools for assessing sperm quality in fish.

The most commonly used parameters for fish sperm analysis were revised by Rurangwa et al. (2004). The percentages of motile (TM) and progressive motile spermatozoa (PM) can provide a general overview about the quality of the sperm sample. TM means any spermatozoa showing any movement while PM is determined by the spermatozoa which swim in an essentially straight line. High correlations have been reported between both parameters and fertilization rates in some fish species (Table 2).

Table 2. Coefficients of correlation between fertilization rates and total (r_1) and progressive (r_2) motility in some marine and freshwater species.

Species	r_1	r_2	**Reference**
Clarias gariepinus	0.76	0.81	Rurangwa el al., 2001
Sparus aurata	0.59	-	Beirão et al., 2011a
Hippoglossus hippoglossus	0.81	-	Otessen et al., 2009
Takifugu niphobles	0.68	0.70	This thesis, Chapter 5
Pagrus major	0.77	-	Liu et al., 2007
Oncorhynchus mykiss	0.73	-	Liley et al., 2002
Cyprinus carpio	0.53	-	Linhart et al., 2000

However, for some authors the most useful parameters are the spermatozoa velocities (Figure 5). In this respect, VCL (curvilinear velocity) is defined as the actual velocity along the real spermatozoon trajectory and VSL (straight line velocity), means the straight line distance between the start and end points of the track divided by the time of the track. In essence, if the trajectory is a straight line, VCL and VSL are identical. Finally, VAP (angular path velocity) is the velocity along a derived smooth path. VAP is actually of little use in most fish because the spermatozoon tracks are generally smooth curves, so VAP and VCL are identical. However, depending on the fertilization microenvironment, the spermatozoa can follow a much more erratic path and in some fish species VCL and VAP are both useful measurements (Kime and Tveiten, 2002). In addition, VAP can generate other related parameters such as the percentage of fast (FA), medium (ME) and slow (SL) spermatozoa, which can provide useful information for assessing the kinetic evolution of spermatozoa over the whole swimming time period (Gallego et al., submitted a).

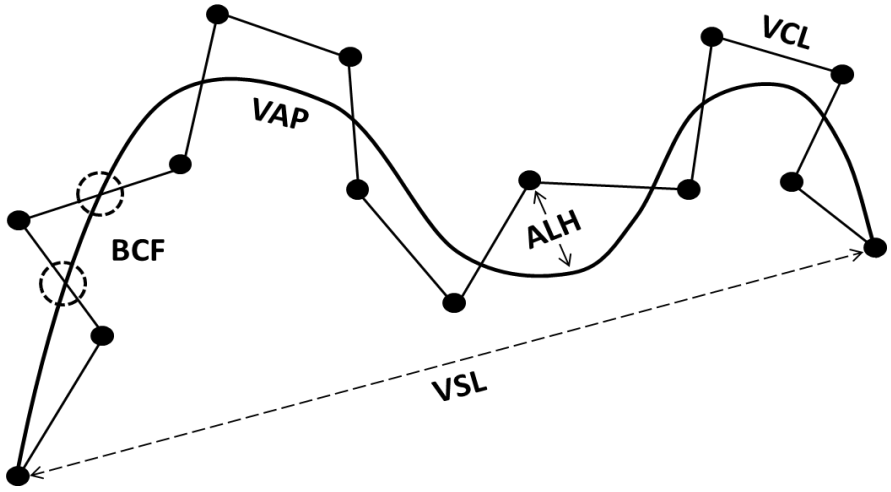

Figure 5. Schematic diagram of some kinetic parameters recorded by CASA system. Black circles represent successive positions of the head of a motile sperm through the video recording. Sperm movement parameters: VCL, curvilinear velocity; VAP, averaged path velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat/cross frequency.

The other motion parameters that are automatically generated by the tracker have not yet provided any additional useful information on sperm quality in fish. Among them, the ratio of the net distance moved to the total path distance (linearity, LIN) and the ratio of the net distance moved to the smoothed path distance (straightness, STR) have been the most often used parameter s for analyzing the curvature of the trajectory.

On the other hand, CASA systems are able to show a huge number of spermatozoa per capture/frame, which means thousands of motion tracks analyzed per sample (Figure 6). However, despite the advantage of working with these extensive databases, sperm motility analyses often show the mean parameter values, considering the whole sperm sample as homogeneous. However, it has been pointed out that the sperm of some species is not a homogeneous mixture, and different sperm subpopulations coexist in the same sample. Although this topic has mostly been studied in mammals (Quintero-Moreno et al., 2003; Dorado et al., 2011), there are some reports in fish which have clearly shown the coexistence of distinct motility-based sperm subpopulations within the same sample : *Solea senegalensis* (Martinez-Pastor et al., 2008), *Oncorhynchus mykiss* (Kanuga et al., 2012) or *Anguilla anguilla* (Gallego el al. , submitted b).

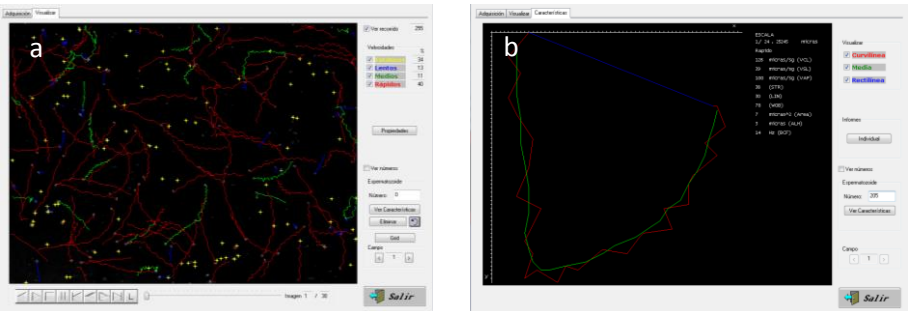

Figure 6. Images obtained by a CASA system. a) Full screen capture of sperm sample; b) Detailed motion trajectory of one spermatozoon.

The CASA systems have been successfully applied in a wide range of aquacultural matters (Table 3), but new fields could be improved by the rapid assessment of sperm quality. In this respect, one of the possible

future applications could be to facilitate the choice of broodstock which in turn would mean the selection of improved quality sperm for the succeeding generations. In fact, it has been reported that genetic selection by growth in Atlantic salmon (*Salmo salar*) alters sperm quality: after a few generations individuals selected by weight displayed a 20 fold reduction in sperm volume produced when compared to wild males (Mylonas et al., 1995). Therefore, a simultaneous selection of several commercial and reproductive traits, taking into account CASA systems sperm quality analysis, could be carried out in fish farms in order to maintain high reproductive quality of the male breeders.

Table 3. Applications for CASA systems in some aquaculture issues over the past few years.

Application	**Reference**
Cryopreservation	Ciereszko et al., 2008; Cabrita el al., 2011
Chilled storage	Babiak et al., 2006; Jing el al., 2009
Activation media	Wilson-Leedy 2009; Kanuga el al., 2012;
Sperm subpopulations	Beirão et al., 2011a; Gallego et al. (submitted b)
Broodstock managment	Felip et al., 2006; Beirão el al., 2011b
Spermiation induction protocols	Asturiano et al., 2005; Radoslaw et al., 2012
In vitro fertilization trials	Liu et al., 2007; Otessen et al., 2009;
Reproductive ethology	Fitzpatrick et al., 2007; Haugland et al., 2009
Environmental contamination	Singh et al., 2008; Dietrich et al., 2010

4. Projects, grants and companies involved in this Thesis

Finally, it is important to note that all the studies carried out in this Thesis have been funded by different projects and grants both at national and international level.

My research career has primarily been supported by, firstly, a 1-year predoctoral fellowship (Programas de Apoyo a la I+D de la UPV) granted by the Universitat Politècnica de València (UPV) and secondly, by a 4-years predoctoral fellowship within the "Formación de Personal Investigador" programme (FPI; BES-2009-020310) granted by the Spanish Ministry of Science and Innovation (MICINN, today MINECO) linked to the Project SELECTBREAM (AGL2007-64040-C03-00) funded by the National Research Projects Programme of the MICINN.

During my PhD studies, I have been also supported by two fellowships granted by the Spanish MICINN´s Personnel Research Training Programme to carry out studies in different research hosting centres. The first training period, which lasted 4 months in 2011, was done in the Department of Molecular Biology in the Universidad de León (León, Spain) under the supervision of Dra. Mª Paz Herraéz (grant reference: EEBB-I-12-05858); and the second training period, which lasted 5 months during 2012, was done in the Misaki Marine Biological Station belonging to the University of Tokyo (Miura, Japan) under the supervision of Dr. Manabu Yoshida (grant reference: EEBB-I-12-05858).

On the other hand, several National and European projects have helped in global terms with the costs necessary to carry out the research for this thesis (animal facilities, equipment, consumables, management costs, staff employed in the lab, overheads, etc.). In this respect, Chapters 1, 2 and 3 have mainly been funded by the Project PRO-EEL (Reproduction of European eel towards s self-sustained aquaculture) by the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology" (grant agreement n°245257); and Chapters 3, 4 and 5 have mainly been funded by the Project SPERMOT (Mecanismos fisiológicos implicados en la espermiación y en la adquisición de motilidad espermática en la anguila europea (*Anguilla anguilla*)) funded by the MICINN (AGL2010-16009). In addition, local support was granted by the Generalitat Valenciana (ACOMP/2011/229) in Chapter 1; and the Generalitat Valenciana (ACOMP/2012/086) in Chapter 2.

With regards to the companies involved, the fish farm Valenciana de Acuicultura, S.A. (Puzol, Spain) supplied all the male eels used in Chapters 1, 2 and 3; and the team of Projectes i Serveis R+D (Proiser S.L.; Paterna, Spain) contributed to the experimental design described in Chapter 1 and performed the task of fractioning the original video sequences in this same chapter (section 3.2).

OBJECTIVES

The chapters presented below have a primary objective in common: the development and improvement of different techniques and protocols with the aim of increasing the knowledge about European eel and pufferfish sperm biology, thereby improving their reproductive performance and the control of their reproduction in captivity. The specific objectives were:

- ✓ To standardize European eel sperm quality assessment by a CASA system in terms of procedural and biological settings, with the aim of defining a standard method to make accurate comparisons in aquaculture/aqualcultural reproductive matters possible.

- ✓ To study the effects on the reproductive performance of three thermal regimes and three hormonal treatments as alternative maturation induction methods of male European eels.

- ✓ To test the changes to the main ions involved in European eel sperm activation, evaluating the spermtozoa intracellular concentrations of Ca^{2+}, K^{+}, and pH by flow cytometry, and establishing the first hypothesis about the role of these ions in the sperm motility activation of this species.

- ✓ To develop a simple chilled storage method able to preserve the pufferfish sperm quality parameters over a short-term period.

- ✓ To evaluate the effects of the composition and osmolality of the sperm activation medium on the motility characteristics of pufferfish spermatozoa, as well as to measure the intracellular concentrations of the main ions involved in its sperm activation.

- ✓ To study the correlations between all the pufferfish sperm parameters provided by a CASA system and the fertilization and hatching rates. Moreover, the effect of the sperm/egg ratio will be analyzed in relation to these parameters in order to estimate the optimum dosage to use in reproductive matters in aquaculture.

CHAPTER 1

Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA software

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Abstract

The development of powerful computerized-assisted sperm analysis software (CASA) has made kinetic studies of spermatozoa possible. This system has been used and validated for several species, but some technical questions have emerged regarding fish sample evaluations (i.e., frame rates, sperm dilution, chamber models, time of analysis, magnification lens, etc.). In the present study, we have evaluated the effects of different procedural and biological settings with the aim to measure sperm quality parameters on the European eel correctly.

The use of different chambers did not affect the sperm motility parameters. However, regarding lens magnification, 10x was the most accurate lens, showing the least variation in the acquired data. Similarly, the frame rate setting resulted in a dramatic effect in some sperm kinetic parameters, primarily in terms of curvilinear velocity, we therefore recommend using the camera's high frame rate setting available. Finally, the reduction in sperm motility over post-activation times suggests that sperm analysis should be performed within the first 60 s after activation of the European eel sperm. In conclusion, some protocol variables of sperm analysis by CASA software can affect the measurement of eel sperm quality parameters, and should be considered before directly comparing results obtained by different laboratories. Moreover, as marine fish species show relatively similar features to sperm kinetic parameters, these results could be considered for the evaluation of sperm motility in other fish species.

1. Introduction

The economic importance and high commercial demand of the European eel, *Anguilla anguilla*, primarily from European and Japanese markets, is well known (Dekker, 2000; Moriarty and Dekker, 1997; Pérez et al., 2004). However, the population of the European eel has declined to such a degree that major concerns have been raised for its long-term survival (Feunteun, 2002; Stone, 2003). Efforts have been made to understand the life cycle and reproductive biology of this species (Tesch, 1978; van Ginneken and Maes,

2005) and we already know it is necessary to use hormones to induce ovulation and spermiation to overcome the lack of normal spawning stimuli in captivity (Asturiano et al., 2005; Pérez et al., 2008). It is particularly advantageous to stimulate the spermiation of male eels so that sperm is available in a short time and in high volume (Gallego et al., 2012). In this respect, knowledge of how to manipulate and preserve eel sperm is essential (Peñaranda et al., 2008, 2009 and 2010) and a reliable and standardized method to analyse its quality is needed.

The evaluation of sperm motility and other kinetic parameters like curvilinear, straight line, and average path velocities, and morphology, is an essential tool in the examination of sperm quality in many fish species (Liu et al., 2007; Marco-Jiménez et al., 2008; Gallego el at., 2012), including the European eel (Gallego el at., 2012; Marco-Jiménez et al., 2006; Asturiano et al., 2007). Despite the fact that for many years optic microscopes have conventionally been used to carry out analyses and evaluations, it is considered subjective method and great variations have been reported (Coetzee et al., 1999). According to Verstegen et al. (2002), when subjective optical microscopic evaluation is used in humans and animals, variations of 30% to 60% have been reported in the estimation of the motility parameters of the same ejaculates. The computer assisted sperm analysis, or CASA, has been used by an increasing number of researchers worldwide and provides an objective, rapid, and multiple-parameter assessment of sperm quality.

To make it possible to compare the results obtained by different laboratories, 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, software name, microscope optic and magnification, etc. (Castellini et al., 2011; Soler et al., 2012; Rosenthal et al., 2009). Unfortunately, in most publications, details of these parameters are not provided, thus reducing the possibility of comparing the results of different laboratories. Furthermore, because there are many different configurations and methods of using CASA, it is important to establish standard methods of enhancing the reliability,

comparability, and applicability of data produced by different research groups (Contri et al., 2010).

Because CASA systems are not ready-to-use devices, they depend largely on the technical settings and standardizing procedures. Thus, the aim of this study was to evaluate different procedural and biological settings such as chamber models, lens magnification, frame rate acquisition, ejaculate portion, and postactivation times to define a standard method to assess the quality of the European eel semen using a CASA system (ISAS v1; Proiser R&D, S.L., Paterna, Spain).

2. Material and Methods

2.1 Fish handling

Sixty adult male eel from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture Laboratory of the Universitat Politècnica de València, Spain. The fish were distributed in three 200L aquaria (approximately 20 male eels per aquarium) equipped with separate recirculation systems, thermostats, and coolers, and covered to maintain constant darkness. The eels were gradually acclimatized to sea water (salinity 37 ± 0.3 g/L) and once a week they were anesthetized with benzocaine (60 ppm) and weighed before receiving the administration of hormones (hCG; 1.5 IU per g of fish body weight) by intraperitoneal injection. The fish were fasted throughout the experiment and were handled in accordance with the European Union regulations regarding the protection of experimental animals (Dir 86/609/EEC).

2.2 Sperm collection and sampling

Sperm samples were collected 24 hours after the administration of the hormone because previous studies (Pérez et al., 2000) have demonstrated that this is moment when the highest sperm quality is found. In preparation for sperm collection, the fish were anesthetized, and after cleaning the genital area with fresh water to avoid the contamination of the samples

with faeces, urine, or sea water, and thoroughly drying the fish, the sperm were collected by abdominal pressure. A small aquarium air pump was modified to obtain a vacuum breathing force and the sperm was collected in a tube. A new tube was used for every fish and distilled water was used to clean the collecting pipette between each fish. Sperm samples were collected between the 6th and 13th week, kept in plastic tubes and refrigerated (4 ºC) during the 1-2 hours before the analyses.

2.3 Sperm motility evaluation, CASA settings and the analysed parameters

Sperm was activated by mixing 1 mL of sperm with 200 mL of artificial sea water (Aqua Medic Meersalz, 37 g/L, with 2% BSA [wt/vol] with pH adjusted to 8.2; Peñaranda et al., 2010a). All motility analyses were performed in triplicate using the motility module of ISAS v1 and an ISAS 782M camera recorder capturing 60 frames per second (fps). At least 400 to 700 spermatozoa were captured in each field adjusting the brightness and contrast in the CASA settings in relation to the microscope light with the aim to clearly define the spermatozoa. Range size particles were defined between 2 and 20 µm in the CASA settings. The counting chamber used in all experiments was the ISAS D4C20 chamber, with the exception of the "chamber trial," in which ISAS and Makler (Sefi Medical Instruments, Haifa, Israel) chambers were compared.

The parameters considered in this study were: total motility (MOT, %); progressive motility, defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast (average path velocity [VAP] >100 µm/s), medium (VAP = 50–100 µm/s), and slow (VAP = 10–50 µm/s) spermatozoa; curvilinear velocity (VCL, in µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; 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; VAP (µm/s), defined as the time/average of sperm head along its spatial average trajectory; straightness (STR, %), defined as the linearity of the spatial average path, VSL/VAP; and beat cross

frequency (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was <10 µm/s. To perform an in-depth analysis, sperm samples were classified into three classes based on the percentage of motile spermatozoa: Class I (C-I), 0 to 25% motile cells; Class II (C-II), 25% to 50% motile cells; and Class III (C-III), >50% motile cells. All trials were carried out using each of these motility classes (except the ejaculate portion trial, in which only C-III class was used).

2.4 Effect of chambers and magnification lens

Different tools can be used for sperm motility evaluation by CASA systems. In this trial, two commercially available chamber models, the ISAS D4C20 disposable chamber (20 µm deep; Proiser R+D) and the Makler reusable chamber (10 µm deep; Sefi Medical Instruments) and two magnification lenses (10x versus 20x in a Nikon E400 microscope, negative phase contrast) were tested.

2.5 Effect of frame rate

To assess the effect of frame rate on the system's ability to describe sperm motion, sperm quality parameters at 20, 30, and 60 fps (Hz) were compared. With the aim of avoiding variations between replicates within the same sample, the original file, captured at 60 fps, was manually modified using video analysis software removing one or two frames from every three original ones within each video file, as such obtaining files of 30 or 20 fps, respectively.

2.6 Effect of ejaculate portion and post activation time

Sperm samples were collected in two portions: the first collectable millilitre (1st mL) was retrieved in a test tube, and the rest of the sperm was collected in another test tube. Sperm quality parameters of C-III class samples were also measured at different postactivation times (30, 60, and

90 seconds), with the aim of assessing the effects of the differences in time from the sperm activation event.

2.7 Statistical analysis

The mean and standard error (SE) were calculated for all the sperm quality parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. The one-way analysis of variance (ANOVA) and Student's *t*-test were used to analyze data with normal distribution. Significant differences between post-activation times were detected using the Tukey multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks and Mann-Whitney *U*-test were used. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

The sperm cell detection parameters used in this study were suitable for fish sperm evaluation. Quality control analysis performed using the playback facility showed that all spermatozoa observable in the field were detected and recorded.

3.1 Effect of chambers and magnification lens.

The different chambers used in this trial did not significantly affect the sperm quality parameters in any motility class (Table 1). However, samples analyzed by the ISAS® disposable chamber showed slightly higher values in almost all the sperm motility parameters (although no significance differences were found). The coefficients of variation (CV) of samples within the same motility class obtained with both chambers were quite similar (Figure 1), with much higher CV's in C-I than in C-II and C-III classes.

Table 1. Mean ± SEM of sperm quality parameters for different chamber models on different sperm classes (C-I, C-II, and C-III) at 30 s postactivation time.

		C-I		C-II		C-III	
		Makler	**ISAS**	**Makler**	**ISAS**	**Makler**	**ISAS**
MOT	%	16.0 ± 1.6	20.8 ± 2.4	38.6 ± 3.2	42.7 ± 3.7	57.6 ± 2.3	62.8 ± 3.6
PM	%	4.3 ± 0.8	5.5 ± 1.0	19.6 ± 2.4	21.1 ± 2.5	22.1 ± 2.2	26.1 ± 2.2
FA	%	7.3 ± 1.2	9.0 ± 1.3	27.0 ± 2.8	30.0 ± 3.4	38.6 ± 2.9	45.3 ± 3.5
ME	%	4.4 ± 0.5	5.9 ± 0.9	6.1 ± 0.8	6.8 ± 0.7	12.7 ± 0.9	11.2 ± 0.9
SL	%	4.4 ± 0.5	5.8 ± 0.9	5.5 ± 0.8	5.9 ± 0.8	6.3 ± 0.6	6.3 ± 0.5
VCL	µm/s	96.1 ± 5.4	94.3 ± 6.3	140.4 ± 7.5	143.0 ± 7.8	136.1 ± 6.3	145.6 ± 6.0
VSL	µm/s	37.0 ± 2.9	38.5 ± 3.7	69.8 ± 5.4	69.6 ± 4.8	62.5 ± 4.2	69.0 ± 4.1
VAP	µm/s	54.3 ± 3.2	55.6 ± 4.2	87.8 ± 5.3	88.7 ± 5.2	85.0 ± 4.6	92.3 ± 4.6
STR	%	66.7 ± 1.5	67.0 ± 1.9	77.4 ± 2.4	77.4 ± 1.5	72.3 ± 1.6	73.8 ± 1.3
BFC	beats/s	21.6 ± 2.2	24.3 ± 2.0	31.9 ± 1.6	34.9 ± 1.3	30.8 ± 1.2	31.1 ± 1.2

Abbreviations: BCF, beat cross frequency; FA, fast; ME, medium; MOT, total motility; PM, progressive motility; SL, slow; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

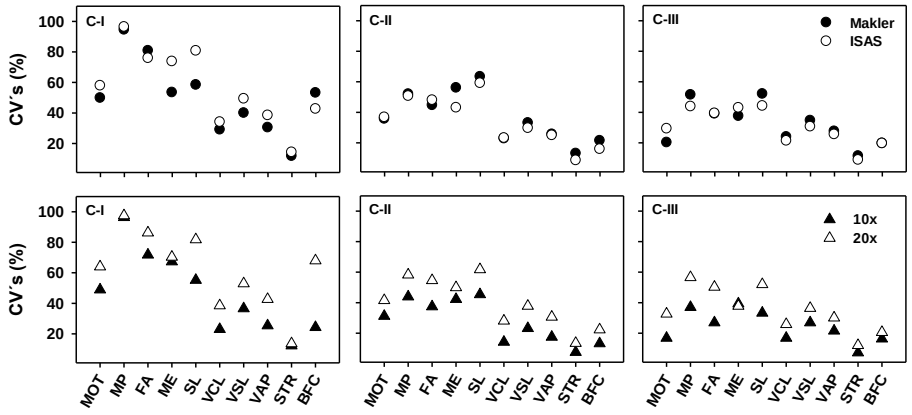

Figure 1. Coefficient of variation (CV) for each chamber model (Makler and ISAS) and each microscopy magnification (10 and 20) on different sperm classes (C-I, C-II, and C-III).

BCF, beat cross frequency; FA, fast; ME, medium; MOT, total motility; PM, progressive motility; SL, slow; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

On the contrary, the different magnification lenses used in this study significantly affected some of the sperm quality parameters in the different sperm classes (Table 2). Samples analyzed using the 20x lens showed lower values than those analyzed using the 10x lens, with more significant differences in C-II and C-III. In addition, the coefficients of variation within the same motility class (Figure 1) with the 20x lens were much higher than with the 10x lens, with much higher CV's in C-I than in C-II and C-III.

Table 2. Mean ± SEM of sperm quality parameters for different magnification lenses on different sperm classes (C-I, C-II, and C-III) at 30 s postactivation time.

