Influence of the ionic and protein environment on sperm motility activation in the European eel

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“Todo lo que somos es el resultado de lo que hemos pensado, y con nuestros pensamientos levantamos el mundo”.

Siddharta Gaut

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SUMMARY

In general, fish spermatozoa are immotile in the testis. Movement is activated due to the osmotic shock (hypo- or hyperosmotic, depending on fish origin: freshwater or sea water species) experienced when they are released into the external medium. However, there is no consensus regarding the mechanisms that occur after activation. The aim of this project was to study the importance of the environmental ions (Ca^{2+}, K^+, Na^+, H^+) and proteins on the activation of European eel sperm, and to apply this knowledge to the improvement of sperm quality preservation. As such, the present study focuses on the importance of Ca^{2+}, K^+, Na^+, H^+ levels both in the seminal plasma and in the activation media, in sperm motility activation. For this purpose, the role of these ions was examined by removing each specific ion from the seminal plasma and the activation media, of European eel sperm. Our results demonstrated that there was a notable reduction in sperm motility when either Na^+ or K^+ was removed from the seminal plasma, but not when they were removed from the activation media. Therefore, our results demonstrated that the presence of Na^+ or K^+ in the seminal plasma is necessary for the preservationof sperm motility in European eel. However, the presence of Na^+ or K^+ in the activation media is not essential for the initiation of sperm activation. In contrast, the presence of the ion Ca^{2+} in the seminal plasma (or the activation media) was not essential for sperm motility activation in this fish species.

Moreover, several authors have hypothesised that the hyperosmotic aquatic environment causes an efflux of water through the spermatozoa membrane, and this efflux causes an increase in the intracellular ion concentration (due to the decrease in cellular volume). However, this hypothesis has never been proven. In this study, sperm size (sperm head area) was studied pre- and post-activation in sea water, and in different conditions. For the first time in a marine fish, a significant decrease in sperm head area post activation in sea water was demonstrated. Also the results of this thesis show a notable reduction in sperm head area when either Na^+ or K^+ was removed from the seminal plasma, as well as a marked reduction in motilityed. Thus, our results demonstrate that the presence of K^+ and Na^+ in the seminal plasma is important for the preservation of sperm motility in the European eel, at least in part by maintaining the right sperm cell volume in the quiescent stage.

In the this study, a method for the quantitative analysis of the intracellular [Na^+] ([Na^+]$_i$) and pH (pH_i) levels of eel sperm was created, and used to study the variations in [Na^+]$_i$ (for first time in a marine fish) and pH_i during motility activation. The pH_i in the quiescent stage was 1.3 units lower than the pH of the seminal plasma, indicating that a H^+ gradient exists in the quiescent stage, with higher [H^+]$_i$ levels than those found in the seminal plasma; an important difference for sperm motility. In contrast, the results show that Na^+ levels are the same both outside and inside the spermatozoa in the quiescent stage.

Our results demonstrate that for sperm motility activation to be successful, the pH_i should be lower than the seminal plasma pH and lower or equal to the activation media pH.

The absolute [Na^+]$_i$ concentration of marine fish spermatozoa was 1.5 times higher after motility activation than in the quiescent stage, and this increase in intracellular Na^+ after activation could be caused both by a cell volume decrease and by the influx of external Na^+.

Regarding the ion Ca^{2+}, our results strongly suggest that an increase in intracellular [Ca^{2+}]

($[Ca^{2+}]_i$) is not a pre-requisite for the initiation of sperm motility in European eel sperm. Nevertheless several sperm velocity parameters (VCL, VSL, VAP) decreased in the absence of Ca^{2+}, thus supporting the theory that Ca^{2+} has a modulatory effect on sperm motility. In addition, it appears that the ion Ca^{2+} is involved in the sperm motility of the European eel through the presence of a sodium/calcium exchanger. This was made evident by the increase in $[Ca^{2+}]_i$ and the decrease in $[Na^{+}]_i$ produced by bepridil, which strongly inhibited sperm motility. The use of specific inhibitors during this thesis has demonstrated that 4-AP-sensitive voltage gated potassium channels and an amiloride-sensitive sodium channel are involved in motility activation of European eel sperm.