		C-I		C-II		C-III	
		10x	**20x**	**10x**	**20x**	**10x**	**20x**
MOT	%	18.5 ± 1.8	18.3 ± 2.3	42.2 ± 3.1	39.1 ± 3.8	63.8 ± 2.1	56.6 ± 3.6
PM	%	4.8 ± 0.9	5.0 ± 0.9	22.1 ± 2.3	18.6 ± 2.6	27.8 ± 2.0*	20.4 ± 2.3
FA	%	8.4 ± 1.2	7.9 ± 1.3	32.0 ± 2.8	25.1 ± 3.2	47.8 ± 2.5*	36.1 ± 3.6
ME	%	5.7 ± 0.7	4.6 ± 0.6	5.6 ± 0.6	7.3 ± 0.9	10.5 ± 0.8*	13.3 ± 1.0
SL	%	4.4 ± 0.5	5.9 ± 0.9	4.6 ± 0.5	6.8 ± 1.0	5.5 ± 0.4*	7.1 ± 0.7
VCL	µm/s	101.2 ± 4.6	89.2 ± 6.7	153.6 ± 5.1*	129.8 ± 8.6	152.3 ± 5.0*	129.4 ± 6.5
VSL	µm/s	39.3 ± 2.8	36.2 ± 3.8	74.9 ± 4.1	64.6 ± 5.7	72.2 ± 3.8*	59.2 ± 4.2
VAP	µm/s	58.3 ± 2.9	51.6 ± 4.3	94.6 ± 3.9	82.0 ± 5.9	95.4 ± 4.0*	81.9 ± 4.8
STR	%	66.4 ± 1.6	67.4 ± 1.8	78.4 ± 1.4	76.4 ± 2.4	74.8 ± 1.1	71.3 ± 1.7
BFC	beats/s	26.2 ± 1.2*	19.7 ± 2.6	35.7 ± 1.1*	31.1 ± 1.6	33.0 ± 1.1*	28.9 ± 1.2

Abbreviations: BCF, beat cross frequency; FA, fast; ME, medium; MOT, total motility; PM, progressive motility; SL, slow; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

3.2 Effect of frame rate setting

The frame rate setting (FR) had no effect neither on the total and progressive motile cells nor on the proportion of fast, medium and slow spermatozoa (data not shown). However, other kinetic values were deeply affected by FR (Figure 2). VCL and BFC showed a progressive increase with significant differences as the FR increased while STR showed a reverse

trend, decreasing as the FR increased. VSL did not show significant differences in any motility class whereas VAP only showed statistical differences in C-II motility class.

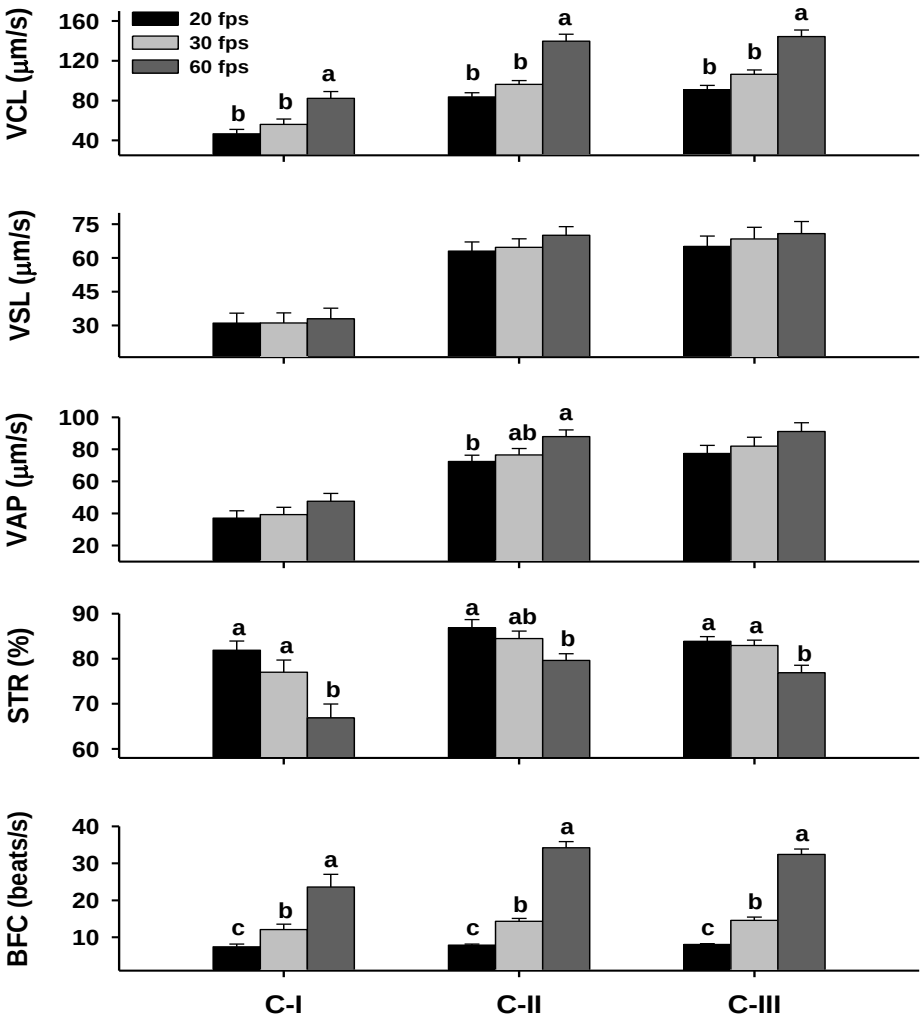

Figure 2. Kinetic parameters at different frame rates (20, 30, and 60 fps) on different sperm classes (C-I, C-II, and C-III). Data are expressed as mean ± SEM and different letters indicate significant differences between fps.

BCF, beat cross frequency; fps, frames per second; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

3.3 Effect of ejaculate portion and post activation time.

Sperm parameters obtained by the 1st mL and the rest of the sperm were similar, and no significant differences were evident (Table 3).

Table 3. Mean ± SEM of sperm quality parameters in the first collectable millilitre (1st mL) and the rest of the sperm (Rest) in high quality sperm samples (C-III) at 30 seconds postactivation time.

		1st mL	Rest
MOT	%	74.75 ± 3.19	73.29 ± 3.78
PM	%	39.36 ± 3.36	36.65 ± 5.49
FA	%	61.16 ± 5.09	58.80 ± 5.26
ME	%	10.39 ± 2.56	10.60 ± 1.93
SL	%	3.20 ± 0.33	3.89 ± 0.60
VCL	µm/s	162.88 ± 7.28	156.95 ± 6.34
VSL	µm/s	87.03 ± 5.77	81.76 ± 6.37
VAP	µm/s	110.58 ± 6.33	104.16 ± 6.03
STR	%	78.41 ± 1.12	77.83 ± 1.87
BFC	beats/s	30.70 ± 1.12	30.44 ± 0.75

Abbreviations: BCF, beat cross frequency; FA, fast; ME, medium; MOT, total motility; PM, progressive motility; SL, slow; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

Regarding changes in sperm parameters after sperm activation, significant differences were found in MOT, and percentage of fast and slow spermatozoa (Fig. 3). The most affected parameter was MOT, with a progressive decrease in motile cells after the activation time with significant differences in the different classes, in which motility value recorded at 90 seconds was lower than motility obtained at 30 seconds. There was a similar trend in percentage of fast spermatozoa, but without significant differences in C-II. There were significant differences in percentage of slow spermatozoa only in the highest motility class (C-III).

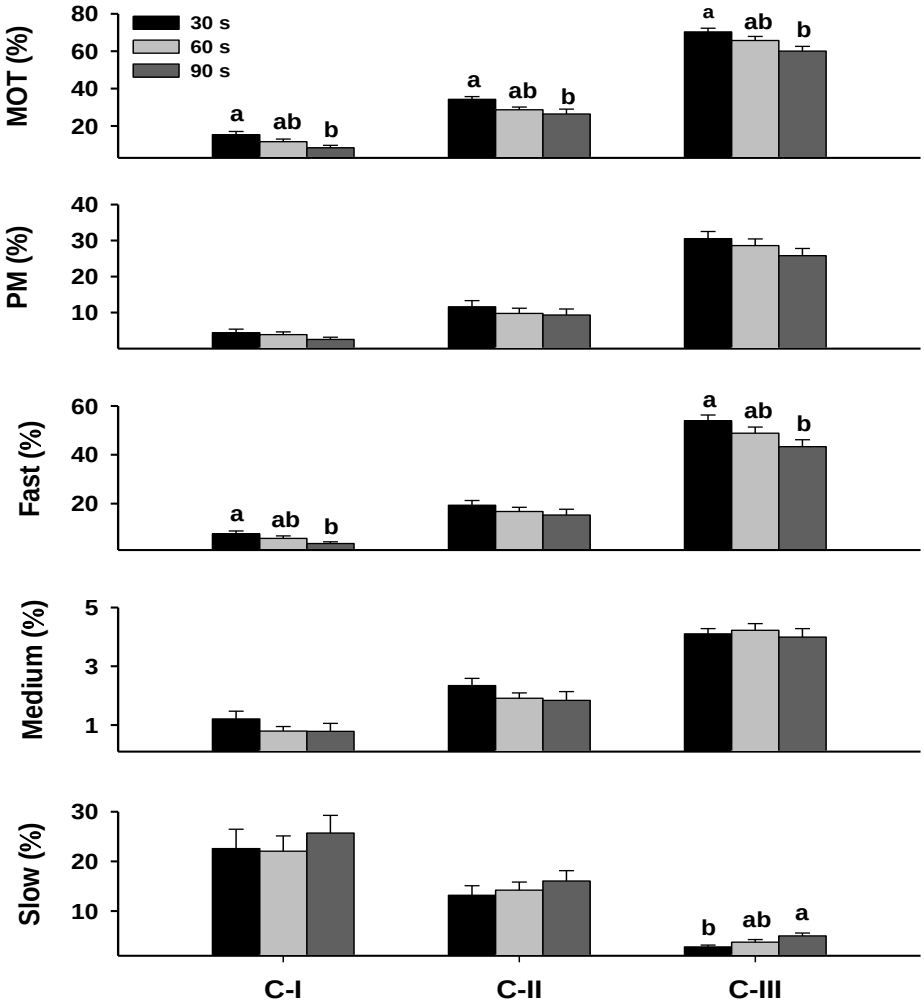

Figure 3. Sperm motility parameters at different postactivation times (30, 60, and 90 seconds) on different sperm classes (C-I, C-II, and C-III). Data are expressed as mean ± SEM and different letters indicate significant differences between times. *MOT, total motility; PM, progressive motility.*

4. Discussion

The subjective sperm quality evaluation, widely used in many laboratories working with male gametes, depends on the skill, perception, and training of the researcher who evaluates the sperm samples (Kime et al. 2001,

Rurangwa et al., 2004; Asturiano et al., 2006). In the past few years, several CASA software systems have been developed with the aim of achieving an objective evaluation of sperm quality parameters (Amann and Katz, 2004). However, although these systems provide the most accurate and repeatable technique currently available, they need to be standardized before their use. Despite the beneficial effects of this standardization process in human andrology (Björndahl et al., 2002), there are few data on domestic animals (Rijsselaere et al., 2003, 2005) and to this day there are no studies about the standardization of procedures in fish species. In this study, we have assessed different technical and biological settings to standardize the sperm quality evaluation of European eel to be used as a sperm model for teleost fish.

Several different chambers can be used for the analysis of spermatozoa using CASA systems. The choice of chamber depends on several factors and in this trial two chamber models were evaluated. The Makler chamber is a round reusable sperm-counting chamber (10 µm depth) loaded by drop displacement, and the ISAS DC420 chamber is a rectangular disposable sperm counting chamber (20 µm depth) loaded by capillarity. All these factors (shape, loading method, depth, etc.) can affect the sperm parameters, as occurs in other species like human (Soler et al., 2012, Kraemer et al., 1998] or bull (Contri et al., 2010, Lenz et al., 2011).

However, in our study, the different chambers did not affect fish sperm quality parameters in any motility class. This result suggests that in the case of eel sperm and, in fish sperm with similar sperm features in general, it is possible to evaluate the sperm quality parameters with different kinds of chambers without compromising the final result.

On the other hand, the different magnification lenses used in this study significantly affected many of the sperm quality parameters. In this case, the result could be related to the sample size, which can affect the results of sperm analysis. If an insufficient number of spermatozoa are analyzed during the video capture an inaccurate measurement of sperm parameters will be obtained because of a higher data variation and/or dispersion (Gallego el al., 2012). In this case, the number of spermatozoa captured by

the 20x magnification lens was much less than those assessed with the 10x lens, therefore the CVs obtained by the 20x lens were much higher than those obtained with the 10x lens within the same motility class. Thus, the results obtained using the 10x lens should be a priori more accurate and precise than the results obtained using the 20x lens.

The number of fps can influence the quality of the acquisition and the sperm quality parameters (Castellini et al., 2011). It has been demonstrated in literature that low frame rates can underestimate the real value of kinetic traits (Wilson-Leedy and Ingermann, 2007; Contri et al., 2010). The higher the quantity of track information available during the sperm capture (increasing fps), the more accurate the reconstruction of the sperm trajectories obtained, more closely resembling the real trajectory. Thus, the reduction in the fps could generate significant variations in several kinetic parameters. In our trial, the frame rate setting had no effect, either on MOT or on progressive motility. However, other sperm quality parameters like VCL, STR, and BCF were affected to a great extent by frame rate. Our results corroborate previous studies (Castellini et al., 2011; Contri et al., 2010), in which it has been suggested that increases in frame rate drastically increase the measured VCL without substantial effect on VAP, resulting in a decrease in STR. In this respect, it seems reasonable to think that the higher number of fps will generate the more “real” spermatozoa trajectory. However, what is the limit of fps? This limit depends on several factors, from the kinetic features of the sperm with which we are working (it is quite different to work with low and linear than with fast and nonlinear spermatozoa movements), to the laboratory’s ability to invest in the best camera available in the market. In this respect, most of the reports about mammal sperm that were carried out using CASA software used an acquisition rate of 50 to 60 Hz (Owen and Kanz, 1993; Mortimer et al., 1998, 1999; Iguer-ouada and Verstegen, 2001). However, this rate seems to be chosen because of hardware and/or software parameters and not because of theoretical considerations. Regarding fish sperm, the problem is magnified because fish spermatozoa are considered to have one of the fastest nonlinear trajectories. Toth et al. (1997) suggested that frame rates >60 fps should be used when analyzing fish sperm. In this sense, Wilson-Leedy and

Ingermann (2007) reported that 97 fps is the lower limit to obtain acceptable trajectories in zebra fish, and Castellini et al. (2011) reported that fish sperm require a frequency of 290 fps to fully trace the movement path. Thus, and to sum up, it is important to take into account that the comparison of results between different laboratories and/or research groups which use a different number of fps might not be valid.

On the other hand, it is well known that marine fish spermatozoa are quiescent in the seminal plasma, and the hyperosmolality of sea water is the trigger that initiates the motility (Morisawa, 2008). However, the ability of spermatozoa to swim is eventually dependent on their previous maturation in the sperm ducts, where some essential processes in acquiring movement capability take place, such as changes in pH and ionic composition of seminal plasma, and the action of the 17a,20β-dihydroxy-4-pregnen-3-one 7 (DHP) (Miura et al., 1992; Schulz et al., 2010). Though in some mammals (Corcini et al., 2012) it has been demonstrated that the portion of ejaculate evaluated can affect the sperm quality parameters, few studies have been developed in fish. For example, in rainbow trout, the spermatozoa collected from the distal portions of the sperm duct display better motility than the spermatozoa collected from the proximal portions Morisawa and Morisawa, 1986). Peñaranda et al. (2010) suggested that the high concentration of lipoproteins (high-density lipoproteins) present in the seminal fluid can interact with the spermatozoa plasma membrane to maintain its lipid composition during storage in the sperm duct. In our trial, significant differences in sperm quality parameters between the 1st mL and the rest of the sperm were not evident. In the case of European eel, an endangered marine species able to produce a high volume of sperm (1–4 mL per 100 g of fish; Asturiano et al., 2005; Gallego et al., 2012), this result confirms the possibility of using sperm produced by male fish given hormonal treatment in artificial fertilizations. This result increases the economical profitability of the relatively expensive hormonal treatment necessary to obtain the sperm (Gallego et al., 2012) and enhances the need for good cryopreservation techniques to reduce the male broodstocks and the hormones required to produce sufficient amounts of sperm.

Moreover, regarding change in movement parameters after sperm activation, MOT was the most affected factor, displaying a progressive decrease in the percentage of motile cells after the activation time. Usually in marine and freshwater species, most of the sperm traits used to characterize motility decline within tens of seconds to a few minutes, depending on the species, and this general decrease leads to an eventual full arrest of spermatozoa by ATP consumption (Cosson et al., 2008b). In the case of European eel, in addition to the reduction in motility, a decrease in the percentage of fast spermatozoa was also evident over time. As such our results suggest that sperm analysis in European eel sperm should be performed within the first 60 seconds after activation.

5. Conclusions

Computer assisted sperm analysis systems are useful tools for carrying out studies about spermatozoa kinetic parameters in fish species. However, some questions have emerged regarding sperm sample evaluations, and as such several procedural and technical settings should be standardized and validated before comparing results obtained by different laboratories. In this study, we have assessed different technical and biological settings to standardize the evaluation of sperm quality in the European eel and use it as a sperm model for teleost fish. We have discovered that some protocol variables in sperm analysis using CASA software (ISAS v1) can affect the measurement of eel (fish) sperm quality parameters. Notably, neither the type of chamber nor the ejaculate portion affected the sperm quality parameters, suggesting that either type can be used for sperm evaluation in European eels. Finally, to carry out a suitable analysis on sperm quality parameters in European eel, we suggest a few recommendations regarding its application: (1) use the lowest available magnification lens, with the aim of avoiding a large spread in data; (2) use the highest available frame rate, with the aim of obtaining the most “real trajectory” of the spermatozoa; and (3) perform the analysis within the first 60 seconds after activation.

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CHAPTER 2

Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation

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Abstract

Since 1960, the European eel (*Anguilla anguilla*) has suffered a dramatic reduction in natural stocks. Breeding in captivity is considered an alternative, but obtaining high quality sperm seems basic on this regard. The main objective of this study was to assess the effects of three thermal regimes (two of them variable: T10 and T15; and one of them constant: T20) and three hormonal treatments with different hormones (hCG, hCG_{rec} and PSMG) on the induction of maturation in European eel males.

In the case of the thermal regimes, our results demonstrated that the onset and progression of spermiation are strongly influenced, and perhaps closely regulated, by water temperature. T20 demonstrated the best results in all the sperm parameters (volume, density, motility, kinetic features, etc.) throughout most weeks of treatment, becoming a reliable and productive method for inducing spermiation in this species. In the case of hormonal treatments, the onset and progression of spermiation in European eel males were influenced by the hormone used. In this respect, hCG_{rec} produced the best results in all the sperm parameters including volume, density, motility, kinetic features, etc., throughout most weeks of treatment, thus becoming an effective alternative treatment to the standard hCG treatment used to induce spermiation in eel species. Moreover, hCG_{rec} gave rise to the best economical profitability, making it possible to obtain good quality sperm samples at a lower price than by using the other two hormonal treatments.

1. Introduction

The European eel (*Anguilla anguilla*) is a teleost fish with a peculiar life cycle: prepubertal eels migrate across the Atlantic Ocean for supposedly 6–7 months to reach the spawning area, in the Sargasso Sea (Tesch, 1978; Van Ginneken and Maes, 2005). In the last years the European eel has suffered a dramatic reduction in its population mainly due to varying factors including overfishing, habitat reduction and contamination (Feunteun, 2002). Therefore, breeding in captivity is the only alternative to save this species,

reducing the pressure on natural populations, and meeting the demands of eel farms.

A good tool for a breeding captivity program is obtaining high quality sperm during a large number of weeks with the aim of synchronizing the gamete maturation and fertilizing the ova produced throughout the breeding season (Jørstad and Navdal, 1996; Roldan and Gomendio, 2009).

In some fish species, reproduction in captivity can be controlled exclusively by environmental factors such as temperature, photoperiod or salinity. However, sometimes it is impractical or even impossible to simulate the environmental factors of the breeding process (i.e., spawning migration, depth, pressure, etc.) so the use of exogenous hormones is the only effective way of inducing maturation and spermiation (Asturiano et al., 2002; Boëtius and Boëetius, 1967; Kagawa et al., 2009; Ohta et al., 1996; Pérez et al., 2000). Eels (*Anguilla* spp.) do not mature spontaneously in captivity, so the maturation of males must be induced with long-term hormonal treatments (Asturiano et al., 2005; Huang et al., 2009; Ohta et al., 1997; Pérez et al., 2000). However, in several studies it has been described that the sexual maturation of non-treated males could be stimulated indirectly by treated males, suggesting the existence of chemical communication (pheromones) between them (Huertas et al., 2006). Despite the effectiveness of these long-term hormonal treatments, little attention has been paid to factors such as the duration of spermiation periods, which has been limited in time, or the variations in sperm quality parameters (Asturiano et al., 2005; Miranda et al., 2005; Mylonas et al., 1998).

A high number of environmental and procedural factors can affect the gonadal development and, consequently, the gamete quality (Mylonas et al., 2010). With regard to environmental factors, the water temperature plays a key role in the gonadal development in many fish species (García-López et al., 2006; Lim et al., 2003; Van Der Kraak and Pankhurst, 1997). In the case of the European eel, the temperature of the hypothetical spawning area is around 20 °C (Boëtius and Boëtius, 1967), and this is probably the reason why both males and females of this species have been matured at this constant water temperature (Asturiano et al., 2006; Pedersen, 2003;

Pérez et al., 2000). However, it has been reported that eels undertake vertical movements during their migration across deep and cold waters (Aarestrup et al., 2009), so it seems probable that the gonadal development, that takes several months, occurs at low temperatures, and the spawning at warm temperatures. Recently it has been shown that in female European eel, variable thermal regimes induce hormonal profiles that resemble the natural ones more closely than those obtained under constant temperatures (Pérez et al., 2011).

Regarding procedural factors, both the type and dosage of hormone used are key factors in the artificial maturation of aquaculture species. Hormonal methods have evolved over time, from the use of pituitaries from mature fish to the use of various synthetic agonists of different hormones (Billard and Marcel, 1980; Rosenfeld et al., 2012; Yaron, 1995). Human chorionic gonadotropin (hCG) has been the hormone most widely used in the maturation and spermiation process in the European eel. However, due to recent problems in the availability of this hormone, new hormones should be tested. In this respect, recombinant hCG (hCG_{rec}), produced by recombinant DNA technology (Satish, 1989), could be a good alternative because it has a similar structure to the native hormone. On the other hand, pregnant mare's serum gonadotropin (PMSG), which is a priori a cheaper choice than hCG and hCG_{rec} hormones, has already been used in other fish species (Brzuska and Ryszka, 1990; Nagahama, 1994; Zakes and Demska-Zakes, 2009) to induce both spermiation and ovulation. Therefore, PMSG can be considered as another option for its use in reproduction studies for eel aquaculture.

Nowadays, the weekly administration of hCG under a constant temperature regime of 20 °C (Asturiano et al., 2005; Peñaranda et al., 2010a; Pérez et al., 2000) has been the most widely used hormonal treatment in European eel males. Despite the good results obtained by this method, the number of weeks in which eels produce a high volume of good sperm is limited, and as such more evolved treatments are necessary to achieve shorter induction times, longer spermiation periods and/or higher volumes of quality sperm. Therefore, the study of alternative hormonal treatments must be an

ongoing task in order to improve the current methods to date. In this respect, the aim of this trial was to assess the effect of the thermal regime (3 thermal regimes, including the standard constant treatment of 20 °C), and the kind of hormone used (3 hormonal treatments, including the standard hCG treatment) on the reproductive performance of European eel males.

2. Materials and methods

2.1 Fish maintenance

Eel males from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture Laboratory at the Universitat Politècnica de València, Spain. The fish were distributed in aquaria equipped with separate recirculation systems, thermostats/coolers (to control the water temperature in the first experiment) and covered to maintain constant darkness. The eels were gradually acclimatized to sea water over the course of 1 week (salinity 37±0.3 g/L), and once a week they were anaesthetized with benzocaine (60 ppm) and weighed before receiving the administration of hormones by intraperitoneal injection. 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 Thermal treatments

A total of 317 adult male eels (mean body weight 100±2 g) were equally and randomly distributed in six 200-L aquaria (approximately 100 males per treatment) and subjected to three thermal regimes: T10, 10 °C (first 6 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15, 15 °C (first 6 weeks) and 20 °C (last 9 weeks); and T20, 20 °C during the whole experimental period (Fig. 1). All the males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Argent Chemical Laboratories, USA) during 13 weeks.