Finally, proteomics science was used to determine the seminal plasma protein composition, in order to explore potential correlations between sperm quality parameters and the seminal protein profile of European eel. In fact, this is the first time that the 2DE protein composition of the seminal plasma of the European eel has been identified, with 14 different proteins corresponding to 9 major families being identitfied. Different protein profiles were observed within the different sperm motility categories, in particular proteins linked to lipid transport (apolipoprotein) and to the immune system (complement C3). The high presence of lipid transport proteins (apolipoproteins) in samples with lower motility suggests that this family of proteins could have some role in the early phases of sperm maturation. In contrast, high levels of complement C3 were observed in samples with higher motility, suggesting they play an immunologic role against microbial infection, especially during final sperm maturation, when the sperm cell is about to be released into the external environment. As a whole, these results suggest that the proteins linked to lipid transport (apolipoprotein) and to the immune system (complement C3) act at different stages of the sperm maturation process.

RESUMEN

En general, los espermatozoides de los peces se encuentran inactivos en los testículos. La activación de los espermatozoides se produce mediante choque osmótico (hipo o hiperosmótico, según se trate de especies de peces de agua dulce o marinos), cuando son liberados en el medio externo. Sin embargo, no existe un consenso sobre el mecanismo fisiológico que ocurre tras la activación espermática. El objetivo de este trabajo es estudiar la importancia de iones (Ca^{2+}, K^+, Na^+, H^+) y proteínas presentes en el medio, en la activación del esperma de la anguila europea, y aplicar éste conocimiento a la mejora de la preservación de esperma. Así pues, este trabajo se centra en estudiar la importancia que tienen los iones Ca^{2+}, K^+, Na^+, H^+ tanto en el plasma seminal como en el medio activador, y su función en la activación de la motilidad espermática. Por lo tanto, el papel de dichos iones fue examinado mediante la eliminación de forma selectiva de los iones tanto del plasma seminal como del medio activador. Los resultados demostraron una importante reducción de la motilidad espermática cuando Na^+ o K^+ fueron eliminados del plasma seminal, pero no así cuando fueron eliminados del medio activador. Así pues, nuestros resultados demostraron que la presencia de Na^+ o K^+ en el plasma seminal (diluyente) fue necesaria para preservar la motilidad espermática en la anguila europea. Sin embargo, la presencia de Na^+ o K^+ en el medio activador no es esencial para la activación espermática. En contra, la presencia del ion Ca^{2+} tanto en el plasma seminal como en el medio activador no es esencial para la activación espermática en la anguila europea.

Además, algunos autores han sugerido que el medio acuático hiperosmótico causa una salida de agua a través de la membrana del espermatozoide, y ésta salida causa un incremento en la concentración de iones intracelulares (provocada por un descenso del volumen celular). Sin embrago, dicha hipótesis no ha sido demostrada. En este trabajo, se ha estudiado los cambios que se producen en el tamaño del espermatozoide (área de la cabeza) durante la pre- y post- activación tanto en agua de mar, como en diferentes condiciones. Por primera vez en una especie de agua marina, se ha demostrado un descenso significativo en el área de la cabeza tras la activación con agua de mar. Además, los resultados de esta tesis también mostraron una importante reducción en el área de la cabeza del espermatozoide cuando Na^+ o K^+ fueron eliminados del plasma seminal, al igual que una importante reducción de la motilidad espermática. Por lo tanto, nuestros resultados demostraron que la presencia de Na^+ y K^+ en el plasma seminal es importante para la preservación de la motilidad espermática en la anguila europea, en parte debido al mantenimiento de un volumen celular adecuado en estado quiescente.

En el presente trabajo, se ha desarrollado un método cuantitativo de análisis de $[Na^+]$ ($[Na^+]_i$) y pH (pH_i) intracelulares para el esperma de la anguila europea, y mediante el uso de esta metodología se han estudiado las variaciones de $[Na^+]_i$ (por primera vez en un pez marino) y pH_i durante la activación espermática en la anguila europea. El pH_i en estado quiescente fue 1.3 unidades menor que el pH del plasma seminal, demostrando la existencia de un gradiente de H^+ en estado quiescente mayor que la $[H^+]_i$ en el plasma seminal, siendo dicha diferencia importante para la motilidad espermática. En cambio, se observó un equilibrio de Na^+ dentro y fuera del espermatozoide en estado quiescente.

Nuestros resultados mostraron que para una correcta activación de la motilidad, el pH_i debería de ser menor que el pH del plasma seminal y menor o igual que el pH del medio

activador.

Después de la activación espermática, la concentración de $[Na^+]_i$ en términos absolutos del espermatozoide de un pez marino fue 1.5 veces mayor que en estado quiescente. Este incremento en Na^+ intracelular después de la activación podría ser causado tanto por un descenso en el volumen celular como por un flujo de entrada de Na+ del exterior.