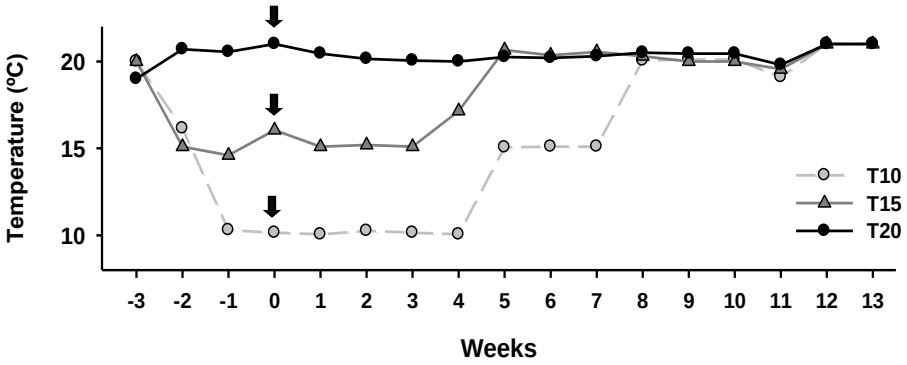

Figure 1. Thermal regimes applied for each treatment (T10, T15 and T20). Arrows indicate the 1st injection with human chorionic gonadotropin (hCG).

2.3 Hormonal treatments

In a second experiment, and after choosing the best thermal regime (T20), a total of 54 adult male eels (mean body weight 81±7 g) were equally and randomly distributed in three 200-L aquaria (18 males per treatment) and submitted to three hormonal treatments: hCG, hCG_{rec} (recombinant hCG; Ovitrelle, Madrid) and PSMG (pregnant mare's serum gonadotropin; Sincropart, Lab CEVA, Barcelona). All hormones were diluted 1:1 (UI/µL) in saline solution (NaCl 0.9%) and a weekly dose of 1.5 IU g^{-1} fish was administered during 20 weeks.

2.4 Sperm collection and sampling

Sperm samples were collected 24 h after the administration of the hormone because previous studies (Pérez et al., 2000) have demonstrated that this is when the highest sperm quality is found. For the sperm collection the fish were anesthetized and after cleaning the genital area with freshwater and thoroughly drying to avoid the contamination of the samples with faeces, urine and sea water, the sperm were collected by abdominal pressure. A small aquarium air pump was modified to obtain a vacuum breathing force and to collect the sperm in a tube. A new tube was used for every male and distilled water was used to clean the collecting pipette between the different males.

To measure sperm density, samples were diluted 1:1000 or 1:10,000 in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, and pH 8.5; Asturiano et al., 2004a). Ten microliters of the dilution was taken for counting in a Thoma hemocytometer and expressed as spermatozoa ×109 mL^{-1}. Sperm volume was measured using graduated tubes and samples were maintained at 4 ºC until analysis and were evaluated in the first hour after extraction.

2.5 Evaluation of motility and kinetic sperm parameters

Sperm was activated by mixing 2 µL of sperm with 200 µL of artificial sea water (Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH adjusted to 8.2; Peñaranda et al., 2010c). All the motility analyses were performed by triplicate at 30 s post-activation by the motility module of ISAS (Proiser R+D, S.L.; Paterna, España) using an ISAS® 782M camera recorder (60 fps; Hz). The chamber used in all experiments was a SpermTrack-10® (Proiser, Paterna, Spain) with 10× negative contrast phase lens in a Nikon Eclipse (E-400) microscope.

The parameters considered in this study were total motility (MOT, %); progressive motility (P-MOT, %), 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 spatial average 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 straight line velocity of over 10 µm/s.

In addition, in order to perform an in-depth analysis of the evolution of sperm quality throughout the weeks of both thermal and hormonal treatments, sperm samples were classified into four classes based on the percentage of motile cells: class I=no motile cells, II ≤25%, III=25–50% and IV>50% of motile cells.

2.6 Economical analysis

To analyze the economical profitability of each hormonal treatment (hCG, hCG_{rec} and PMSG) three factors were taken into account: sperm volume, motility class and amount of hormone used. The essential aim was to relate the level of investment with the amount of good quality sperm produced using each hormonal treatment.

2.7 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. One-way analysis of variance (ANOVA) and Student's t-test were used to analyze data with normal distribution. Significant differences between treatments were detected using the Tukey multiple range test ($P \leq 0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks and Mann–Whitney U-test were used. All statistical analyses were performed by the statistical package SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Thermal treatments

With regard to the percentage of spermiating males (Fig. 2A) the T10 treatment generated lower percentages (with maximum values around 70%) than the T15 and T20 treatments, which reached 100% spermiating males in several weeks. In addition, T10 males did not begin to produce sperm until the 10^{th} week of treatment, whereas T20 and T15 males began spermiating earlier, in the 5^{th} and 6^{th} weeks, respectively.

In all the thermal treatments, there was an increasing trend in sperm volume in the first weeks of spermiation (Fig. 2B). T20 males showed higher volumes than T15 and T10 males in the majority of the weeks, with significant differences in the 8^{th} and 11^{th} weeks.

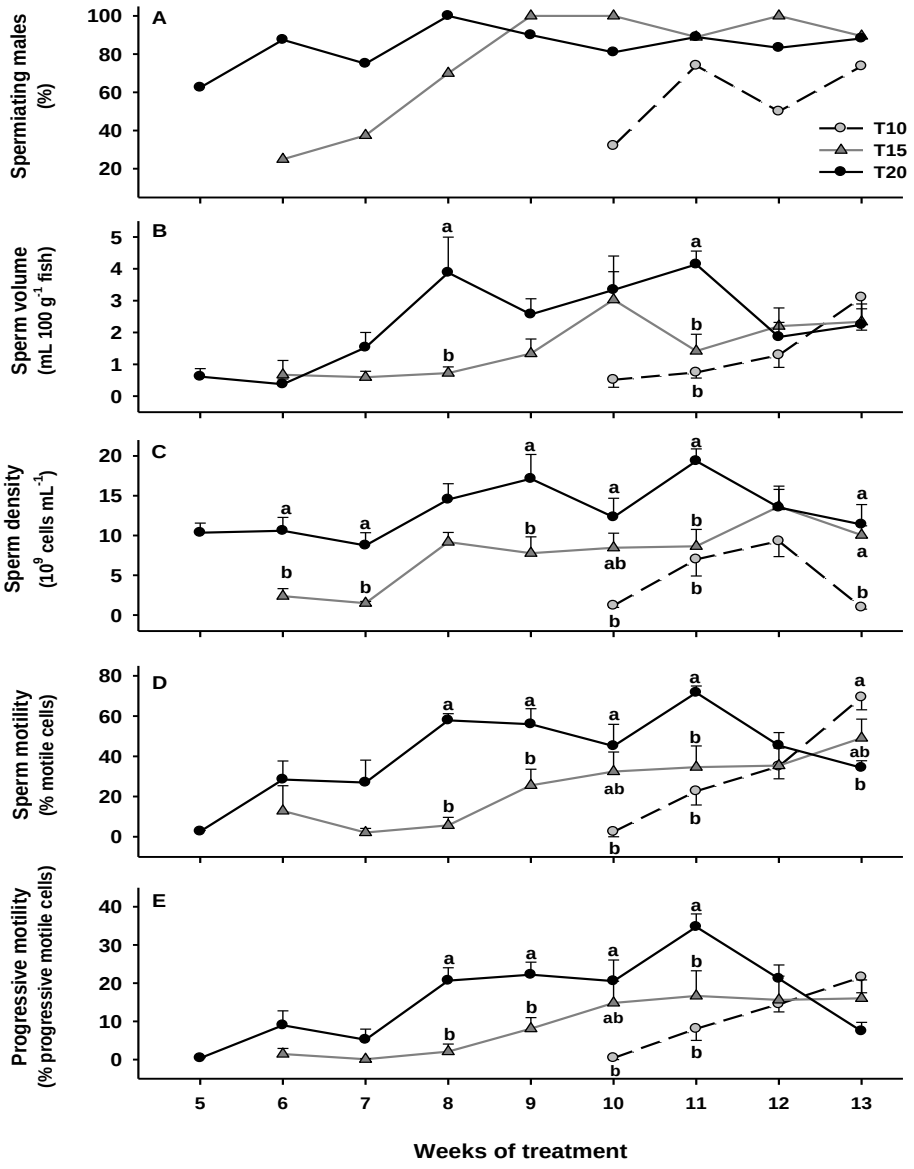

Figure 2. Evolution of sperm quality parameters throughout the temperature treatments (T10, T15 and T20): A) percentage of spermiating males; B) sperm volume; C) sperm density; D) percentage of motile cells and E) percentage of progressive motile cells. Data are expressed as mean±SEM and different letters indicate significant differences between treatments at each week of treatment.

The highest density values were observed in T20 males, with significant differences in most of the weeks (Fig. 2C). Similar density patterns were observed in T20 and T15 males, while T10 males showed an increase in their first 3 weeks of spermiation (10^{th} to 12^{th} week) followed by a marked decrease in the last week of the experiment (13th week). Regarding total and progressive motility (Fig. 2D and E), T20 treatment demonstrated the highest values, reaching maximum values of 75 and 35%, respectively at week 11. From this week to the end of treatment, T20 males displayed a marked decrease in total and progressive motility. Males subjected to the T10 thermal regime showed a significant but delayed increase in motility parameters from its first spermiation week (week 10) to the end of the experiment, and ended up exceeding the values obtained with the T15 and T20 treatments due to the fact that this coincided with the final reduction of motility in these treatments in the last week of treatment.

With regard to the sperm motility classes, it was observed that the T10 and T20 treatments displayed better volume profiles (with relative volumes of maximum quality sperm (class IV) of 60% and 70%, respectively) than the T15 treatment, which showed values of around 50% in this same motility class (Fig. 3). In addition, the T20 treatment resulted in a higher number of weeks (7) with good quality samples, compared to either the T10 or T15 treatment, which only induced these good sperm samples for 3 and 5 weeks, respectively (Fig. 4).

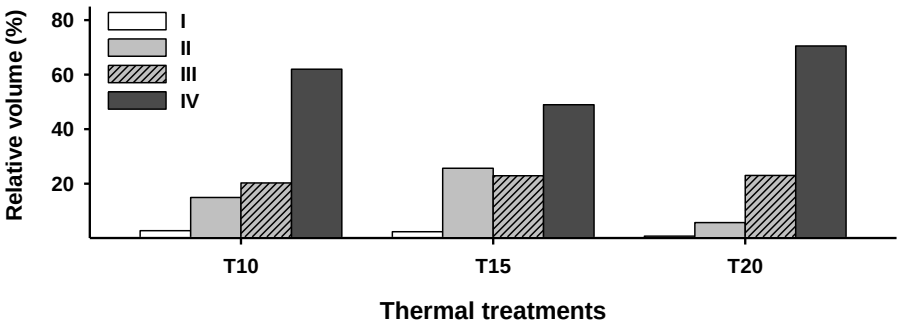

Figure 3. Percentage of total volume for each motility class (I–IV) in thermal treatments. I=no motile cells; II ≤25%; III=25–50% and IV>50% of motile cells.

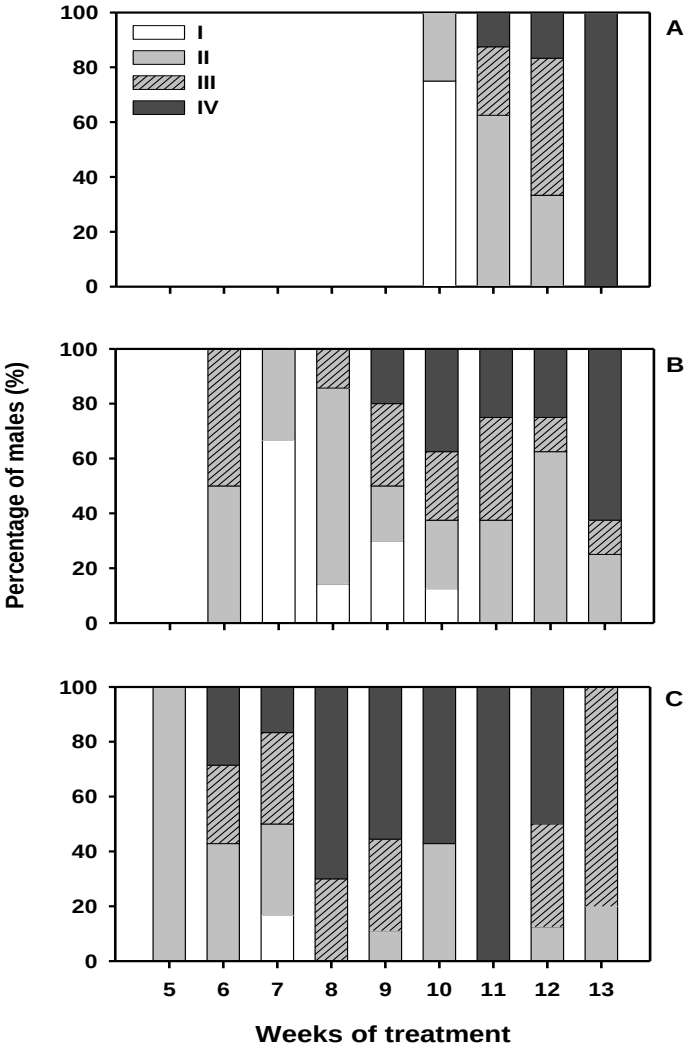

Figure 4. Percentage of spermiating males from each motility class (I–IV) in each week throughout the thermal treatments: A) T10; B) T15 and C) T20. Class I=no motile cells; II≤25%; III=25–50% and IV>50% of motile cells.

The kinetic parameters (Table 1) of the sperm cells showed a similar evolution in all thermal treatments, increasing as the weeks advanced. T20 induced the highest values in the three velocity parameters (VCL, VSL and VAP), with significant differences in several weeks.

Table 1. Kinetic parameters (VCL, VSL and VAP) of sperm cells throughout the thermal treatments (T10, T15 and T20). Data are expressed as mean (SEM) and different letters indicate significant differences between treatments.

	VCL						VSL						VAP					
Week	**T10**		**T15**		**T20**		**T10**		**T15**		**T20**		**T10**		**T15**		**T20**	
5					65.5	(12.0)					25.5	(6.2)					39.6	(7.3)
6			127.8	(43.6)	95.9	(16.2)			61.0	(31.7)	39.0	(9.2)			83.0	(37.8)	56.1	(10.4)
7			24.1	(24.1)	77.2	(16.8)			5.4	(5.4)	27.6	(6.0)			12.1	(12.1)	43.8	(9.5)
8			82.2	(23.5)	140.0	(9.0)			36.3	(12.3) b	63.3	(6.2) a			48.6	(14.8) b	85.7	(5.9) a
9			85.6	(22.1) b	142.6	(9.0) a			39.9	(10.3) b	65.7	(5.3) a			53.7	(13.4) b	88.1	(5.9) a
10	33.5	(33.5)	117.7	(20.9)	126.0	(10.8)	6.7	(6.7) b	51.2	(14.4) ab	63.9	(6.0) a	15.7	(15.7) b	69.9	(15.8) ab	83.0	(7.1) a
11	122.1	(13.6)	122.7	(17.5)	149.9	(6.4)	49.1	(7.1)	57.5	(11.2)	72.2	(4.8)	69.8	(8.3)	76.0	(13.0)	94.3	(5.6)
12	119.2	(9.8)	123.1	(11.4)	121.0	(8.4)	57.8	(4.7)	52.8	(9.5)	57.0	(4.2)	77.0	(6.3)	71.5	(9.5)	73.5	(5.0)
13	133.2	(13.6) a	113.6	(12.4) ab	74.1	(5.1) b	56.4	(7.8)	49.1	(8.8)	30.2	(4.2)	79.7	(9.4) a	67.9	(9.4) ab	45.8	(3.8) b

3.2 Hormonal treatments

PMSG treatment induced lower percentages of spermiating males (with maximum values around 50–60%) than hCG and hCG_{rec} treatments (Fig. 5A), both of which reached 100% in several weeks. In addition, PMSG males did not begin to spermiate until the 8^{th} week of treatment, while hCG and hCG_{rec} males began spermiating earlier, in the 5^{th} week.

An increasing trend in sperm volume (Fig. 5B) was displayed in all the hormonal treatments and hCG- and hCG_{rec}-treated males showed higher values than PSMG-treated males, with significant differences in the 10^{th}, 11^{th}, 14^{th} and 18^{th} weeks. Maximum values for PMSG, hCG and hCG_{rec} treatments were obtained in the 15^{th}, 19^{th} and 20^{th} weeks, respectively.

Sperm density showed high variability under all the hormonal treatments (Fig. 5C). Samples from males treated with hCG_{rec} demonstrated an increase in the first 5 weeks of treatment reaching maximum values of 18×10^{9}, followed by a decrease from the 10^{th} week until the end of treatment. However, in the first 5 weeks of spermiation hCG-treated males displayed a progressive decrease and a gradual decline until the end of the treatment, reaching minimum values in the 19^{th} week. PMSG-treated males generated the highest values at the end of the treatment, with significant differences in the 15^{th}, 17^{th}, 18^{th} and 19^{th} weeks.

With regard to motile and progressive motile cells (Fig. 5D and E), hCG_{rec} treatment generated the highest values, reaching maximums of 70 and 35% (motile and progressive motile cells, respectively) in the 9^{th} week. Males treated with hCG showed a similar motility pattern to hCG_{rec} males in the first weeks of treatment, but displayed a decrease in both parameters from the 11^{th} week to the end of treatment. PMSG-treated males showed an upward trend from the 11^{th} week, reaching motility and progressive motility values similar to hCG_{rec}-treated males in the last 7 weeks of treatment.

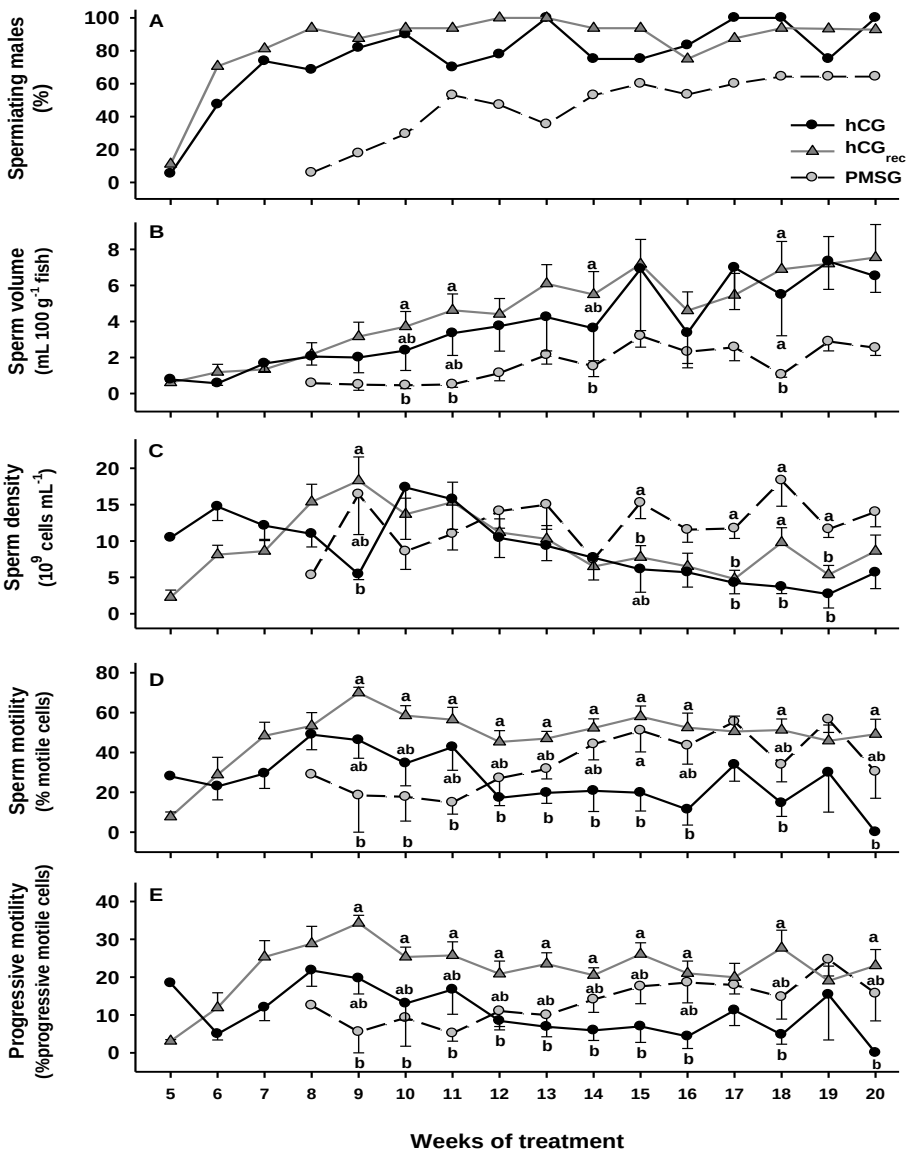

Figure 5. Evolution of sperm quality parameters throughout the hormonal treatments (hCG, hCG_{rec} and PMSG): A) percentage of spermiating males; B) sperm volume; C) sperm density; D) percentage of motile cells and E) percentage of progressive motile cells. Data are expressed as mean±SEM and different letters indicate significant differences between treatments at each week of treatment.

With regard to the sperm motility classes, hCG_{rec} treatment induced good quality samples (IV) in every week of treatment (except in the 5th week), while hCG and PMSG treatments showed some weeks without good sperm samples (weeks 6 and 3, respectively) throughout the treatment (Fig. 7). hCG_{rec} and PMSG treatments produced better volume profiles (with class IV sperm volumes around 70% and 60%, respectively) than hCG treatment, which resulted in values of less than 30% for the same motility class (Fig. 6).

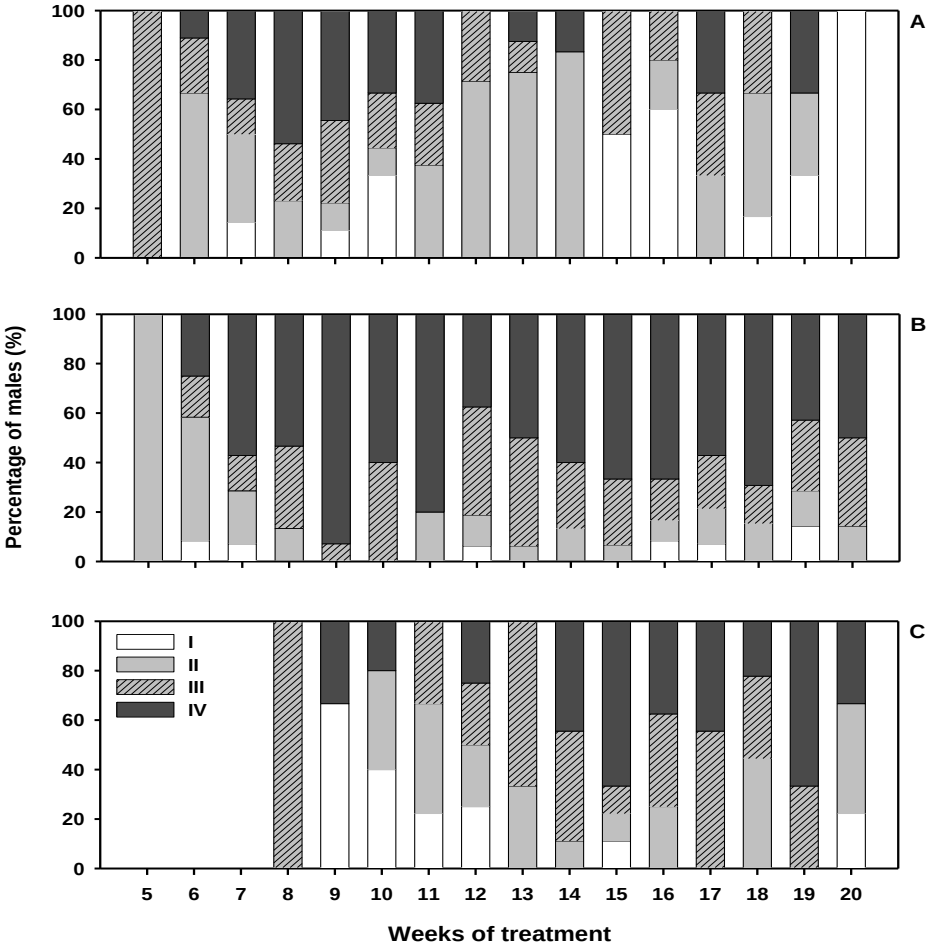

Figure 6. Percentage of spermiating males from each motility class (I–IV) in each week throughout the hormonal treatments: A) hCG; B) hCG_{rec} and C) PMSG. Class I=no motile cells; II≤25%; III=25–50% and IV>50% of motile cells.

Table 2. Kinetic parameters (VCL, VSL and VAP) of sperm cells throughout the hormonal treatments (hCG, hCGrec and PMSG). Data are expressed as mean (SEM) and different letters indicate significant differences between treatments at one sampling time.

	VCL						VSL						VAP					
Week	hCG		hCG_{rec}		PMSG		hCG		hCG_{rec}		PMSG		z		hCG_{rec}		PMSG	
5	121.–	(0.0)	113	(14.1)			63.–	(0.0)	55.–	(13.7)			77.4	(0.0)	67.2	(14.1)		
6	86.5	(6.6)	108	(12.9)			30.–	(5.1) b	50.–	(7.1)			49.9	(5.0)	67.5	(8.8)		
7	111.–	(14.9)	127	(13.2)			48.–	(7.5)	66.–	(7.1)			66.5	(9.4)	85.3	(8.6)		
8	129.–	(8.5)	141	(10.5)	109.–	(0.0)	60.–	(5.7)	72.–	(7.0)	50.–	(0.0)	80.9	(6.4)	91.6	(7.9)	66.5	(0.0)
9	141.	(18.7)	166	(4.1) a	36.	(36.5) b	63.	(8.5) ab	86.	(3.9)	14.	(14.9) b	85.3	(11.3) a	110.	(3.6) ab	21.9	(21.9)
10	96.5	(24.4)	147	(7.8) a	67.	(29.6) b	46.–	(12.2)	72.	(5.0)	31.–	(14.7) b	59.7	(15.4)a	94.9	(5.7) a	41.2	(18.9)
11	119.	(13.4) a	137	(7.0) a	65.	(14.2) b	52.	(9.2) ab	70.	(5.4)	31.	(8.3) b	73.6	(10.2) a	90.6	(6.0) a	42.9	(10.4)
12	118.	(5.2) ab	130	(10.7)	82.	(19.5) b	59.	(5.5)	68.	(6.9)	40.	(10.9)	74.8	(5.6)	86.9	(7.8)	53.1	(13.5)
13	112.–	(10.4)	145	(7.6) a	107.–	(10.9) b	43.–	(6.5) b	75.	(5.7)	48.–	(8.3) b	63.4	(7.1) b	94.9	(5.9) a	66.2	(8.8) b
14	129.	(9.5)	159	(7.2)	132	(12.3)	54.	(5.0) b	76.	(4.9)	56.	(6.7) ab	75.9	(6.1) b	100.	(5.0) a	78.9	(8.0)
15	71.9	(33.2) b	171	(7.7) a	130	(20.9)	32.	(16.0) b	86.	(5.8)	57.	(10) ab	44.2	(20.8) b	110.	(5.9) a	80.1	(13.8)
16	56.2	(34.9) b	148	(15.6)	150.–	(11.4) a	26.–	(16.8)	73.–	(8.3)	68.–	(8.5)	35.7	(22.3)	95.6	(9.9)	90.8	(9.1)
17	127.	(16.2)	155	(12.8)	153	(8.3)	59.	(10.4)	71.	(7.0)	69.	(5.3)	78.4	(11.5)	94.8	(8.4)	95.7	(6.3)
18	108.–	(23.6)	156	(12.1)	133.–	(15.0)	39.–	(9.7) b	83.	(8.9)	59.–	(10.6)	60.9	(13.8) b	103.–	(9.2) a	81.2	(11.7)
19	109.–	(54.9)	116	(15.7)	150.–	(7.8)	48.–	(26.6)	56.–	(8.2)	71.–	(5.5)	65.2	(33.8)	73.9	(10.3)	94.4	(6.5)
20	0.0	(0.0) b	140	(9.5) a	123	(26.2) a	0.0	(0.0) b	69.	(7.4)	60.–	(13.8) a	0.0	(0.0) b	90.2	(7.7) a	78.1	(17.2)

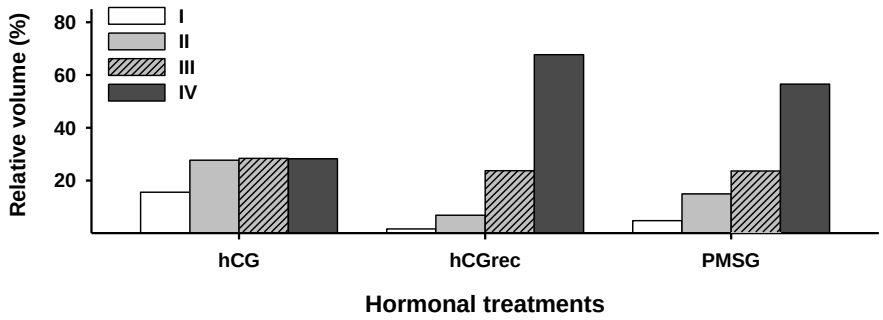

Figure 7. Percentage of total volume for each motility class (I–IV) in hormonal treatments. I=no motile cells; II ≤25%; III=25–50% and IV>50% of motile cells.

The hCG_{rec} and hCG treatments produced the highest values in VCL, VSL and VAP throughout the first weeks of spermiation (Table 2). However, from the 14th week of treatment hCG-treated males displayed a sharp decline in these parameters, whereas values of hCG_{rec}-treated males remained constant until the end of treatment.

3.3 Economical analysis

The investment needed to obtain mature males was quite different in each hormonal treatment (Table 3). hCG_{rec} treatment signified the highest investment per male and an amount of 19.5 € was necessary in order to mature each animal throughout the 21 weeks. hCG and PMSG treatments represented a smaller investment per male (7.5 and 10.6 €, respectively).

Table 3. Profitability of hormonal treatments (hCG, hCG_{rec} and PMSG) in relation to economic investment and production of high quality sperm.

		hCG	hCG_{rec}	PMSG
Hormone price	€/IU	0.003	0.008	0.004
[a]Dose price	€/$g_{\text{treated fish}}$	0.005	0.012	0.007
[b]Investment per male	€/male	7.5	19.5	10.6
[c]Sperm (class IV) price	€/mL	0.7	0.5	1.8

[a] *Hormone Price x 1.5 IU g^{-1}*
[b] *Investment to maturate one male during 21 weeks of treatment.*
[c] *Total Investment / Total Volume of Sperm (class IV)*

However, the total volume of class IV sperm obtained from hCG_{rec}-treated males was much higher, therefore the final profitability of this hormone was the best, as it was possible to obtain 1 mL of the highest quality sperm for the lowest price (0.5 €/mL). The other hormones produced worse economic results, and PMSG was found to be the most expensive treatment (1.8 €/mL).

4. Discussion

4.1 Thermal treatments

Temperature is one of the most important environmental factors affecting aquatic wildlife organisms, where seasonal changes in this parameter, interacting with the photoperiod signal, can regulate the sexual maturation process (Dorts et al., 2012; Van Der Kraak and Pankhurst, 1997).

In terms of aquaculture production, obtaining spermiating males weeks in advance means minimizing costs and risks in fish handling. In this respect, T20 males began spermiating earlier and also demonstrated higher percentages of spermiating males in all weeks, compared to the alternative thermal treatments (T15 and T10). In addition, it was observed that warm temperatures were strictly necessary in inducing sperm production in European eel males: fish that underwent both T10 and T15 thermal regimes did not begin to produce sperm until they had spent 1–2 weeks at 20 °C. Therefore, it seems that the lower temperatures used during the first weeks of T10 and T15 treatments were capable of preventing the spermiation process and thus, the production of sperm. In a previous study about the temperature effect in the sexual maturation on the European eel, Boëtius and Boëtius (1967) could obtain males in an earlier stage of maturity, in which the lumen of their spermatic tubules is being filled of spermatozoa, from a wide temperature range of 13 to 25.5 °C after 10 weeks of treatment. However, the sperm volume obtained in this trial was not good, and a mathematical analysis of the temperature/maturation period data revealed an optimum temperature of about 20 °C.

In this respect, it is well known that water temperature can modulate the enzymatic activity necessary for the synthesis of steroids and its receptors and so, the different thermal treatments applied in this study could be regulating all stages of spermatogenesis and spermiogenesis throughout the gonadal development (Billard et al., 1982; Peñaranda et al., 2011; Schulz and Miura, 2002). Previous studies have demonstrated the effects of temperature on the spermatogenesis of fish: in rainbow trout (*Salmo gairdneri*) low temperatures stimulate the first stages of this process, while warm temperatures stimulate the latter stages (Breton and Billard, 1977); in Nile tilapia (*Oreochromis niloticus*) higher temperatures accelerate spermatogenesis, whereas at lower temperatures it takes longer (Vilela et al., 2003).

On the other hand, parameters such as volume and density are usually analyzed in sperm studies in order to report information on the amount of sperm available for use in reproductive events like artificial gamete fertilizations, etc. In the present study, the volume data agrees with values obtained by other authors of European eel studies (1–4 mL 100 g^{-1} fish; Asturiano et al., 2005; Pérez et al., 2000), and the sperm density values obtained were significantly higher than those obtained in these same experiments (values around 1–2×10^9 mL−1). In this respect, it would be important to find out the minimum sperm-to-egg ratio needed for successful fertilization, but this parameter is only known for a limited number of species and few studies have been developed with regard to the European eel (Sørensen et al., 2013).

On the other hand, total motility and progressive motility are recognized as important sperm traits for male fertility and sperm competition, because significant correlations were found between the number of motile spermatozoa and fertilization rates in some fish species (Liu et al., 2007; Ottesen et al., 2009; Rurangwa et al., 2004). In our study, T20 was the treatment that demonstrated the best values throughout most of the weeks, whereas alternative thermal regimes (T10 and T15) did not reach such high values, except for T10 males that did show values of around 70% of motile cells in the last week of treatment. Therefore, T20 was the most

effective treatment with regard to these parameters (total and progressive motility), resulting in good quality samples (class IV) in almost every week of treatment, from the 5th week onwards.

In addition to percentage of motile spermatozoa as a good tool to predict fertilization ability, kinetic sperm parameters (VCL, VSL or VAP) provided by CASA software may also serve as prognostic indicators of the fertilization potential of sperm (Donnelly et al., 1998; Gage et al., 2004; Rudolfsen et al., 2008). In our study, T20 males showed higher velocity values than T15 and T10 males in most of the weeks of treatment, and VCL values were comparable with data previously reported in European eel: 134 μm/s (Gibbons et al., 1985), 160 μm/s (Woolley, 1998) or 125 μm/s (Asturiano et al., 2005). However, VSL values obtained under these thermal treatments were significantly higher than those reported in the cited literature. With VSL being one of the most important kinetic parameters (probably because spermatozoa with faster straight line speeds have more chance of contacting an oocyte in the natural environment), sperm samples induced in the present study showed better quality spermatozoa than those demonstrated in previous reproduction studies of this same species (Gibbons et al., 1985; Woolley, 1998).

In summary, our results demonstrate that the onset and progression of spermiation in European eel males are strongly influenced, and perhaps closely regulated, by changing water temperature. The T20 regime showed the best results in all of the sperm parameters (volume, density, motility, kinetic features, etc.) throughout most weeks of treatment, becoming a reliable and productive method for inducing spermiation in this species.

4.2 Hormonal treatments

Our results indicate that the type of hormone used affected significantly the onset and progression of spermiation in European eel males. Recombinant hCG (hCG_{rec}) produced the best results in almost all the sperm parameters throughout the weeks of treatment, becoming an effective and alternative

treatment to that of the standard hCG used to induce the spermiation in European eel at 20 °C.

With regard to the onset of spermiation, both hCG- and hCG_{rec}-treated males began to produce sperm in the 5th week of treatment, and both treatments induced a high percentage of spermiating males in the following weeks. Previous studies have reported similar results with this hormonal treatment both in European eel, where eel males usually begin producing sperm in the 4th–5th weeks of treatment (Asturiano et al., 2006; Peñaranda et al., 2010a; Pérez et al., 2009), and in Japanese eel, where males usually begin producing sperm in the 5th–6th weeks of treatment (Ohta et al., 1996, 1997). However, males induced by PMSG began to spermiate later, in the 8th week, showing a lower percentage of spermiating males throughout all the treatments. It appears that this hormone caused a delay in the gonadal development and thus, late spermiation.

These different responses found in eel males regarding the different hormonal treatments could be explained by the biological activity of each hormone. This bioactivity depends on dimerization and glycosylation, which are processes occurring in the rough endoplasmic reticulum and Golgi apparatus (Ulloa-Aguirre et al., 2001). There are different degrees and types of glycosylation, and depending on these types, gonadotropins will show more or less bioactivity (Hearn and Gomme, 2000; Ulloa-Aguirre et al., 1999). In the present study, the hormones used to induce the maturation in the European eel had different characteristics and origins: hCG is a hormone produced during human pregnancy and purified from urine, while hCG_{rec} is a recombinant version of endogenous hCG produced in Chinese hamster ovary (CHO) cultured cells by recombinant DNA technology. Both hormones (hCG and hCG_{rec}) are analogues of the luteinizing hormone (LH). On the other hand, PMSG is a complex glycoprotein obtained from the serum of pregnant mares and acts like a follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Therefore, considering the different nature and origins of these hormones, it is possible that each hormone has different degrees and types of glycosylation and thus performs the stages of maturation in a different way.

On the other hand, sperm volume and density are important parameters that have traditionally been used for the assessment of semen quality. Customarily, sperm volume obtained from the artificially maturated eel males has been unsuitable compared with the volume of eggs obtained from eel females (Ohta and Unuma, 2003). In this trial, milt volume increased gradually under all the treatments as the number of injections increased, probably due to the cumulative effect of hormones in the first weeks (Asturiano et al., 2005; Ohta et al., 1996; Pérez et al., 2000) and finally, due to the hydration controlled by the maturation-inducing steroids (MIS) in the last weeks (Asturiano et al., 2004b). hCG_{rec}- and hCG-treated males showed the highest volumes throughout the treatment (>5 mL 100 g^{-1} fish from the 15th week onwards), exceeding previous values obtained both in European (Pérez et al., 2000) and Japanese (Ohta and Unuma, 2003) eel males. A reverse trend was evidenced in density and volume from the 10th week of treatment: as the sperm volume increased, the density values decreased. This opposite pattern may be explained by the fact that high densities are probably necessary in order to compensate for the small volumes and, in the opposite case, low densities need to be compensated by high volumes of sperm production.

On the other hand, with regard to motility and progressive motility, hCG_{rec} was the treatment that produced the best values (both high and stable) in every week. In eel species, the potential of sperm motility is usually acquired during the period between the 7th and 9th injections (Ohta and Unuma, 2003; Pérez et al., 2000). In this trial hCG_{rec}- and hCG-treated males showed a similar trend, while PSMG-treated males did not show high percentages of motile cells until the 15th week. hCG_{rec} treatment generated good quality samples for the largest number of weeks, displaying samples of this kind (class IV) in almost every week of treatment. The kinetic parameters showed a similar trend to that of the motility data and hCG_{rec} was the treatment that induced higher velocities throughout all the sampling weeks. hCG- and PMSG-treated males also showed good values in these parameters, but only at the beginning and end of treatment, respectively.

Our results demonstrate that the onset and progression of spermiation in European eel males are influenced by the hormone used. In this respect, hCG_{rec} showed the best results in all the sperm parameters (volume, density, motility, kinetic features, etc.) throughout most weeks of treatment, becoming an effective and alternative treatment to the standard hCG treatment used to induce spermiation in eel species.

4.3. Economic analysis

From a practical point of view, the best hormonal treatment is one which is able to provide samples with high values of volume, density, motility and kinetic parameters for as many weeks as possible. However, due to the current economic crisis, the aquaculture sector is going through a delicate situation and cheaper and more effective treatments are becoming more and more necessary. In the present study, hCG_{rec} treatment generated the best results, improving on the results obtained by hCG hormone, which nowadays is the most widely used method of inducing male maturation in eel species. In addition, despite the greater investment required for hCG_{rec}-treated males, the final profitability of this hormone was demonstrated to be the best, making it possible to obtain 1 mL of good quality sperm for a lower price than possible using either of the other two hormonal treatments (hCG and PSMG).

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CHAPTER 3

Intracellular changes in Ca^{2+}, K^{+} and pH after sperm motility activation in the European eel (*Anguilla anguilla*)

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Abstract

Although it is widely accepted that osmolality and ion fluxes are the main factors triggering sperm motility in fish, a complex universal mechanism for sperm motility initiation does not exist in fish, and studies of marine fish species are even more scarce. Therefore, the main goal of this study was to estimate the intracellular variations in the main ions involved in sperm activation for the first time in European eel, in order to provide additional new data about this little-known process.

It was observed that levels of intracellular Ca^{2+} and K^{+} sperm ions increased significantly 30 s after the hyperosmotic shock compared to baseline levels, and remained at this level until 120 s post-activation. In contrast, the intracellular pH remained constant during the first 30 s, and decreased gradually at 60 and 120 s post-activation. Our data agree with the current main theory for explaining motility initiation in marine fish, in which internal fluctuations of Ca^{2+} and K^{+} seem to participate in sperm activation. In addition, fluorescent images showed that both Ca^{2+} and K^{+} were concentrated in the apical area of the sperm head, which corresponds to the location of the eel mitochondria, suggesting this organelle plays an important role in sperm motility activation.

1. Introduction

In marine teleosts, the spermatozoa are quiescent in isotonic solutions, such as seminal plasma, and become motile when the sperm is diluted in hypertonic solutions, suggesting that motility is suppressed by the osmolality of the seminal plasma, and initiated by exposure to hypertonic seawater at spawning (Morisawa and Suzuki, 1980, Cosson, 2004, Morisawa, 2008). The osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water between intracellular compartments and external medium (Oda and Morisawa, 1993; Zilli et al., 2009). It has been proposed that Ca^{2+} and K^{+} are the main ions involved in sperm motility initiation in marine fish (see reviews of Morisawa, 2008, Cosson, 2008), but the exact mechanism

through which this happens is still unknown. Although both in marine and freshwater fish species an increase in intracellular Ca^{2+} has been observed after osmotic sperm motility activation, it is not clear if that increase comes from an influx of Ca^{2+}, as has been proposed in seawater tilapia (*Oreochromis mossambicus*; Linhart et al., 1999), if it comes from intracellular stores, as it has been proposed in the case of puffer fish (*Takifugu niphobles*) and salmonid sperm (Krasznai et al., 2003; Takei et al., 2012), or from a decrease in cell volume due to the water efflux, as has been proposed by Zilli et al. (2008) in sparids species sperm. The first step to elucidate this mechanism in European eel sperm is studying intracellular Ca^{2+} variations in quiescent sperm and then hyperosmotic activated motility.

Changes to intracellular potassium levels after sperm activation have been measured in a limited number of freshwater fish species: two salmonids (Tanimoto et al., 1994), and common carp (Krasznai et al., 2003) sperm, where a K^+ efflux or a decrease in $[K^+]_i$ after hypoosmotic activation was observed. In marine fish species, $[K^+]_i$ changes after sperm activation have only been studied in the case of the pufferfish, and an increase in $[K^+]_i$ was observed after hyperosmotic activation (Takai & Morisawa, 1995, Krasznai et al., 2003). It is unknown whether hyperosmotic activation in the sperm of other marine fish causes an intracellular increase in K^+, like in pufferfish, or a decrease, like in trout and carp.

In sea urchin (Lee et al., 1983) and mammals (Wong et al., 1981; Babcock et al., 1983), the alkalinization of intracellular pH induces sperm activation. In carp a pH increase was also observed after hypoosmotic sperm activation (Krasznai et al., 1995), but trout sperm undergo an acidification upon hypoosmotic activation (Boitano and Omoto, 1991). An increase in pH_i was observed at motility initiation in the sperm of several marine fish species (Oda and Morisawa, 1993). Therefore, there is no current consensus on the intracellular pH changes related to sperm activation in fish species. We observed that extracellular pH was important for sperm motility in European eel; while acidic pH extenders (pH 6.5) induced a reversible

motility inhibition, that was not observed when sperm was maintained in an extender at physiological pH (8.5) (Peñaranda et al., 2009).

Thus, this study was designed to observe the changes in intracellular Ca^{2+}, K^{+}, and pH after motility activation in European eel spermatozoa. In this study we used flow cytometry to describe the variations of these factors from the immotile stage to the motile stage, at different times after the initiation of sperm motility, with the aim of establishing the first hypothesis on motility activation in this species.

2. Materials and methods

2.1 Fish handling and sampling

Fifteen adult eel males (100±2 g; 40±5 cm) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to the aquaculture facilities at the Universidad de León (Spain). The fish were distributed in three 60-L aquaria (5 males per aquarium) equipped with separate recirculation systems, thermostats and covered to maintain constant darkness. The eels were gradually acclimatized to artificial seawater (Aqua Medic Meersalz, 37 g/l) and once a week they received an intraperitoneal injection of hCG (1.5 IU g^{-1} fish; Argent Chemical Laboratories, USA), after being anesthetized (benzocaine, 60 ppm) and individually weighed. The total treatment lasted 14 weeks, and sperm samples were obtained from the sixth week until the end of the treatment. The fish were fasted 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 Sperm collection and evaluation of motility

Sperm samples were collected 24 h after the administration of the hormone to obtain the highest quality sperm (Pérez et al., 2000). The eels were anesthetized, and the genital area was first cleaned with distilled water and then thoroughly dried to avoid contaminating the sperm with faeces, urine or seawater. Then, the sperm was collected by applying gentle

abdominal pressure with the help of a small modified aquarium air pump which produced a vacuum breathing force to help collect the sperm in plastic Falcon tubes. Sperm samples were kept at 4 ºC until the motility analyses, which were performed less than 1 hour after collection. Sperm motility was assessed subjectively in triplicate by a trained observer, after mixing 1 µl of sperm with 200 µl of artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2) and observed under a microscope in a glass slide. Samples having >50% of motile cells were selected to study intracellular ionic changes. They were diluted 1:100 in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, and pH 8.5; Peñaranda et al., 2010) and maintained at 4 ºC until the flow cytometry analysis.

2.3 Flow citometry

The relative amounts of different ions were determined by flow cytometry using a CyAn ADP Flow Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser. Slightly angled scattered front light was used for the electronic gating of data collection, allowing us to exclude dead cells from the analyses.

2.3.1 Incubation protocol

For Ca^{2+}, a stock solution of 1mM Fluo-4 AM (Invitrogen F14201) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm was incubated with 0.5 µl Fluo-4 AM (final concentration 5 µM) at RT (20 ºC) for 30 minutes. For K^+, a stock solution of 1mM PBFI AM (Invitrogen, P1267) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm was incubated with 0.5 µl PBFI AM (final concentration 5 µM) at RT (20 ºC) for 90 minutes. For pH_i, a stock solution of 1mM Snarf-5F AM (Invitrogen, S23923) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm was incubated with 0.5 µl Snarf-5F AM (final concentration 5 µM) at RT (20 ºC) for 45 minutes.To exclude dead cells from the analysis, the spermatozoa were also incubated with TO-PRO®-3 (Invitrogen T7596) to reach a final concentration of 2 µM. Final DMSO concentrations in sperm

were less than 0.05 % in all the cases, and therefore a DMSO effect on motility could be discarded.

2.3.2 Determination of intracellular Ca^{2+} and K^{+} and pH

After the incubation time, 5 µl of sperm sample was added to a tube containing P1 medium (500 µl) to measure the fluorescence emitted by the specific ion in the quiescent stage. Later, 5 µl of sperm sample was added to a tube containing activation medium (seawater, 500 µl) and the fluorescence emitted by sperm cells at 30, 60 and 120 s after activation was recorded. Fluo-4 AM and Snarf-5F AM were both excited by the blue laser (488 nm), and their fluorescence was read with the FL1 (530/40BP filter) and FL4 (680/30BP filter) photodetector, respectively. PBFI AM was excited by ultraviolet light (340 nm) and its fluorescence was read with the FL6 photodetector (450/50BP filter). TO-PRO-3 was excited by the use of red laser (635 nm), and its red fluorescence was read with the FL8 photodetector (665/20BP filter). The fluorescence data was displayed in logarithmic mode. Ten thousand events were collected per sample, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis.

2.4 Location of Ca^{2+} and K^{+} in quiescent spermatozoa

An aliquot of sperm samples incubated with Fluo-4 AM or PBFI AM (see section 2.3.1) was used to obtain microphotographs through a Nikon Eclipse E600 microscope. UV-2A (ultraviolet excitation and blue emission) and B-2A (blue excitation and green emission) filters were used to obtain Ca^{2+} and K^{+} images, respectively.

2.5 Statistical analysis

Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by flow cytometry. After removing dead spermatozoa (TO-PRO®-3) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) was obtained from each sample. Statistical analyses were performed using

the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyze data with normal distribution. Significant differences were detected using the Tukey multiple range test (P<0.05). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks was used.

3. Results

3.1 Intracellular concentrations of Ca^{2+}, K^+ and H^+

The fluorescence emitted by intracellular Ca^{2+}, K^+ and H^+ were estimated for quiescent sperm and after motility activation. Intracellular [Ca^{2+}] increased significantly 30 s after sperm activation compared to the baseline levels, and remained at this level until the end of activation (120 s) (Figure 1A).

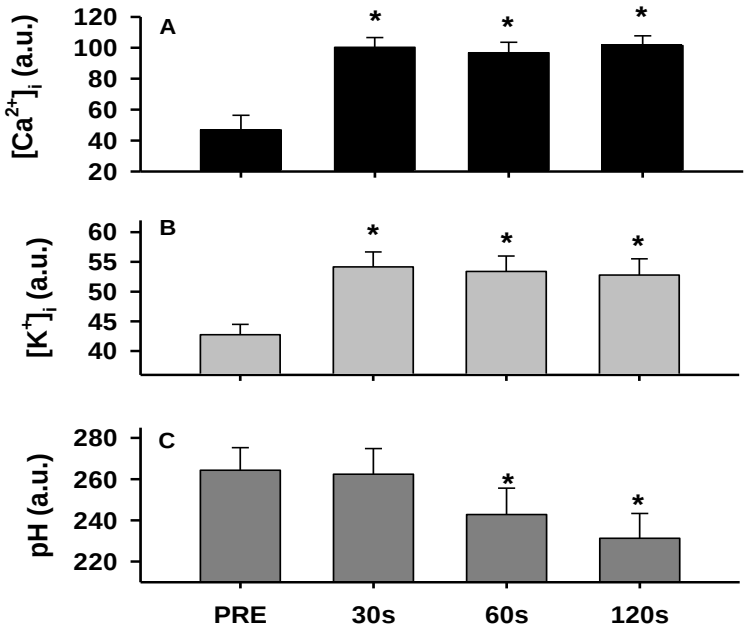

Figure 1. Intracellular ion concentrations on pre- and post-activation times (30, 60 and 120 s) in European eel spermatozoa: a) Ca^{2+}; b) K^+ and c) pH. Asterisks indicate significant differences with baseline pre-activation levels.

A similar pattern was observed with $[K^+]_i$ (Figure 1B), which increased significantly after sperm activation and remained constant thereafter. However, the $[Ca^{2+}]_i$ increase was higher than the $[K^+]_i$ increase. In contrast to Ca^{2+} and K^+, the intracellular pH (Figure 1C) level remained constant after sperm activation (30 s), only showing a significant decrease at 60 and 120 s post-activation.

3.2 Intracellular distribution of Ca^{2+} and K^+

Figure 2 shows the fluorescence emitted by the intracellular calcium and potassium located in quiescent eel spermatozoa. High fluorescence intensity for both Ca^{2+} and K^+ can be observed in the apical zone of the sperm head, which corresponds to the location of the eel mitochondria.

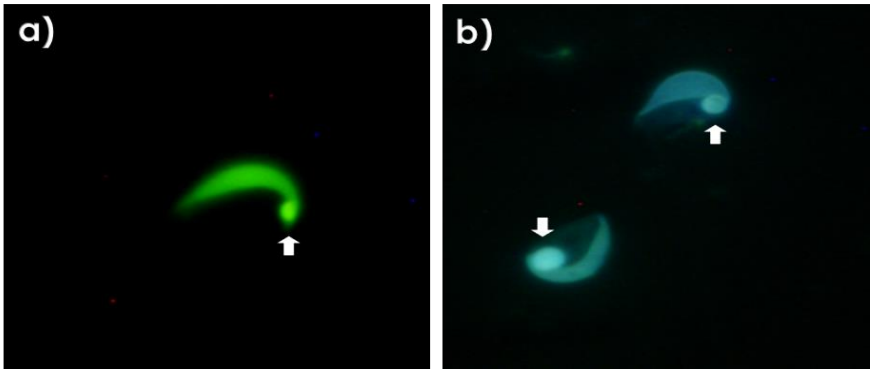

Figure 2. Pictures show (a) Ca^{2+} and (b) K^+ distribution on quiescent European eel spermatozoa. Arrows indicate mitochondrion.

4. Discussion

There is little understanding of the molecular mechanism which happens in marine sperm cells after hyperosmotic shock, and ion fluxes or variations in the concentration of several ions could act as triggers of sperm motility. We have shown for the first time that intracellular calcium and potassium levels increased with the hyperosmotic activation of sperm motility in European eel. Such increases after osmotic shock have previously been observed in

another marine fish species, the pufferfish (Oda and Morisawa, 1993), but in salmonid species (Tanimoto et al., 1994) and carp (Krasznai et al., 2003), an increase in $[Ca^{2+}]_i$, but a decrease in $[K^+]_i$ was observed after hypoosmotic sperm activation. So, internal Ca^{2+} and K^+ fluctuations seem to participate in motility initiation in European eel sperm, agreeing with the Morisawa's (2008) hypothesis which explains motility initiation in marine fish.

Regarding Ca^{2+}, it has been reported that this ion plays an important role in the control of the axonemal movement in some marine species (Zilli et al., 2012). The flagellum can change its beating pattern in response to Ca^{2+} concentrations, and thus, Ca^{2+} fluctuations could regulate the spermatozoa's kinetic features (Brokaw, 1991; Cosson et al., 2008). Oda and Morisawa (1993) reported that in the case of pufferfish the addition of Ca^{2+} ionophore to quiescent spermatozoa induced motility initiation in the same manner as in the seawater, suggesting that an increase in $[Ca^{2+}]_i$ is sufficient for the induction of sperm motility.

Regarding K^+, there are not many studies about the effect of this ion on sperm motility in marine fish species. In Atlantic croaker (*Micropogonias undulatus*), K^+ channel blockers reduced the percentage of motile cells (Detweiler and Thomas, 1998). In puffer fish it has been recently demonstrated that $[K^+]_i$ increased after sperm activation even after activation in potassium-free activation media (Gallego et al, 2013). Therefore, the increase of $[K^+]_i$ at the initiation of eel sperm motility could be one of the triggers for sperm motility initiation, as occurs in puffer fish, while in carp a decrease in intracellular K^+ was observed after hypoosmotic activation (Krasznai et al., 2003).

In relation to intracellular pH (pH_i), the baseline levels remained constant after 30 s post-activation and a gradual decrease was observed after 60 s post-activation. Our results agree with the data published by Oda and Morisawa (1993) about two marine fish species, in which after an initial increase in intracellular pH, the levels return to the baseline levels 30 s post-activation. Our first pH_i reading was taken 30 s post-activation. Therefore, we do not know if a pH increase occurs in the first few seconds

after activation in eel sperm, as is the case with pufferfish. Also, our results agree with those of Boitano & Omoto (1991), regarding rainbow trout sperm, where an intracellular acidification upon sperm hypoosmotic activation was observed. These authors considered that changes in intracellular pH do not regulate trout sperm motility, contrarily to what happens in other species. The slow decrease in pH over time observed in their study suggests that this change could be a consequence of motility activation, rather than a trigger for sperm motility initiation. The observed pH_i decrease might simply be the result of the mitochondrial sperm respiration during the active movement.

Finally, regarding the ion distribution in quiescent European eel spermatozoa, fluorescence images showed that Ca^{2+} and K^+ were located mainly in the mitochondrion, which in this species is a single, small and round organelle, located in the apex of the spermatozoa head, opposite the axoneme (Marco-Jiménez et al., 2006). The accumulation of calcium stores in the mitochondrion has previously been observed in human sperm (Costello et al., 2009), but potassium accumulation has not been reported in any other species studied so far. However, the existence of specific potassium channels in the mitochondria of several animal tissues (liver, heart, brain, kidney, skeletal muscle, human T lymphocytes and amoeba; review in Szewczyk et al., 2009) indicates that a differential concentration of K^+ between this organelle and the cytoplasm is possible in these tissues. It is not known whether sperm mitochondria possess such K^+ channels, but the accumulation of the ion in this organelle therefore suggests an important role of the eel sperm mitochondria in the function of the sperm.

In conclusion, intracellular concentrations of Ca^{2+} and K^+ ions in sperm increase after hyperosmotic sperm activation in European eel, with a progressive decrease in intracellular pH. Fluorescence images suggest an accumulation of both ions in the mitochondrion, which could represent ion stores. However, further studies including the use of ion channels blockers, activation media with/without specific ions, as well as changes in cell volume, may be necessary to determine the fluxes of these ions and their role in motility initiation on the European eel sperm.

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CHAPTER 4

Study of pufferfish (*Takifugu niphobles*) sperm: development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca^{2+} and K^{+} in the initiation of sperm motility.

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Abstract

The first goal of this study was the development of a short-term storage method for pufferfish (*Takifugu niphobles*) sperm. In this respect, the best results were obtained by diluting the sperm in a seminal-like solution and keeping it in a Petri dish in chilled storage (4 ºC). This method was successful in preserving sperm quality parameters without resulting in differences in fresh sperm for a relatively long-term period (7 days), for use in aquaculture matters. The addition of bovine serum albumin (BSA) to the medium did not improve the results.

On the other hand, both the osmolality and the ion composition of the media are essential factors which can modulate the sperm motility parameters. The osmolality of the activating medium was the most important factor in triggering pufferfish sperm motility, and osmolalities of 750-825 mOsm/kg were necessary to initiate this process, demonstrating that it appears to be a dose-independent mechanism. Regarding the ion composition of the activation media, this study has shown that despite the spermatozoa being able to initiate movement without any ion in the activation medium, the absence of ions can negatively affect the kinetic parameters of the spermatozoa. Finally, in natural conditions (seawater), the activation of sperm motility generates intracellular increases in Ca^{2+} and K^{+}, suggesting that these ions play an essential role in the activation mechanism of pufferfish sperm.

1. Introduction

The pufferfish (*Takifugu niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean, most commonly around Japan, Taiwan and Vietnam. This species is one of around 24 pufferfish species in the tetraodontine genus *Takifugu*, and it displays interesting features for its own preservation: $_{i}$) it is placed on the IUCN Red List due to the fact that its current population is not well known, making it a possible endangered species (Roberts, 1996); and ii) another closed species, like *Takifugu rubripes*, is widely-kept by scientists as a model organism (Aparicio et al.,

2002) so *Takifugu niphobles* could be used like this due to its small and similar genome (Brenner et al., 1993). Reproduction of Takifugu spp. involves the collection/handling of sperm samples, and often it is necessary to store this sperm during a relatively long period, from a few hours to several days. Different media for the cold storage (4 ºC) of fish sperm have been developed to improve the sperm handling of several freshwater and seawater teleosts, including rainbow trout, *Oncorhynchus mykiss* (Billard, 1981), zebra fish, *Danio rerio* (Jing et al., 2009), sturgeon, *Acipenser oxyrinchus desotoi* (Park and Chapman, 2005), striped bass, *Morone saxatilis* (Jenkins-Keeran and Woods, 2002), walking catfish, *Clarias macrocephalus* (Vuthiphandchai et al., 2009) and the European eel, *Anguilla anguilla* (Peñaranda et al., 2010c,d). In terms of the pufferfish sperm, a seminal-like solution has usually been used as the standard dilution agent (Krasznai et al., 2003). Despite the fact that this diluent has only ever been used on the day of sperm collection, its preservation capability over a longer period (a few days) has never been checked. Therefore, the main aim of this study was to develop a simple method for cold storage able to preserve the sperm quality parameters of *Takifugu niphobles* spermatozoa over a short-term period.

On the other hand, it is well known that spermatozoa of teleost species are immotile in the male reproductive organ, or in electrolyte or nonelectrolyte solutions with a similar osmolality to that of the seminal plasma (Alavi and Cosson, 2006). In marine teleosts, such as pufferfish, the increase in environmental osmolality is the main factor determining the activation of sperm motility (Cosson, 2004; Morisawa, 2008; Takai and Morisawa, 1995). The osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid influx/efflux of ions/water between intracellular and extracellular spaces. In this respect, the increase in intracellular concentrations of Ca^{2+} and K^{+} ions has been proposed as the trigger for the initiation of sperm motility in marine fishes (Morisawa, 2008). However, neither the origin/nature of these ions (from the extracellular medium or intracellular stores) nor their specific effects on motility and the kinetic parameters measured by CASA systems have been described.

In addition, different results can be found in literature for several species. In the case of seawater tilapia (*Oreochromis mossambicus*), it has been reported that extracellular Ca^{2+} as well as osmotic pressure are both essential factors for sperm activation (Linhart et al., 1999); however, Krasznai et al. (2003) showed that extracellular Ca^{2+} was not necessary for sperm activation in pufferfish, but rather a hyperosmotic shock is required to release Ca^{2+} from the intracellular stores; in the case of the European eel it has been reported that intracellular Ca^{2+} and K^{+} ions increase upon activation, and may have an important role in the initiation of spermatozoa motility; and finally, in the case of Atlantic croaker (*Micropogonias undulatus*), in addition to Ca^{2+} and K^{+}, Na^{+} and Cl^{-} ions seem to be involved in sperm activation (Detweiler and Thomas, 1998).

Thus, the second goal of this study was to evaluate the effects of environmental factors, including the composition and the osmolality of the medium, on the motility characteristics of spermatozoa, as well as to measure the intracellular concentrations of the main ions involved in sperm activation in pufferfish.

2. Materials and methods

2.1 Fish handling, sperm collection and sampling

The pufferfish displays a unique spawning behaviour at Arai Beach near the Misaki Marine Biological Station (MMBS, Japan). Large schools of fish arrive to the beach with the spring tide around the new moon during the spawning season between June and July (Yamahira, 1996). Spawning takes place repeatedly from 2 hours before the sunset to that moment, and during that time, pufferfish males were caught and moved to the MMBS facilities. Fish were kept in running seawater tanks at 18 °C and were fed a commercial pellet throughout the experiment. All the experiments were carried out in compliance with the animal guidelines of the University of Tokyo on Animal Care.

Fish in captivity were able to produce sperm several days after stocking and sperm samples were collected periodically over the sampling days. Before

carrying out sperm collection the genital area was cleaned with freshwater and thoroughly dried to avoid contamination of the samples with faeces, urine or seawater. Sperm samples (approximately 1 mL) were collected by applying gentle pressure to the fish abdomen, they were then maintained at 4 ºC until analysis and evaluated in the first hour after extraction.

2.2 Evaluation of motility and kinetic sperm parameters

Sperm was diluted (1:50) before the activation process in seminal plasma-like solution (SLS) consisting in 130 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM $CaCl_2$, pH adjusted to 7.5 (Krasznai et al., 2003). Sperm was activated by mixing 0.5 µl of this dilution with 4 µl of artificial seawater (ASW100) comprised of 460 mM NaCl, 10 mM KCl, 36 mM $MgCl_2$, 17 mM $MgSO_4$, 9 mM $CaCl_2$, and 10 mM HEPES, with 1% BSA (w/v) and pH adjusted to 8.2. The sperm-seawater mix was put in a SpermTrack-10® chamber and observed (Proiser R+D, S.L.; Paterna, Spain). Video sequences were recorded through a high-sensitivity video camera (HAS-220; 50 fps) mounted on a phase contrast microscope (Olympus BX51) with a 10x objective lens (Olympus Splan NH). All the motility analyses were performed by triplicate using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain).

The parameters considered in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in a essentially straight line; curvilinear velocity (VCL, µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; 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; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its average spatial trajectory; the percentage of fast (FA; VAP > 100 µm/s), medium (ME; VAP = 50-100 µm/s) and slow (SL; VAP = 10-50 µm/s) spermatozoa; straightness (STR, %), defined as the linearity of the average spatial path; linearity (LIN, %), defined as the linearity of the curvilinear trajectory; wobble (WOB, %), defined as the trajectory

oscillation along its average spatial path; amplitude of lateral head displacement (ALH, µm), defined as the measure of lateral displacement of a sperm head along its average spatial trajectory; and beat cross frequency (BCF, beats/s), defined as the time-average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was lower than 10 µm/s.

2.3 Experimental design for short-term storage

Sperm samples collected from pufferfish were stored in 4 different ways: $_{i}$) 40 µl of undiluted fresh sperm was kept in an open 500 µl Eppendorf microtube (EP); ii) 40 µl of undiluted fresh sperm was kept in a 5 ml closed Petri dish (PD); iii) 40 µl of fresh sperm was diluted in 1960 µl of SLS (1:50) and kept in a closed 5 ml Petri dish, and finally, iv) 40 µl of fresh sperm was diluted in 1960 µl of SLS (1:50) containing 2% BSA (w/v) and kept in a closed 5 ml Petri dish. All the samples were stored in a refrigerator at 4 ºC during the whole experimental period.

2.4 Trials of different activation media

In the first trial, different activation media (Table 1) with different osmolalities and ionic compositions (obtained using different dilutions of ASW100, described in section 2.2) were tested in the activation process of pufferfish sperm samples. In the second trial, a non-electrolyte activation medium (GLU; 1100 mM Glucose, 5 mM HEPES and 5 mM EGTA) with an osmolality of around 1000-1100 mOsm/Kg was compared to the standard activation medium (ASW100, with an osmolality of around 1000-1100 mOsm/Kg). With the aim of avoiding any kind of ion contamination during sperm handling before activation, sperm was washed three times with a non-electrolyte solution (NEM, consisting in 300 mM Glucose, 5 mM HEPES and 5 mM EGTA, pH 7.5), as follows: sperm was diluted 1: 50 in NEM, centrifuged (5 min, 700 g) and the precipitate was resuspended and incubated in NEM solution for 5 min. This step was done in triplicate. Finally, the washed sperm was activated with ASW100 or GLU solutions (pH=8.2; 1% BSA (w/v)).

Table 1. Activation media used for the trial about medium´s osmolality. The osmolalities were calculated theoretically through the medium dilution/composition.

Activation media	Dilution ASW100:DW	Osmolality (mOsm/Kg)
ASW100	1:0	1000-1100
ASW075	3:1	750-825
ASW050	1:1	500-550

2.5 Determination of intracellular Ca^{2+} and K^{+} concentrations

Two sperm washing protocols, with SLS or NEM, were used before measuring the relative amounts of different ions in the pre- and post-activation times. In the first protocol (a), sperm samples were diluted 1:50 in SLS, then centrifuged (5 min, 700 g) and the precipitate was resuspended in 500 µl of SLS solution and incubated for 5 min. This step was repeated three times. In the second protocol (b), sperm samples were diluted 1:50 in NEM, centrifuged for 5 min at 700 g and the precipitate was resuspended in 500 µl of NEM solution and incubated for 5 min (this step was repeated three times). Finally, the washed sperm was activated with both ASW100 and GLU solutions (pH=8.2; 1% BSA (w/v)).

The relative intracellular amounts of calcium ($[Ca^{2+}]_i$) and potassium ($[K^{+}]_{i,}$) were analysed by a fluorescent spectrophotometer (650 10-S, Hitachi, Japan). To carry out $[Ca^{2+}]_i$ analysis , the spermatozoa were loaded with Fluo-4 AM indicator (Dojindo F312) for a final concentration of 5 µM for 30 min using an excitation/emission wavelength of 480/525 nm; to carry out $[K^{+}]_i$ analysis, the spermatozoa were loaded with PBFI AM indicator (Invitrogen P1267) for a final concentration of 5 µM for 30 min using an excitation/emission wavelength of 370/500 nm; in both cases the sperm incubation with the fluorescent dyes was done at room temperature. The ion concentrations in sperm were measured before motility activation and 5, 30 and 60 s after the addition of activation media.

2.6 Statistical analysis

The mean and standard error were calculated for all sperm quality parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) and the Student's t-test were used to analyze data with normal distribution. Significant differences were detected using the Tukey multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks and Mann-Whitney U-test were used. 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 Short-term storage

Different storage methods for preserving the sperm throughout the storage time were tested using the seminal like solution (SLS) as a diluent medium. Fresh sperm showed excellent motility values due to the fact the samples were collected in the middle of the breeding season.

The sperm quality parameters of undiluted samples, stored both in Petri dishes or microtubes, decreased significantly after just 1 day of incubation when compared to fresh samples (Fig. 1). In this respect, the undiluted sperm samples stored in Petri dishes showed a dramatic decrease in motility, and neither progressive motile cells nor any motile cells were found after 1 and 2 days of incubation, respectively. For this reason, motility of undiluted sperm stored in Petri dishes was not measured further in the rest of the experiment. Microtubes generated the best sperm quality parameters results within the undiluted samples, reaching maximum values of around 16 and 10% of total and progressive motility on day 1, respectively. These values decreased to 5 and 3% at 4 days, and no motile cells were found after 7 days of incubation.

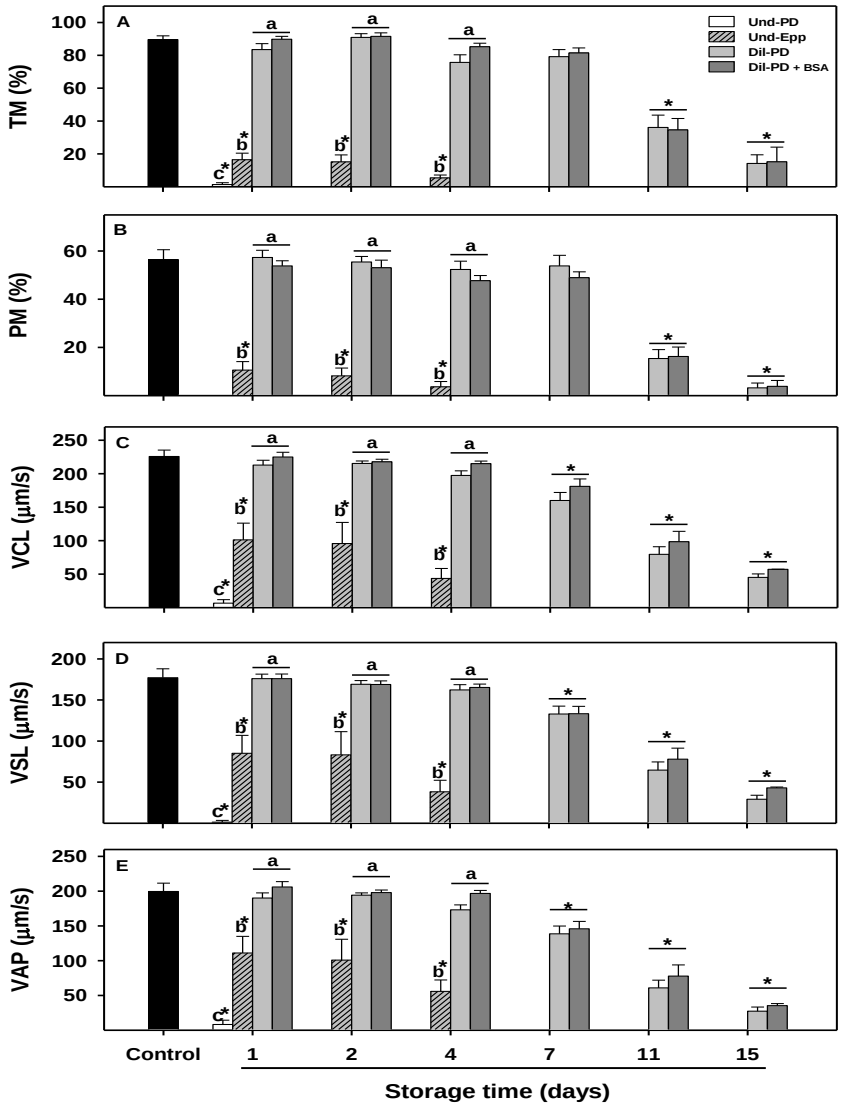

Figure 1. Evolution of sperm quality parameters in the different tested storage-ways compared with control samples after different incubation times (Und-PD, undiluted sperm stored in Petri dishes; Und-Epp; undiluted sperm stored in Eppendorf´s microtubes; Di-PD, diluted sperm stored in Petri dishes and Di-PD+BSA, diluted sperm containing 2% BSA stored in Petri dishes. Data are expressed as mean ± SEM (n=10). Different letters mean significant differences between storage-way at the same incubation time and the asterisk indicates significant differences with control samples.

On the other hand, diluted samples maintained in Petri dishes showed the best results, and no significant differences in any sperm quality parameter when compared to fresh samples were found until 7 days of incubation. The first significant differences were found in VCL, VSL and VAP after 7 days (Figs. 1C, D and E) and all the samples showed lower motilities than fresh samples after 11 days of incubation. Finally, diluted samples maintained in Petri dishes displayed the highest sperm quality parameter values of all the storage methods, showing significant differences compared to the undiluted samples at all incubation times. No differences were registered between diluting media with or without BSA.

3.2 Effect of different activation media

Different activation media with different osmolalities and ionic compositions were tested on fresh sperm samples with a view to analyzing the effects on motility. The initial motility values were lower than those from the short-term storage trial, as samples were collected at the end of the breeding season.

TM and PM of samples activated with ASW100 and ASW075 media showed significantly higher motility values at 10 and 20 s than samples activated with ASW050, which displayed the lowest values (Fig. 2A, B). Forty seconds after activation, a sharp decrease in these parameters resulted in samples activated with ASW100 and ASW075 media, and no significant differences (except in TM) were found in the samples activated with the ASW050 media. The values remained constant from this post-activation time until the end of the sperm motility analysis (80 s).

With regards to VCL, VSL and VAP (Fig. 2C, D and E), ASW100 and ASW075 media resulted in the best values at 10 s post-activation. However, from 40 s after activation this pattern changed, with velocity values decreasing in the media with the highest osmolality. ASW050 produced the highest velocity values, reaching a maximum level at 80 s after activation, with significant differences between ASW100 and ASW075.

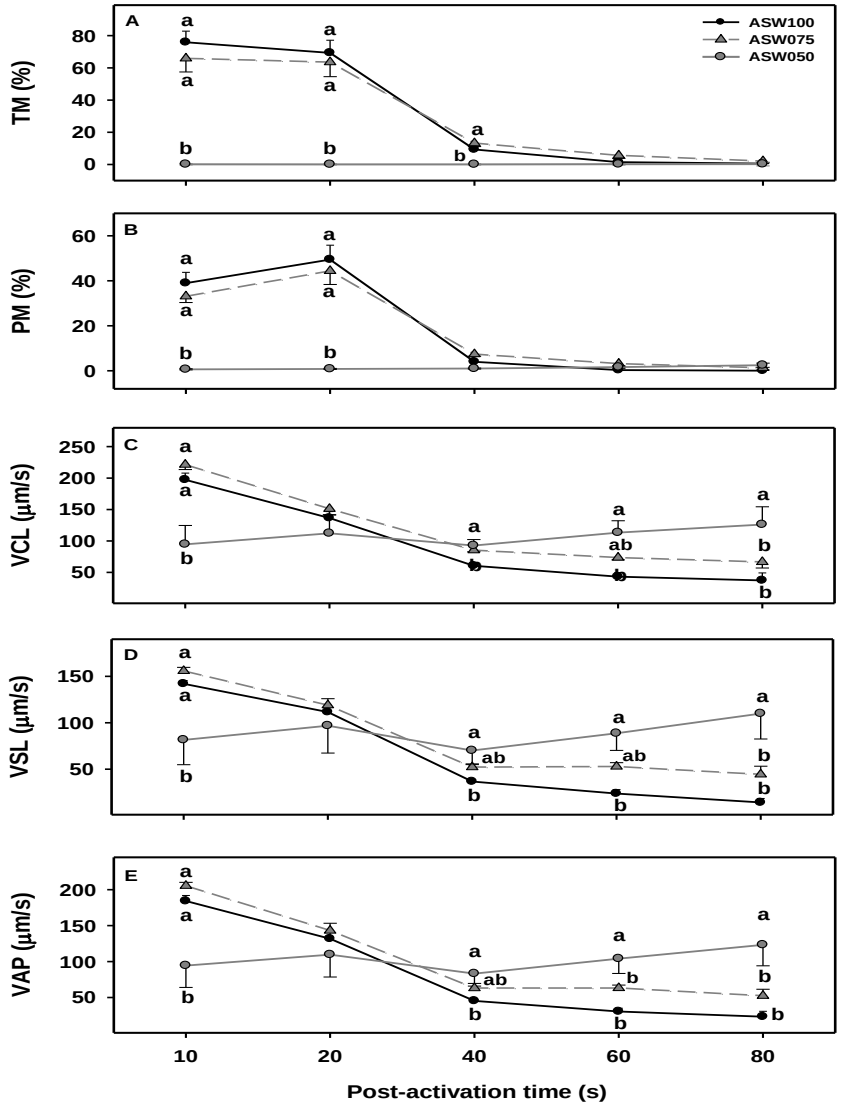

Figure 2. Effect of the osmolality on motility and velocity parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

Abbreviations: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Table 2. Effect of the osmolality on the sperm quality parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

		T10			T20			T40			T60			T80		
		ASW100	ASW075	ASW050	ASW100	ASW075	ASW050	ASW100	ASW075	ASW050	ASW100	ASW075	ASW050	ASW100	ASW075	ASW050
FA	%	66.2 ± 8.8a	61.0 ± 9.1a	0.7 ± 0.3b	50.8 ± 7.6a	55.2 ± 9.5a	0.6 ± 0.3b	1.3 ± 0.2b	4.5 ± 0.5a	0.6 ± 0.1b	0.1 ± 0.1	2.4 ± 1.3	1.4 ± 0.5	0.1 ± 0.1b	0.2 ± 0.1b	2.2 ± 0.9a
ME	%	4.3 ± 1.0a	3.2 ± 0.5a	0.2 ± 0.1b	15.0 ± 5.0a	7.0 ± 1.3ab	0.4 ± 0.1b	4.1 ± 1.0a	5.3 ± 0.8a	0.6 ± 0.2b	0.4 ± 0.1	1.6 ± 0.6	0.6 ± 0.3	0.2 ± 0.1	1.4 ± 1.1	0.8 ± 0.5
SL	%	5.4 ± 3.1	1.7 ± 0.5	0.7 ± 0.3	3.5 ± 1.7	1.3 ± 0.3	0.5 ± 0.2	3.9 ± 0.4a	3.4 ± 0.8a	0.7 ± 0.2b	1.0 ± 0.2	1.6 ± 0.3	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	0.8 ± 0.3
LIN	%	72.9 ± 3.7	71.1 ± 3.8	72.4 ± 12.3	82.0 ± 1.7	78.9 ± 1.7	79.6 ± 5.4	60.7 ± 1.1	61.1 ± 2.6	72.3 ± 11.6	54.4 ± 4.6b	71.5 ± 3.2a	74.1 ± 5.7a	35.7 ± 8.5c	64.4 ± 4.8b	84.5 ± 2.7a
STR	%	77.6 ± 2.7	76.1 ± 2.7	73.3 ± 12.4	84.9 ± 1.1	83.6 ± 1.5	84.4 ± 4.3	80.8 ± 0.9	82.4 ± 1.0	76.7 ± 10.5	75.6 ± 2.2b	83.3 ± 1.1a	83.3 ± 1.9a	55.7 ± 10.5b	82.6 ± 2.8a	87.7 ± 1.2a
WOB	%	93.7 ± 1.5	93.0 ± 1.5	84.6 ± 14.1	96.6 ± 0.8	94.3 ± 1.4	94.0 ± 2.7	75.1 ± 1.3	74.0 ± 2.6	87.3 ± 8.7	71.6 ± 4.8b	85.7 ± 2.7a	88.4 ± 5.4a	54.9 ± 12.1b	77.6 ± 3.6a	96.2 ± 1.8a
ALH	µm	1.6 ± 0.1a	1.7 ± 0.1a	0.9 ± 0.2b	1.2 ± 0.0	1.3 ± 0.1	1.0 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.4 ± 0.3	0.8 ± 0.2b	1.3 ± 0.2ab	1.6 ± 0.2a	0.2 ± 0.1b	1.2 ± 0.3a	1.2 ± 0.1a
BFC	beats/s	16.8 ± 0.4a	17.8 ± 0.2a	10.0 ± 3.0b	14.8 ± 0.2	15.6 ± 0.4	11.4 ± 3.1	20.3 ± 1.0a	22.5 ± 1.4a	9.1 ± 2.3b	9.5 ± 2.9b	13.1 ± 1.5ab	20.1 ± 2.3a	2.3 ± 1.5b	12.8 ± 2.6a	18.0 ± 2.2a

Abbreviations: FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; LIN; linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 3. Effect of the ion composition of the activation media on the sperm quality parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Asterisks indicate significant differences between the different media at the same post-activation time.

		T10		T20		T40		T60		T80	
		ASW100	GLU	ASW100	GLU	ASW100	GLU	ASW100	GLU	ASW100	GLU
FA	%	60.5 ± 10.6	48.6 ± 8.2	53.2 ± 9.0	30.6 ± 7.9	4.3 ± 0.9*	1.6 ± 0.6	0.0 ± .	0.1 ± .	0.2 ± 0.1	0.1 ± 0.1
ME	%	4.7 ± 1.0	6.5 ± 1.2	11.5 ± 3.7	23.1 ± 6.4	8.6 ± 1.4	6.2 ± 1.0	1.6 ± 0.3	0.9 ± .4	0.2 ± 0.1	0.3 ± 0.1
SL	%	2.0 ± 0.6	1.8 ± 0.4	1.1 ± 0.3	2.6 ± 0.4*	4.3 ± 0.5	4.4 ± 0.8	2.2 ± 0.6	1.3 ± 0.3	0.5 ± 0.1	0.7 ± 0.2
LIN	%	78.7 ± 3.6	82.9 ± 2.6	82.8 ± 2.6	82.1 ± 2.5	62.2 ± 1.7	57.5 ± 2.8	54.3 ± 1.6	49.7 ± 3.8	47.1 ± 2.8	36.7 ± 5.1
STR	%	81.9 ± 2.5	85.1 ± 1.7	85.2 ± 1.9	86.0 ± 1.5	81.7 ± 1.3	79.0 ± 2.3	76.9 ± 1.6	72.6 ± 3.3	71.3 ± 4.4*	46.0 ± 7.7
WOB	%	95.9 ± 1.5	97.4 ± 1.2	97.0 ± 0.9	95.4 ± 1.2	76.1 ± 1.2	72.6 ± 1.7	70.6 ± 1.5	68.3 ± 3.3	66.5 ± 3.0	82.2 ± 4.0*
ALH	µm	1.5 ± 0.1*	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.1 ± 0.3	0.8 ± 0.3	0.8 ± 0.4	0.5 ± 0.2
BFC	beats/s	16.0 ± 0.6*	13.9 ± 0.2	14.9 ± 0.3	14.4 ± 0.2	21.3 ± 0.5	19.0 ± 1.1	11.5 ± 2.3	6.8 ± 2.7	2.9 ± 1.6	1.1 ± 1.1

Abbreviations: FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; LIN; linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

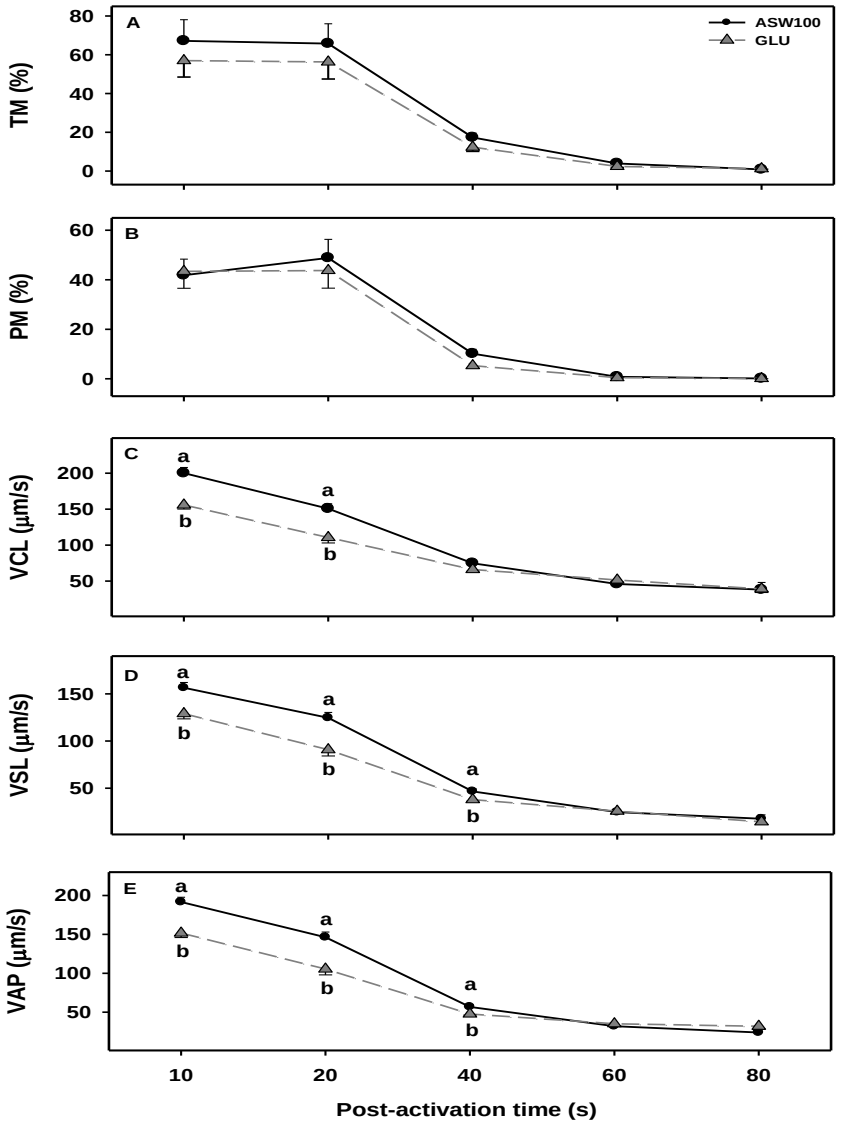

Figure 3. Effect of the ion composition of the activation media on motility and velocity parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

Abbreviations: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Other sperm quality parameters (Table 2) showed significant differences at different post activation times. FA, ME, ALH and BFC values obtained once activated with ASW100 and ASW075 media were significantly higher than those obtained with ASW050 at 10 s post-activation. However, from 40 s after activation this pattern changed and the highest values were found with the ASW050 medium, with significant differences compared to ASW100 and/or ASW075 at 60 and 80 s.

On the other hand, regarding electrolyte (ASW100) and non-electrolyte (GLU) media, TM and PM were not strongly affected by the ion composition of the medium, and decreased progressively after sperm activation with an sharp decline found at 40 s (Fig. 3A,B).

In terms of kinetic traits (Fig. 3C, D and E), significant differences were found in VCL and VSL between ASW100 and GLU activation media at 10 and 20 s, with the obtained values being significantly higher with the electrolyte media. A progressive decrease was seen in spermatozoa velocities with the addition of hyperosmotic medium (ASW100 or GLU), with values falling to close to zero at 80 s post-activation.

Other sperm quality parameters (Table 3) showed occasional significant differences at different post activation times. In this respect, ALH and BFC values were significantly higher when activated with ASW100 rather than GLU media at 10 s after activation.

3.3 Intracellular concentration of Ca^{2+} and K^{+}

The intracellular concentrations of Ca^{2+} and K^{+} in pre- and post-activated sperm cells were estimated using different activation media and sperm washing protocols. When applying protocol A (see section 2.5), $[Ca^{2+}]_i$ increased significantly 5 s after the sperm activation compared to $[Ca^{2+}]_i$ in quiescent sperm using both ASW100 and GLU media, and it remained this way until 60 s (Fig. 4A). This $[Ca^{2+}]_i$ increase rose significantly when the sperm was activated with ASW100 rather than with the non-electrolyte medium. In relation to the increase in K^{+} (Fig. 4C), $[K^{+}]_i$ increased significantly after the sperm activation using both activation media,

although the activation media did not significantly affect the levels of this ion within the cell after the sperm activation. Finally, the increase in $[Ca^{2+}]_i$ was more than twice higher than the increase in $[K^+]_i$ when the sperm was activated with ASW100 (340 against 170, ASW100 and GLU, respectively).

On the other hand, when applying the protocol B (see section 2.5), where the sperm was washed with a glucose-based media, there was no increase in $[Ca^{2+}]_i$ compared to the baseline levels after being activated with the non-electrolyte activation medium (GLU), and significant differences in $[Ca^{2+}]_i$ pre- and post-activation were only detected when the sperm was activated with the electrolyte medium (Fig. 4B). In contrast, $[K^+]_i$ increased significantly compared to the baseline levels after the sperm activation using both ASW100 and GLU media (Fig. 4D). However, the activation media affected the levels of this ion within the cell at 30 and 60 s after the sperm activation, with the values obtained by ASW100 being higher than those found with the GLU medium.

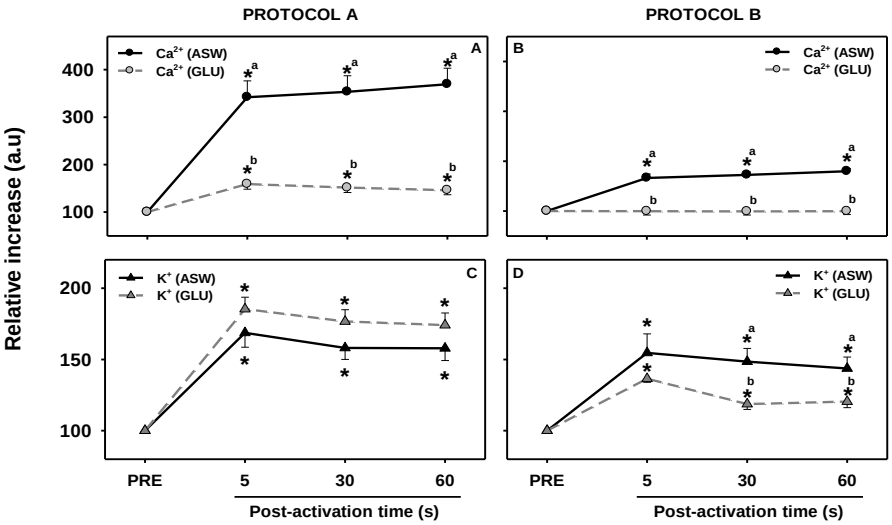

Figure 4. Intracellular concentrations of $Ca^{2+}$and K^+ at pre- and post-activation times (5, 30 and 60 s) using different activation media (ASW100 or GLU) and handling sperm protocols (protocol A: graphs A and C; protocol B: graphs B and D). Data are expressed as mean ± SEM (n=9). Asterisks mean significant differences with baseline levels and different letters indicate significant differences between the different activation media.

4. Discussion

4.1 Short-term storage

With a view to seeking a way to improve the handling of fish sperm used for aquaculture, ecological (repopulations) or scientific purposes, several chilled storage methods have been developed to preserve sperm integrity and quality over time. However, several factors such as the incubation temperature, the composition of the diluent, the dilution ratio or environmental conditions should be taken into account when designing a proper storage protocol (Peñaranda et al., 2010c,d).

The first step of this process involves deciding whether the sperm will be preserved undiluted or, on the contrary, diluted in a medium. It has been reported that fish sperm samples stored undiluted tend to show poorer motility values than diluted sperm samples (Babiak et al., 2006; DeGraaf and Berlinsky, 2004; Peñaranda et al., 2010c). Our results agree, with undiluted pufferfish sperm showing significantly lower values in the sperm quality parameters than diluted sperm samples at all the incubation times. On the other hand, within the undiluted samples, microtube vials generated better results than PD storage. This could be explained at least in part by the effect of the aerobic and anaerobic spermatozoa pathway. While the spermatozoa stored in microtube would have been subjected to an atmosphere with poor oxygen levels due to the shape of the vial, thus inducing the use of the anaerobic pathway; the spermatozoa stored in Petri dishes would have been in a richer oxygen atmosphere, thus using the aerobic pathway. It has been reported that aerobic pathway produce higher levels of oxygen free radicals (ROS, Kowalowka et al., 2007), which have been associated with defective sperm function in fish spermatozoa (Bansal and Bilaspuri, 2010; Martínez-Páramo et al., 2012; Pérez-Cerezales et al., 2009). Therefore, the use of the aerobic pathway of undiluted spermatozoa stored in Petri dishes would generate a higher level of ROS and thus, a sharp decrease in sperm quality parameters.

On the other hand, the second step to designing an optimum short-term storage method involves selecting a suitable diluent medium, with a proper

dilution ratio and an optimum incubation temperature. Seminal plasma in almost all teleost fish is composed of a mix of ions including Na^+, Ca^{2+}, K^+, Cl^-, etc., with an osmolality of 300-350 mOsm⁄kg (Asturiano et al., 2004b; Morisawa, 2001, 2008; Pérez et al., 2003). In this respect, we used the common dilution medium used in pufferfish sperm studies (Krasznai et al., 2003; Takai and Morisawa, 1995) applying a dilution ratio of 1:50 (v/v), which had been tested in other fish species with good results (Ohta and Izawa, 1996; Peñaranda et al., 2010d). In the present trial, the first significant differences between the diluted samples and the fresh samples were found after 7 days of incubation in the velocity parameters and, after 11 days, all diluted samples showed lower motility values than fresh samples. In this respect, the diluent acted by prolonging the quality of stored spermatozoa, providing better control of the physicochemical conditions during storage through avoiding negative effects such as desiccation, contamination and unbalanced gas exchange (Babiak et al., 2006). It is worth highlighting this result as it allows the preservation and use of pufferfish sperm during a short-term period for aquaculture matters. Regarding temperature, it has been reported that low temperatures result in better motilities than high temperatures for ATP-spending reasons (Alavi and Cosson, 2005), thus a temperature of 4 ºC was used in this trial.

Finally, in addition to the choice of diluent and its dilution ratio, there is the possibility of adding some reagents such as membrane protectors to the medium. In this respect, bovine serum albumin (BSA) has been used with good results in other species such as gilthead seabream (Cabrita et al., 2005b), European sea bass (Zilli et al., 2003) and European eel (Peñaranda et al., 2010c). However, no differences were registered between the diluting media with or without BSA in our experiment, therefore we do not recommend the use of this reagent in the chilled storage of pufferfish sperm because it can increase the chances of bacterial growth.

4.2 Effect of the osmolality on sperm quality parameters

In the natural environment seawater has an osmolality of 1000-1100 mOsm/kg, with a high variety of ions. However, in order to find out the

essential mechanisms which trigger and regulate the sperm activation process, it is necessary to assay with different artificial media and consider both the osmolality and the ion composition of the media. In marine fish, sperm activation can occur within a wide range of osmolalities, below or above that of seawater (Chauvaud et al., 1995; Linhart et al., 1999; Suquet et al., 1994), and the optimal osmolality is species-specific (Cosson et al., 2008a). In the present study, we analysed the effect of activating medium with different osmolalities on pufferfish sperm, using the CASA system. Our results showed that osmolalities around 500-550 mOsm/Kg did not activate pufferfish spermatozoa and, values of at least close to 750-825 mOsm/Kg were necessary to reach high percentages of motile and progressive motile spermatozoa. Cosson et al. (2008) reported similar results in cod, in which the motility was activated in seawater solutions with osmolalities above 700 mOsm/kg, and twice-diluted seawater did not activate sperm motility. The highest values obtained in spermatozoa velocities (VCL, VSL and VAP) with the lowest osmolality medium ASW050, at 60 and 80 s, would have been due to the gradual activation of a few spermatozoa over time. Finally, regarding the duration of sperm motility in relation to the medium´s osmolality, significant differences have been reported both in marine and freshwater species (Dreanno et al., 1999; Perchec et al., 1996). In fact, a previous study with pufferfish (Morisawa and Suzuki, 1980) showed that the duration of sperm motility was longer with lower osmolality than when induced by seawater. However, it is worth noting that this study only showed the duration in time and not the percentage of motile cells. In this respect, we have demonstrated that despite the duration of motility appearing to be longer with lower osmolalities, the values of all sperm parameters, in particular the total and progressive motility, are not appropriate when carrying out fertilization trials.

4.3 Role of Ca^{2+} and K^{+} ions in sperm motility activation

In addition to hyperosmotic shock as the main trigger in initiating sperm motility in marine fish (Morisawa and Suzuki, 1980), the ion composition of the activation medium is considered the second most important factor able to modulate/regulate the sperm activation process (Alavi and Cosson,

2006). Several studies in marine fish (pufferfish, *Takifugu niphobles* (Morisawa and Suzuki, 1980); halibut, *Hippoglossus hippoglossus* (Billard et al., 1993); European sea bass, *Dicentrarchus labrax* (Dreanno et al., 1999) and cod, *Gadus morhua* (Cosson et al., 2008c) have shown that sperm activation can be induced by hypertonic sugar (non-electrolyte) solutions; but in other species such as seawater tilapia (Linhart et al., 1999) or Pacific herring, *Clupea pallasii* (Vines et al., 2002), it has been reported that extracellular Ca^{2+} is essential for sperm activation. However, scarce data are published regarding the effect of the ion composition of activation medium on sperm quality parameters obtained by the CASA system.

Our results showed that whether the medium had electrolytes or not had no bearing on the motility values, even though significant differences were found in curvilinear and straight line velocities. These results suggest that, although the absence of ions in the extracellular medium does not affect the percentage of motile and progressive motile cells, spermatozoa could use both Ca^{2+} and K^{+} from the extracellular medium to increase/improve the kinetic features. Detweiler and Thomas (1998) reported similar data in Atlantic croaker, where the depletion of Ca^{2+} in the activation medium through the addition of EGTA caused a decline in spermatozoa speed; and Cosson et al. (2008b) showed that internal Ca^{2+} was able to regulate axonemal motility, governs the asymmetry of beating, resulting in the control of the spermatozoa curvilinear velocity. Therefore, an ion-rich medium would contribute by providing more resources to the sperm cells and thus, increasing the spermatozoa velocities.

Regarding the ion levels after sperm activation using different activation media and sperm handling protocols, scarce studies have been published in marine fish (Oda and Morisawa, 1993; Takai and Morisawa, 1995). Nowadays, the widely accepted model about freshwater (Krasznai el al., 2003) and marine fish (Morisawa, 2008) suggests that a hypotonic and hyperosmotic shock, respectively, would cause a spermatozoa membrane depolarization, causing in turn a Ca^{2+} and K^{+} increase inside the cell. Recent studies in European eel (Gallego et al., 2011) corroborate this theory and also show that intracellular stores of these ions may be stored in the

mitochondria (located in the apix of the spermatozoon head), suggesting this cellular compartment has an important role in the activation mechanism of fish sperm. The present study showed that if there is no free Ca^{2+} neither in the activation medium nor in the sperm diluent (using protocol B; with EGTA), there is no increase in $[Ca^{2+}]_i$ after activation with a non-electrolyte medium (Fig. 4B), even if motility starts. However, if any Ca^{2+} remains in the activation medium and/or the sperm diluent, the spermatozoa will be able to use it, in line with its availability, and incorporate it inside the cell, thus increasing the $[Ca^{2+}]_i$ levels (Fig 4A).

On the other hand, $[K^+]_i$ increased after sperm activation regardless of the media or the washing protocol used. In this respect, there are three main hypotheses about the origin/nature of the ions after sperm activation $_i$) the first hypothesis maintains that after activation there is an influx of Ca^{2+} and/or K^+ through ion channels from the external environment (Morisawa, 2008); ii) the second hypothesis maintains that after activation the ions are released from intracellular stores (Morisawa, 2008); and finally iii) the last hypothesis maintains that after activation there is water efflux through specific proteins called aquoporins, and this efflux causes the increase of ion intracellular concentrations (Zilli et al., 2009, 2011). Our results suggest that the Ca^{2+} ion is of an extracellular origin, due to the fact that $[Ca^{2+}]_i$ increased only when there was free calcium in the activation medium. However, these results do not agree with data previously published by Oda and Morisawa (1993), who found an increase in $[Ca^{2+}]_i$ even in the absence of this ion in the extracellular medium. However, we must taken into account the fact that in their study Ca^{2+} chelator was not used in the activation medium, thus trace amounts of this ion could be masking the different results. In this respect, Krasznai et al. (2000) showed that levels of $[Ca^{2+}]_i$ in fish spermatozoa seminal plasma are particularly low (40-70 nM) and negligible remains of extracellular Ca^{2+} could be enough to interact with spermatozoa. Regarding the K^+ ion, our data show that $[K^+]_i$ increased regardless of the composition of the activation media and therefore, the K^+ ion would be of an intracellular origin. However, intracellular potassium stores in animal cells have not been proven, so more studies may be necessary to determine the origin/nature of this ion in marine fish sperm.

5. Conclusions

Some conclusions regarding different issues of pufferfish sperm have emerged from this study:

i) Through the use of diluents, it is possible to preserve pufferfish sperm for a short-term period (up to 7 days without differences compared to fresh sperm) for use in aquaculture matters.

ii) Medium osmolality is the most important factor in triggering trigger sperm motility, and values of around 750-825 mOsm/kg are necessary to activate this process in pufferfish, which seems to be a non-dose-dependent mechanism in terms of osmolality.

iii) The ion composition of the activation media is able to modulate the sperm activation process. Despite spermatozoa being able to initiate movement without any ion in the activation medium, the presence/absence of these ions can affect the kinetic parameters of spermatozoa.

iv) In the natural environment, the activation of sperm motility generates an increase in intracellular Ca^{2+} and K^{+}, suggesting these ions have an important role in the activation mechanism of marine fish sperm.

Acknowledgements

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CHAPTER 5

Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*)

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Abstract

The use of high quality gametes from both males and females during in vitro fertilization (IVF) trials is an essential step in order to achieve high fertilization and hatching rates. Although aquaculture hatcheries have focused more on egg rather than spermatozoa quality, some studies have demonstrated that sperm quantity and quality have a great influence both on fertilization/hatching success.

In this study we have demonstrated that sperm/egg ratio and sperm quality are factors strongly related to each other in the pufferfish (*Takifugu niphobles*). Our results suggest that both factors should be taken into account as unique interrelated elements, making possible to obtain high fertilization rates using a successful combination of small amount of high quality sperm or high amount of low quality sperm.

In addition, coefficients of correlation and determination among all the sperm motion parameters provided by a CASA system and fertilization/hatching rates were estimated for the first time in a marine species. Positive significant correlations were found in some parameters such as total and progressive motility (0.68 and 0.70 respectively). However, curvilinear velocity (VCL), straight line velocity (VSL) and average velocity (VAP) showed the highest coefficients of correlation (0.82, 0.80, and 0.81, respectively). In this respect, spermatozoa velocity appears to be a key factor in the fertilization process, especially when the number of spermatozoa per egg is limited in the aqueous environment.

1. Introduction

The pufferfish (*Takifugu niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean. This species is one of around 24 pufferfish species in the tetraodontine genus Takifugu, and it presents own interesting features to preserve it: i) it is placed on the IUCN Red List due to the fact that its current population is not well known, making it a possible endangered species (Roberts, 1996); and ii) another closed species, like

Takifugu rubripes, is widely-kept by scientists as a model organism (Aparicio et al., 2002) so *Takifugu niphobles* could be used like this due to its small and similar genome (Brenner et al., 1993).

Breeding in captivity of *Takifugu* spp. involves the handling of captive fish broodstocks and the collection of gametes both from males and females for the application of in vitro fertilization (IVF). This technique is essential for the reproductive manipulation of some fish species such as *T. niphobles*, which displays an exclusive and complex spawning behavior because spawning does not happen spontaneously under cultured conditions.

The general hatchery protocol for IVF in fish involves mixing quiescent spermatozoa with a batch of oocytes, followed by activation through the addition of seawater and finally, the incubation of the fertilized eggs in order to promote embryo development and hatching (Yasui et al., 2012). The use of high quality gametes both from males and females during this process is essential in order to achieve both suitable fertilization and hatching rates. In this respect, despite the fact that aquaculture hatcheries have focused more on egg rather than sperm quality (Snook, 2005), some studies have demonstrated that spermatozoa quality has a great influence both on fertilization success and the subsequent development of the embryo and larvae (Butts et al., 2011 and Ottesen and Babiak, 2007).

From a practical viewpoint, the sperm quality can be measured by any quantifiable parameter which is directly correlated related to fertilization capacity. Spermatozoa motility has been the most commonly used parameter in evaluating sperm quality (Kime et al., 2001), but other features such as sperm density, plasma composition, head morphology or ATP concentration can be also useful tools to measure sperm quality (Fauvel et al., 2010). The gradual appearance of the computer assisted sperm analysis (CASA) has allowed an objective, rapid and accurate assessment of fish sperm samples, including *T. niphobles* (Gallego et al., Chapter 4). This kind of software is able to provide a high number of parameters, which can be related to fertilization ability and thus, to sperm quality. However, despite the fact that there are some studies about the relationship between the percentage of motile cells and fertilization and

hatching ability, scarce research has been carried out regarding other sperm kinetic parameters assessed by computerized automatic systems.

On the other hand, the sperm/egg ratio is another essential factor which needs to be taken into account in IVF trials. Usually, an excess of sperm is used in these trials, but an appropriate combination of the number of spermatozoa per oocyte should be used in order to optimize reproductive efficiency in fish farms. Improvements in this area would allow a rational use of gametes, limiting the number of breeding fish in culture stations and reducing production costs (Sanches et al., 2011).

Therefore, the main goals of this study were (i) to analyze the effect of the sperm/egg ratio on the sperm quality parameters assessed by CASA system and (ii) to study the correlations between these sperm parameters and the fertilization and hatching rates in the pufferfish.

2. Materials and methods

2.1 Fish handling and gamete collection

Pufferfish demonstrate a characteristic spawning behavior at Arai Beach near Misaki Marine Biological Station (MMBS, Japan). Large schools of fish arrive to the beach around the new moon at spring tide during the spawning season which occurs between June and July (Yamahira, 1996). Spawning takes place repeatedly from 2 hours before the sunset to that moment, and during that time, male and female pufferfish were caught and moved to the MMBS facilities. Fish were kept in running seawater tanks at 18 °C and all trials were carried out under the approval of the animal guidelines of the University of Tokyo on Animal Care.

Before gamete collection the genital area was cleaned with freshwater and thoroughly dried to avoid the contamination of the samples with faeces, urine or seawater, and gentle abdomen pressure was applied to obtain the gametes both in males and females. Fresh sperm was diluted 1:50 in seminal plasma-like solution (consisting in 130 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM $CaCl_2$, pH adjusted to 7.5; (Krasznai et al., 2003))

immediately after its extraction sperm samples were maintained at 4 °C until motility analysis. Eggs were collected just before the fertilization assay.

2.2 Assessment of sperm quality parameters

Sperm were evaluated in the first hour after extraction mixing 0.5 µl of diluted sperm with 4 µl of artificial seawater. The sperm-seawater mixture was placed and observed in a chamber SpermTrack-10® (Proiser R + D, S.L.; Paterna, Spain). Video sequences were recorded using a high-sensitivity video camera (HAS-220; 50 fps) mounted on a phase contrast microscope (Olympus BX51) with a 10x objective lens (Olympus Splan NH). All the motility analyses were performed by triplicate using the motility module of ISAS (Proiser R + D, S.L.; Paterna, Spain).

The parameters assessed in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in an essentially straight line; curvilinear velocity (VCL, µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; 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; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its spatial average trajectory; the percentage of fast (FA; VAP > 100 µm/s), medium (ME; VAP = 50-100 µm/s) and slow (SL; VAP = 10-50 µm/s) spermatozoa; straightness (STR, %), defined as the linearity of the spatial average path; linearity (LIN, %), defined as the linearity of the curvilinear trajectory; wobble (WOB, %), defined as the trajectory oscillation along its spatial average path; amplitude of lateral head displacement (ALH, µm), defined as the amount of lateral displacement of a sperm head along its spatial average trajectory; and beat cross frequency (BCF, beats/s), defined as the average-time rate at which the curvilinear sperm trajectory crosses its average path trajectory. Some of these kinetic parameters are plotted on Fig. 1. Spermatozoa were considered immotile if their VAP was lower than 10 µm/s.

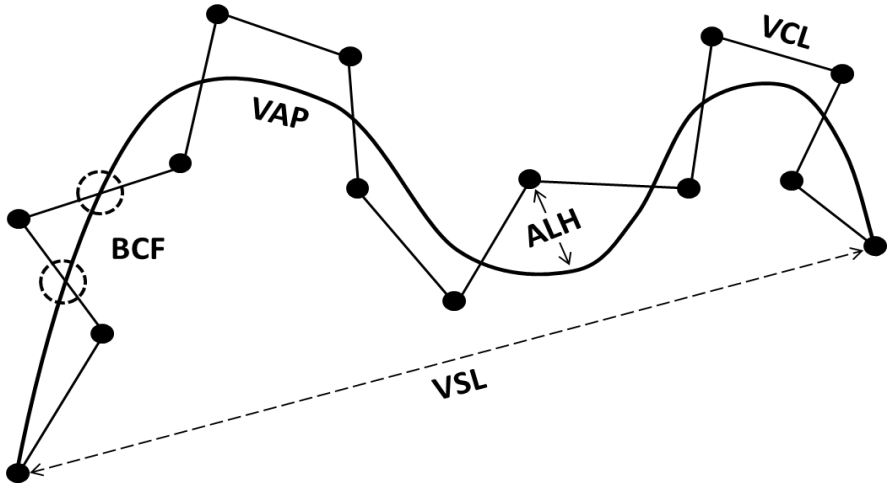

Figure 1. Schematic diagram of some kinetic parameters recorded by CASA system. Black circles represent successive positions of the head of a motile sperm through the video recording. Sperm movement parameters: VCL, curvilinear velocity; VAP, averaged path velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat/cross frequency.

2.3 Experimental setup: fertilization and hatching trials.

This study was divided into two trials as described in Fig. 2. In Trial 1, gametes from five males and two females were separately used for fertilizations assays, using 5 of the 10 possible combinations (1 male, 1 female), which were simultaneously assayed in three different sperm/egg ratios (10^3, 10^4 and 10^5 spermatozoa/egg) and using sperm activated after three different times (5, 20 and 40 s).

TRIAL 1									TRIAL 2		
Sperm/egg (10^3)			Sperm/egg (10^4)			Sperm/egg (10^5)			Sperm/egg (10^4)		
5s	20s	40s	5s	20s	40s	5s	20s	40s	5s	20s	35s
M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M6xF3	M6xF3	M6xF3
M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M7xF3	M7xF3	M7xF3
M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M8xF3	M8xF3	M8xF3
M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M9xF3	M9xF3	M9xF3
M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M10xF33	M10xF3	M10xF3

Figure 2. Experimental crosses for Trial 1 and 2 (n=15). M: male; F: female.

Eggs from two females were divided into batches of 80-90 eggs and placed into 60 × 15 mm Petri dishes using a micropipette with the tip cut off to prevent compression of the eggs. A known aliquot of sperm (adjusting the volume according to the calculated sperm/egg ratio) was added to 5 ml of seawater and then the sperm-water solution was added to the corresponding batch of eggs at different post-activation times. After an incubation period of 10 min, the eggs were transferred into a clean Petri dish for incubation with 8 mL of clean seawater. The eggs were then incubated in darkness at a controlled temperature of 20 °C. Fertilization rates were evaluated between 1-2 hrs after insemination by counting the percentage of embryos which reached the 4-cell stage in relation to the total number of eggs used.

In Trial 2, using the 104 sperm/egg ratio, new batches of 80-90 eggs from one female were separately fertilized with sperm from five males at different post-activation times (5, 20 and 35 s) with the goal of establishing correlations between sperm quality parameters and fertilization and hatching rates. The gamete collection and the artificial insemination were carried out in the same manner as in Trial 1 and the hatching rates were calculated as the percentage of hatched larvae in relation to the total number of eggs. Dead eggs and larvae were removed and counted when detected during daily inspections, and seawater was exchanged once a day.

2.4 Statistical analysis

The mean and standard error were calculated for all the sperm quality parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyse the data. Significant differences were detected using the Tukey´s multiple range test ($P < 0.05$). Pearson´s correlation, coefficient of determination and linear regression analysis were used to find the relationship between the different sperm quality parameters and fertilization/hatching rates. 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 Effect of sperm/egg ratio and sperm quality parameters on fertilization rates

Sperm quality parameters from samples used in Trial 1 are shown in the Table 1. A time-dependent effect was found, lower values in spermatozoa velocities were registered (VCL, VSL and VAP) the longer the post-activation time. However, spermatozoa motilities (TM and PM) and other sperm parameters such as FA, LIN, STR, WOB and ALH did not show significant differences until 40 s post-activation. Finally, ME, SL and BFC did not show significant differences over time.

Table 1. Sperm quality parameters of sperm samples used in Trial 1 at different post-activation times (5, 20 and 40 s). Data from 5 males of Trial 1 was used and values are expressed as mean ± SEM (n = 5). Different letters mean significant differences between post-activation times at the same parameter.

		Post-activation time		
		5s	**20s**	**40s**
TM	%	80.30 ± 2.06 a	79.39 ± 2.50 a	14.47 ± 2.70 b
MP	%	65.63 ± 1.19 a	70.90 ± 2.46 a	8.00 ± 1.49 b
FA	%	78.04 ± 2.49 a	71.18 ± 6.08 a	3.74 ± 0.86 b
ME	%	1.28 ± 0.33	8.79 ± 4.41	6.90 ± 1.33
SL	%	0.97 ± 0.30	2.42 ± 0.43	3.83 ± 0.83
VCL	µm/s	238.49 ± 1.99 a	152.84 ± 7.43 b	74.83 ± 3.22 c
VSL	µm/s	191.39 ± 2.83 a	133.59 ± 6.94 b	44.86 ± 2.70 c
VAP	µm/s	232.30 ± 1.94 a	151.19 ± 8.03 b	54.72 ± 2.69 c
LIN	%	80.27 ± 1.14 a	87.24 ± 0.75 a	59.93 ± 2.86 b
STR	%	82.37 ± 0.82 a	88.43 ± 0.46 a	71.64 ± 1.49 b
WOB	%	97.42 ± 0.47 a	98.65 ± 0.68 a	73.25 ± 2.48 b
ALH	%	1.53 ± 0.03 a	1.15 ± 0.01 ab	1.08 ± 0.09 b
BFC	beats/s	15.67 ± 0.23	15.10 ± 0.54	14.66 ± 1.61

Abbreviations: TM, total motility; PM, progressive motility; FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral displacement; BCF, beat cross frequency.

The sperm was used to fertilize egg batches at different post-activation times and with different sperm/egg ratios (Fig. 3). When the spermatozoa activated after 5 s were used to fertilize the egg batches, no significant differences were found between the 10^4 and 10^5 ratios (inducing over 94% fertilized eggs in both cases). However, the lowest sperm/egg ratio (10^3) produced significantly lower values (approx. 85%) in comparison to the highest ratios.

Regarding the spermatozoa activated after 20 s, significant differences in fertilization rates were found between the different assayed ratios. While the highest sperm/egg ratio showed the highest fertilization rate (approx. 97%), 10^3 and 10^4 sperm/egg ratios showed significantly lower fertilization rates. Finally, using the sperm activated after 40 s, negligible fertilization rate values were obtained irrespective of the sperm/egg ratio, and even the highest (10^5) showed very low values (3.8%).

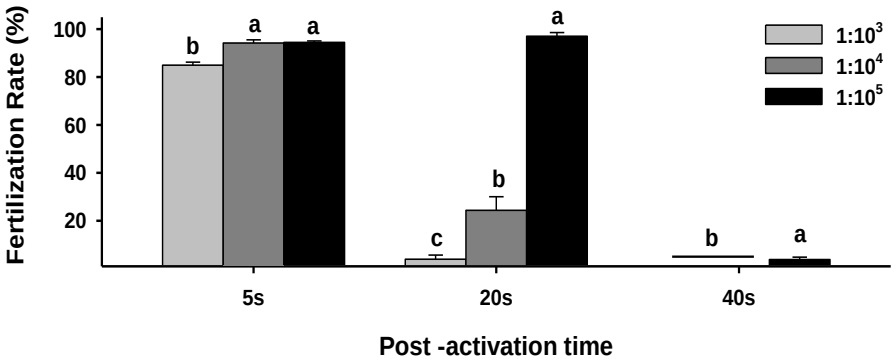

Figure 3. Fertilization rates at different post-activation times and sperm/egg ratios. Data from Trial 1 was used and values are expressed as mean ± SEM (n=5). Different letters mean significant differences between sperm/egg ratios at the same post-activation time.

3.2 Relationship between the sperm motility parameters and fertilization/hatching rates

Coefficients of correlation (*r*) and determination (*r*-squared) between the sperm motility parameters and fertilization/hatching rates are shown in

Table 2. Positive significant correlations between fertilization/hatching rates and several parameters such as TM, PM, FA, VCL, VSL VAP, LIN and WOB were found, although correlation values were slightly lower with hatching rates. The sperm parameters showing the highest positive correlations were TM, PM, VCL and VSL, shown in Fig. 4, where a linear regression equation was calculated for each parameter.

Table 2. Coefficients of correlation (*r*) and determination (*r*-squared) between the sperm motility parameters and fertilization or hatching rates (n = 15; data from Trial 2 was used). Asterisks indicates significant correlations between parameters (*, p-value < 0.05; **, p-value < 0.01).

	Fertilization rate		**Hatching rate**	
	r	***r*-squared**	***r***	***r*-squared**
TM	0.68**	0.47	0.67**	0.45
MP	0.70**	0.49	0.68**	0.47
FA	0.75**	0.56	0.74**	0.54
ME	-0.80**	0.64	-0.79**	0.63
SL	-0.49	0.24	-0.48	0.23
VCL	0.82**	0.68	0.81**	0.66
VSL	0.80**	0.63	0.78**	0.61
VAP	0.81**	0.65	0.80**	0.63
LIN	0.52*	0.27	0.51	0.26
STR	0.16	0.02	0.14	0.02
WOB	0.59*	0.34	0.57*	0.33
ALH	-0.19	0.04	-0.18	0.03
BFC	-0.44	0.19	-0.42	0.18

Abbreviations: TM, total motility; PM, progressive motility; FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral displacement; BCF, beat cross frequency.

Regarding coefficient of determination (*r*-squared; Table 2), which shows the goodness of fit of a model and represents the proportion of variability in a data set that is accounted by the statistical mode, spermatozoa velocities (VCL, VSL and VAP) showed the highest values both in fertilization and hatching rates.

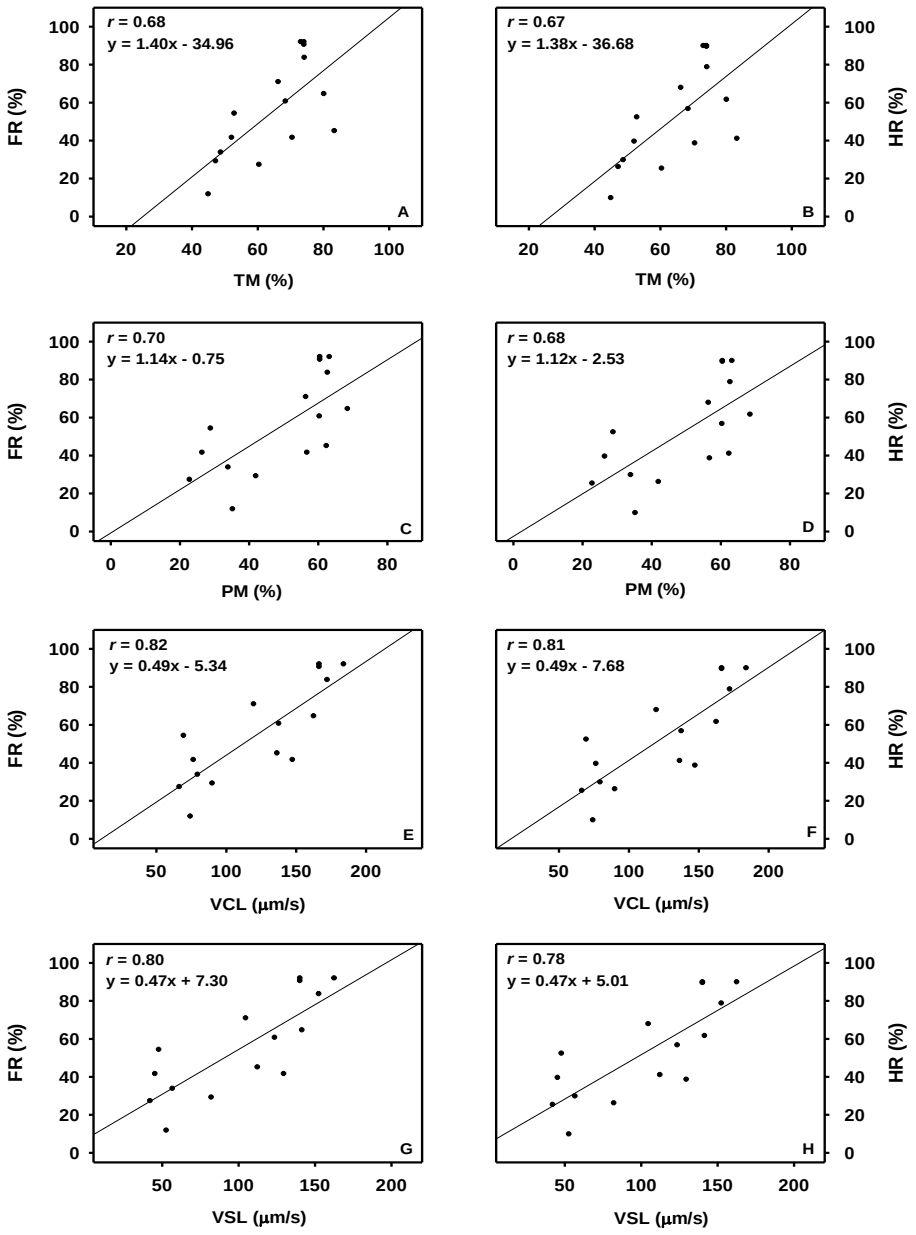

Figure 4. Relationship between the sperm motility parameters with fertilization (FR) and hatching (HR) rates (n=15). Data from Trial 2 was used to determine the correlations. Linear regression equation was calculated for each parameter. *Abbreviations: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity.*

Finally, fertilization and hatching rates showed a high and significant correlation between them (Fig. 5).

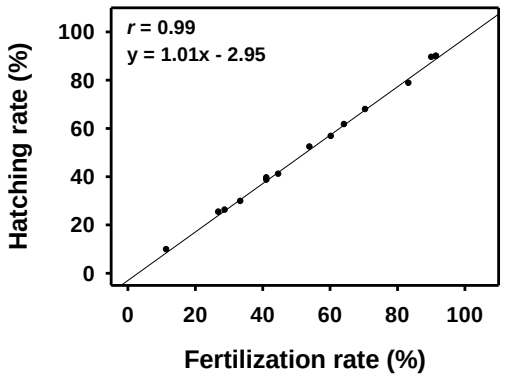

Figure 5. Relationship between fertilization and hatching rates (n=15; the experimental unit is each Petri Dish). Data from Trial 2 was used to determine the correlations and linear regression equation was calculated.

4. Discussion

Sperm/egg ratio and sperm quality are the main factors affecting fertilization and hatching success in artificial insemination trials (Linhart et al., 2008 and Trippel and Neilson, 1992). During the present study, the unavailability of low quality fresh sperm samples forced us to work with spermatozoa at different post-activation times with the aim of mimicking sperm samples of different qualities. Thus, during this study the sperm activated after 5 s represent the high-quality sperm samples (high motility and velocity values) while the sperm activated after 20 (high motility and intermediate velocity values) and 40 s (low motility and velocity values) resemble the medium and low quality sperm samples, respectively. However, it must be noted that the swimming time/speed of sperm activated after 20 and 40 s may be shorter than medium and low quality fresh samples.

In this study we have demonstrated that sperm/egg ratio and sperm quality are factors which are strongly related to each other: when high-quality

sperm samples (sperm activated after 5 s) were used in the IVF trials, all the different sperm/egg ratios tested produced high fertilization rates (> 80%). However, when lower quality sperm samples (sperm activated after 20 or 40 s) were used, the amount of sperm became an essential element in reaching suitable fertilization rates. These results suggest that both sperm/egg ratio and sperm quality should be taken into account as unique interrelated factors, with it possible to obtain high fertilization rates using a successful combination of high/low quality sperm in high/low volumes.

Nowadays, the aquaculture sector is going through a delicate situation and improvements in some issues with regards to IVF should be carried out (Gallego et al., Chapter 2). In this respect, fixing an appropriate combination of the number of spermatozoa per oocyte seems to be a useful tool to optimize the reproductive efficiency in fish farms. However, little data has been reported in marine species about the optimum sperm/egg ratio, which seems to be a species-specific parameter, finding ratios from 10^3 in Atlantic croacker (*Micropogonias undulatus*, (Gwo et al., 1991)) to 10^6 in northern pike (*Esox lucius*, (Zhang et al., 2011)). In this study, we have demonstrated that sperm/egg ratios from 10^3 to 10^5 can be used to achieve proper fertilization rates in pufferfish. However, the optimal sperm/egg ratio depends on the sperm sample quality, so, when good sperm samples are available, it would be possible to use low sperm/egg ratios ($\leq 10^3$); while when sperm samples with high motilities and velocities values are not available, it would be necessary to use higher sperm/egg ratios ($\geq 10^5$) to achieve high fertilization rates in this species.

On the other hand, the use of high quality gametes both from males and females is the other essential factor to reach suitable fertilization and hatching rates both for aquaculture and scientific purposes. With regards to male´s gametes, the percentage of motile cells has been the most used parameter to estimate sperm quality. However, for many years the conventional method of motility evaluation has been subjective, and the current appearance of CASA systems has made it possible to estimate a higher number of sperm parameters by an objective, rapid and accurate technique (Gallego et al., Chapter 1). In this study we have estimated, for

the first time, the relationship between all the parameters provided by a CASA system and the fertilization and hatching rates in a marine fish species. Total motility (TM) and progressive motility (PM) are recognized as important sperm traits for male fertility and sperm competition in fish (Rurangwa et al., 2004). In the present study, high correlations were found between these parameters and FR and HR ($r \sim 0.7$), the same as occurs in some fish species such as Atlantic halibut (*Hippoglossus hippoglossus*; (Ottesen et al., 2009)), red seabream (*Pagrus major*, (Liu et al., 2007)) or common carp (*Cyprinus carpio*, (Linhart et al., 2000)). However, parallel studies about this relationship in other species have given conflicting results, as negligible correlations were found between TM or PM and fertilization potential (Bozkurt and Secer, 2006). In this respect, it is worth highlighting that fertilization trials should be carried out both with an optimal sperm/egg ratio and using a wide range of sperm motility values in order not to mask the real correlations between the motility values and the fertilization and hatching rates (Moccia and Munkittrick, 1987).

On the other hand, in addition to the percentage of motile spermatozoa as a good tool to predict fertilization ability, spermatozoa velocities may also serve as prognostic indicators of the fertilization potential of sperm (Liljedal et al., 2008). In fact, in our study the highest coefficients of correlation and determination were found for VCL, VSL and VAP, which showed better correlations with FR and HR than the parameters traditionally used to define sperm quality (TM and PM). This result can be explained through logical hypothesis: at the gamete level, the egg-sperm contact could be influenced by several factors such as the amount of spermatozoa, the number of motile spermatozoa, sperm velocity and sperm longevity. When in IVF trials the number of spermatozoa becomes a limiting factor (tight sperm/egg ratio), increases in spermatozoa velocities will enable spermatozoa to look for the egg and penetrate the micropyle at a faster rate per time unit, increasing in this way fertilization success (Gage et al., 2004 and Linhart et al., 2005). In this respect, the results obtained in Trial 1 using 10^3 and 10^4 sperm/egg ratios support this hypothesis, and sharp decreases in fertilization rates were found when the sperm used showed significantly lower velocities but similar motilities (Fig 3).

Similar data has been reported in other marine species such as Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) or green swordtail (*Xiphophorus helleri*), in which spermatozoa velocity seems to be the major component that determines fertilization success and the proportion of the paternity through the sperm competition (Gage et al., 2004, Gasparini et al., 2010 and Rudolfsen et al., 2008). In this respect, new approaches in relation to male´s broodstock selection through sperm kinetics features can be used from this perspective. Improvements in the aquaculture sector could optimize the reproductive efficiency in the fish farms, making rational use of gametes possible, limiting the number of breeding fish and, thus, reducing production costs. However, it is important to highlight that breeding fish programs involves a lot of factors and, reducing the number of breeders we could also be decreasing the genetic diversity/basis of broodstock. Therefore, the proper application of several factors among these programs will define the further improvements in aquaculture sector.

Finally, it is important to highlight that besides the sperm/egg ratio and sperm kinetic parameters other factors involved in IVF can modulate fertilization and hatching rates. For example, variations in the spawning environment, oocyte quality, oocyte size or even the micropyle closing time can determine the final results. Therefore, it appears that it is critical to maintain identical fertilization conditions when sperm doses for IVF trials must be estimated, with the aim of avoiding masking effects through the experimental variables (Chereguini et al., 1999).

To sum up, this study showed that both sperm/egg ratios and some sperm kinetic parameters provided by CASA system play a crucial role in the fertilization and hatching success in pufferfish. These kinetic parameters have been defined throughout the IVF trials, and linear regression equations have been developed to the most important parameters with the aim of determining proper values of fertilization and hatching rates. In this respect, spermatozoa velocity seems to be a key factor in this event, especially when the number of spermatozoa per egg is limited in the aqueous environment. This kind of study can serve as a basis for improved efficiency in broodstock management fish reproduction.

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GENERAL DISCUSSION

1. Improvements to the control of European eel reproduction

The control of the reproduction of the European eel has become the focus of much research due to the severe decline in the natural stocks. Nowadays breeding in captivity seems to be the only alternative both in order to reduce the pressure on natural populations and to meet the demands of the eel farms (Feunteun, 2002).

In a breeding captivity program, the first step is to achieve high quality gametes in order to reach high fertilization and hatching rates (Bozkurt et al., 2006). During this thesis, alternative treatments for the induction of maturation were carried out with the aim of improving several male reproductive traits in the European eel such as the onset and the duration of the spermiation period and the quantity and quality of sperm (see Chapter 2).

The most used method until now in this species, which involves the weekly injection of hCG (1.5 IU g^{-1} fish) under a constant thermal regime of 20 ºC (Pérez et al., 2000), has previously shown successful spermiation results in closely related species such as the Japanese eel (*Anguilla japonica*; Ohta el al., 1997), American eel (*Anguilla rostrata*; Sorensen and Winn, 1984) or New Zealand eel (*Anguilla australis*; Todd, 1981), thus that treatment is able to induce in an effective way both spermatogenesis and spermiogenesis in eel species.

The results obtained in this study using this standard treatment agree with previously published reproductive data on European eel such as the onset of spermiation, the percentage of spermiating males or sperm volume (Asturiano et al., 2005; Pérez et al., 2000). However, the total motility values from our experiment (described in Chapter 2) were much higher than those obtained in previous studies where an identical maturation method had been applied. In this respect, numerous factors such as i) the variability between eel batches, ii) the hormone provider, and especially iii) the way of evaluating the sperm samples (subjective against objective methods) could explain these differences. Nowadays, thanks to the use of an objective and accurate previously standardized CASA system (see

Chapter 1), the way of evaluating the sperm samples does not handicap the assessment of the sperm quality, meaning it is possible to compare a wide range of aquacultural matters in a more effective and accurate way.

The water temperature, which plays an important role in the sexual maturation of several fish species, was chosen as the first factor to improve the reproductive performance of male European eels. Our results demonstrate that the onset and progression of spermiation were strongly influenced, and probably closely regulated, by changing water temperature. However, the alternative treatments tested in this study (T10 and T15) did not improve the results of the samples induced by the constant regime (T20), and a greater number of weeks were necessary in the alternative treatments both to induce the onset of spermiation and to achieve high quality sperm samples. Therefore, a constant temperature of 20 ºC is recommended in order to achieve the best spermiation performance during induced maturation of male European eels.

Nevertheless, it is necessary to highlight that when we talk about "best results", we do so from an aquaculture point of view, trying to get spermiating males and high quality sperm weeks in advance. From a biological viewpoint, we observed that treatments T10 and T15 were also able to induce both a high percentage of spermiating males and high quality samples, but several weeks later. The warm temperatures during these alternative treatments (T10 and T15) were strictly imperitive in inducing final sperm production, thus this fact could indicate that gonadal development in eel males could be slowed down by the cold waters they encounter before arriving at the warm Sargasso Sea, and then relaunched once there in order to synchronize gamete maturation and the fertilization of the eggs produced from the females. In fact, it has been reported that variable thermal regimes in both European and Japanese eel females induce hormonal profiles that more closely resemble the natural ones than those obtained under constant temperatures (Dou et al., 2008; Pérez et al., 2011). Therefore hormonal data from male eels undergoing variable thermal regimes are being estimated in order to evaluate the hormonal

profiles induced by each thermal treatment (Peñaranda et al., *in preparation*).

After selecting the best thermal regime (T20), the kind of hormone used for the induction of maturation was chosen as the second factor for the improvement of reproductive performance. Although successful maturation and spermiation by hCG has been reported by several authors in the last half century (Boëtius and Boëtius, 1967; Bleniarz and Epler, 1977; Amin, 1997), scarce attempts have been made to optimize previously used hormonal treatments (Asturiano et al., 2005). Our results have shown that the type of hormone used significantly affects the onset and progression of spermiation in European eel males, and a recombinant version of the hCG (hCG_{rec}) produces the best results in almost all the sperm parameters.

Until the present study, only Asturiano et al. (2005) had carried out trials with the aim of achieving shorter induction treatments, longer spermiation periods and/or higher sperm quality. In their experiment, five hormonal treatments based on hCG, modifying both the doses and timing of injection, were tested for the induction of maturation and spermiation in male European eels, reaching a good reproductive performance. Even so, the results produced by the hCG_{rec} treatment in the present trial far exceed those reached by Asturiano et al. (2005), thus making it an effective and alternative treatment for inducing spermiation in male European eels.

To sum up, a new hormonal treatment which improves the reproductive performance of male European eels has been set up and is ready for applying in eel hatcheries. This treatment, based on the weekly administration of hCG_{rec} under a constant temperature regime of 20 °C, has been able to induce a high percentage of spermiating males with good quality samples for a high number of weeks. In addition, other tools such as short and long-term sperm storage methods have specifically been developed for this species in recent years (Peñaranda et al., 2008, 2009, 2010). With regards to short-term sperm storage methods, recent studies have come up with good results in terms of fertilized eggs and hatched larvae during *in vitro* fertilization trials. However, although it was possible

to obtain the first European eel “cryolarvae”through cryopreserved spermatozoa, the low percentages achieved in terms of fertilization and hatching rates mean that some improvements in the cryopreservation techniques for this species are necessary (Asturiano et al., 2013).

However, new methods are needed to enable the production of viable eggs and larvae from broodstock in a regular and predictable way. In this respect, further challenges include the identification of suitable larval rearing conditions and initial feed in order to establish feeding cultures of European eel larvae.

2. Sperm quality biomarkers: which, how and where to apply?

The use of high quality gametes from both males and females in the field of aquaculture is essential in order to achieve a production of viable fertilized eggs and larvae from broodstock. In this respect, sperm quality biomarkers have proven themselves a powerful tool which is ready to use, making itossible to predict fertilization rates in IVF trials. In this thesis, we have demonstrated that both the number of spermatozoa and their “swimming” parameters (time, velocity, direction, etc.) have a great influence on fertilization and hatching success in pufferfish, thus sperm motion parameters can be used as sperm quality biomarkers in this species. However, it is important to note that although certain sperm quality biomarkers can be used in different fish species, sometimes spermatozoa motion features are species-specific and depends on spawning microenvironment, reproductive strategy, etc., thus more studies on different fish species are necessary to analyze the specific requirements of each one in order to define their own sperm quality biomarkers.

On the other hand, a single sperm biomarker by itself often does not provide enough data, and a complete assessment of several biomarkers generates the right information. In fact, we have shown how sperm/egg ratio and sperm motion must be taken into account as unique factors, making it possible to apply a successful combination of high/low quality sperm in low/high volumes in order to achieve fertilized oocytes. There are

more studies on multifactorial sperm biomarkers. For example, Lahnsteiner el al. (1998) reported that in rainbow trout motile spermatozoa, seminal plasma pH and sperm metabolism were able to explain a high percentage of variance in fertilization rates. In fact, some sperm traits in that trial were linked, and subsequently high quality semen was characterised by high sperm motility (≥75%), medium sperm swimming velocities (100–120 µm/s) and optimal seminal fluid protonic composition (pH of 8.0–8.2). In this respect, it suggests that in-depth knowledge of as many sperm traits as possible allows us to establish the relationships between them, making it possible to assess sperm quality though a small set of species-specific biomarkers able to predict both fertilization and hatching rates.

Following on from this basic idea, CASA systems are able to provide us with many spermatozoa motion parameters, and each one can be correlated with the spermatozoa ability to fertilize the oocyte. In fact, positive correlations between fertilization and hatching rates and many CASA parameters (8 out of 13) were found in pufferfish (see Chapter 5). The subsequent questions are: which should we choose? Which are the most important? Really, the answer is to choose those which show the highest correlations, and checking a so far not very extensive bibliography on this topic in fish can help us make this choice. However, another approach could be to study sperm subpopulations. This kind of study uses data mining techniques such as cluster analysis, grouping the spermatozoa in subpopulations that have a biological meaning (Martinez-Pastor et al., 2011). In this respect, although there are scarce reports in fish, other recent trials have reported the coexistence of several subpopulations in different species. For example, 3 sperm subpopulations have been described in European eel sperm, and they have been used to evaluate different treatments for the induction of maturation in this species (Gallego et al., submitted). In this case, subpopulations were made by taking some CASA parameters, not often used for assessing directly the sperm quality, as a basis (LIN, WOB, ALH and DNC). For this reason, it is important to note that all the sperm parameters analyzed by CASA systems must be taken into account in order to generate powerful and useful information to detect new sperm quality biomarkers in fish species.

Sperm motion parameters become a helpful tool not only for aquaculture purposes, but also regarding other diverse issues. For example, it has been reported that environmental pollution from cadmium or zinc, can affect sperm motility parameters and thus, reproduction success (Kime et al., 1996). In this respect, it would be possible to detect toxic discharges in aquatic environments simply by assessing some fish sperm motion parameters. Another powerful application is that of ethological matters: some fish species show complex social relationships between males, resulting in a different mating tactics, and it is astonishing to observe how sperm motion is complexly defined by the status of the male (Fitzpatrick et al., 2007; Gallego et al., unpublished results).

To summarize, the study of the sperm motion parameters assessed by a CASA system during these IVF trials has made some of them useful fish sperm quality biomarkers. Which, how and where to apply them it depends directly on the species and the aim of the research, but sperm biomarkers have proven themselves to be useful tools which can be applied in a wide range of fields such as aquaculture, and for scientific or ecological purposes.

3. New data about the sperm activation process in marine fish

Spermatozoa from external fertilizers are usually released in a hostile external medium, where they have to cope with extremely harmful conditions on their way to reach and fertilize the oocyte (Cosson et al., 2004). In this respect, the features of the aquatic environment will define, in addition to the sperm activation, both the swimming time period and the motion patterns of the spermatozoa.

In this thesis we have shown that both the osmolality and the availability of certain ions (Ca^{2+} and K^{+}) in the activation media are able to affect the normal motion patterns of spermatozoa. Understanding how these factors affect sperm motility could be key in improving spermatozoa performance for aquaculture matters. Regarding osmolality, partly diluted sea water has been used in some marine species to induce good motility activation without exposing sperm to extreme osmotic conditions, increasing thus the

sperm motility patterns and the period of fertility (Billard, 1978). However, our data has shown that sperm motility parameters do not improve when using partly diluted seawater in pufferfish, so the common activation medium (not diluted seawater) is recommended when carrying out reproduction trials in this species.

Regarding the ion composition of the activation media, we have reported that despite the fact that pufferfish spermatozoa were able to initiate movement without any ion in the activation medium, the presence/absence of Ca^{2+} and K^{+} affected kinetic parameters such as velocities. We also observed that spermatozoa velocities (VCL, VSL and VAP) were highly correlated to the fertilization success and hatching rates. It can therefore be assumed that the presence of these ions in the activation media helps the fertilization process, thus the proper ion composition of the activation/fertilization media should be taken into account in order to reach suitable fertilization and hatching rates in both laboratory trials and commercial hatcheries.

However, while in the majority of marine fish species studied sperm motility can be induced by hyperosmotic non-ion solutions (using sugars, i.e.), in species such as tilapia (*Oreochromis mossambicus*) certain ions such as calcium are essential for sperm activation (Linhart et al., 1999). Thus, it seems clear that there is a wide interspecific variability in terms of the role of extracellular ions and their effect on the sperm motion parameters, so more studies focusing this topic are necessary in order to understand the sperm motility activation process in marine fish.

On the other hand, although it is widely accepted that firstly, the osmolality, and secondly, the ion composition of the activation media are the main factors triggering sperm motility in marine fish, little is known about the molecular mechanisms that enable environmental stimuli to determine the activation of the axoneme and the intracellular ion changes occurring after sperm activation (Zilli et al., 2012). In this thesis we have reported that when using an ion-rich medium (seawater) for activation of the sperm, intracellular Ca^{2+} and K^{+} increased both in European eel and

pufferfish spermatozoa, as occurs in other marine fishes (Oda & Morisawa, 1993; Morisawa, 2008). However, when using a free-ion activating medium, there was an increase in $[K^+]_i$ but not in $[Ca^{2+}]_i$, even when motility started, and the same has recently been observed in European eel sperm (data not published). These results pose a tricky question because nowadays, the widely accepted model regarding sperm not only from fish, but also from mammals and marine invertebrates (see Darszon et al., 2005, 2011; Espinal et al., 2011), indicates that an increase in intracellular Ca^{2+} is necessary for sperm motility activation. However, in the case of pufferfish (see Chapter 4) and European eel (unpublished results), this intracellular calcium increase seems unnecessary, at least for triggering motility, thus other mechanisms could be involved in the initiation of sperm motility in this species.

On the other hand, seawater also contains significant amounts of other ions such as Cl^-, $HCO3^-$, Na^+ and Mg^{2+}, and all of them have important functions in cell biology, and can affect sperm motility in marine fish. For example, Na^+ has been studied in freshwater fish sperm (Alavi & Cosson, 2006), and preliminary studies in European eel also suggest that Na^+ fluxes happen at motility activation. In mammals, sperm motility hyperactivation involves the activation of sperm-specific Ca^{2+}, and K^+ channels (CatSper, KSper), a specific Na^+/HCO3 co-transporter (sNHE), a proton channel (Hv), and a Na^+/Ca^{2+} channel (Darszon et al., 2011; Krasznai et al., 2006). In sea urchin, sperm motility involves the participation of at least 8 different ion channels, controlling Ca^{2+}, K^+, Cl^-, Na^+, and H^+ fluxes in the sperm membrane (Espinal et al., 2011). We are far from having a similar understanding of sperm activation in marine fish, where a complex universal mechanism for sperm motility initiation does not seem to exist, and different species-specific activation mechanisms could be acting among marine species.

Therefore, new studies involving the use of specific channel blockers, the intracellular measure of other ions (like Na^+, Cl^-, HCO_3^-), and the use of patch-clamp techniques would help to understand the specific mechanisms which occur during motility activation in marine fish sperm.

CONCLUSIONS

i. The foundations of a standardized and accurate method of assessing fish sperm quality through the CASA system have been established, allowing the comparison of both inter and intra laboratory sperm studies.

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

iii. A short-term sperm storage method for puffer fish sperm was developed, preserving the sperm quality parameters for a relatively long-term period (7 days) compared to fresh sperm samples.

iv. Both the osmolality and the ion composition of the activation media had a significant impact on the sperm motion parameters, consequently playing an essential role in the initiation of the sperm motility of pufferfish and, probably, of other marine fish.

v. The intracellular concentrations of Ca^{2+} and K^{+} increased upon sperm activation both in pufferfish and European eel, suggesting that these ions play an important role in the initiation of sperm motility, as occurs in other marine and freshwater teleosts.

vi. The sperm/egg ratio and the sperm quality were strongly linked, suggesting that both should be taken into account as unique interrelated elements during *in vitro* fertilization trials.

vii. The coefficients of correlation between all the sperm motion parameters provided by a CASA system and the fertilization and hatching rates were estimated for the first time in a marine species. Spermatozoa velocities showed the highest coefficients (≥0.80), suggesting that kinetics of spermatozoa is a key factor in fertilization trials.

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