En cuanto al ion Ca^{2+}, nuestros resultados sugieren de forma fehaciente que el incremento de la concentración de Ca^{2+} intracelular ($[Ca^{2+}]_i$) no es un pre-requisito para la inducción de la motilidad espermática en la anguila europea, aunque determinadas velocidades espermáticas (VCL, VSL, VAP) disminuyeron en ausencia de Ca^{2+}, por lo tanto es posible que Ca^{2+} tuviera un efecto modulador en la motilidad espermática. Además, el ion Ca^{2+} tiene una función en la motilidad espermática de la anguila europea debido a la presencia del intercambiador sodio/calcio, el cual ha sido demostrado por el incremento de $[Ca^{2+}]_i$ y el descenso de $[Na^+]_i$ producido por el bepridil, el cual inhibió de forma drástica la motilidad espermática. El uso de inhibidores específicos durante ésta tesis ha demostrado que el canal de potasio sensible a 4-AP y controlado por voltaje, y el canal de sodio sensible a amiloride están involucrados en la activación de la motilidad espermática.

Por último, estudios en proteómica se han usado durante esta tesis para realizar una completa descripción de la composición proteica del plasma seminal, con el fin de estudiar posibles correlaciones entre los parámetros de calidad espermática y el perfil proteico del plasma seminal en la anguila europea. Para ello, y por primera vez, se ha realizado el perfil proteico en 2DE del plasma seminal de la anguila europea, mostrando 14 proteínas diferentes correspondientes a 9 familias mayoritarias. Diferentes perfiles proteicos has sido observados dependiendo de las categorías de motilidad espermática, en concreto, las proteínas relacionadas con el transporte de lípidos (apolipoproteinas) y con el sistema inmune (complemento C3). La elevada presencia de proteínas transportadoras de lípidos (apolipoproteinas) en muestras con baja motilidad, sugiere que dicha familia de proteínas podría tener algún papel en fases tempranas de la maduración espermática. En cambio, las proteínas del complemento C3 mostraron elevada presencia en muestras con elevada motilidad, lo que sugiere un papel inmunológico contra infecciones bacterianas, especialmente durante la fase final de maduración espermática, cuando la célula espermática se encuentra cerca de ser liberada al medio externo. Por lo tanto, estos resultados sugieren que las proteínas relacionadas con el transporte de lípidos (apolipoproteinas) y con el sistema inmune (complemento C3) podrían llevar a cabo sus funciones durante diferentes fases del proceso de maduración espermática.

RESUM

En general, els espermatozoides dels peixos es troben inactius als testicles. L'activació dels espermatozoides es produeix per mitjà d'un xoc osmòtic (hipo o hiperosmòtic, segons es tracte d'espècies de peixos d'aigua dolça o marins) quan són alliberats a l'exterior. No obstant això, no hi ha un consens sobre el mecanisme fisiològic que ocorre després de l'activació espermàtica. L'objectiu d'aquest treball fou estudiar la importància dels ions (Ca^{2+}, K^+, Na^+, H^+) i proteïnes presents en el mig en l'activació de l'esperma d'anguila europea, i aplicar aquest coneixement a la millora de la preservació de l'esperma. Així doncs, aquest treball es va centrar en estudiar la importància que tenen els ions Ca^{2+}, K^+, Na^+, H^+ tant en el plasma seminal com en el mig activador, i la seua funció en l'activació de la motilitat espermàtica. El paper d'aquests ions va ser examinat per mitjà de l'eliminació de forma selectiva dels ions tant del plasma seminal com del mig activador. Els resultats van demostrar una important reducció de la motilitat espermàtica quan Na^+ o K^+ van ser eliminats del plasma seminal, però no així quan van ser eliminats del mig activador. Així doncs, els nostres resultats van demostrar que la presència de Na^+ o K^+ en el plasma seminal va ser necessària per a preservar la motilitat espermàtica en anguila europea. No obstant això, la presència de Na^+ o K^+ en el mig activador no fou essencial per a l'activació espermàtica. En contra, la presència de l'ió Ca^{2+} tant en el plasma seminal com en el mig activador no fou essencial per a l'activació espermàtica en anguila europea.

A més, alguns autors han suggerit que el medi aquàtic hiperosmòtic causa una eixida d'aigua a través de la membrana de l'espermatozoide, i aquesta eixida causa un increment en la concentració d'ions intracel•lulars (provocada per un descens del volum cel•lular). No obstant, aquesta hipòtesi encara no ha sigut demostrada. En aquest treball, s'han estudiat els canvis que es produeixen en la grandària de l'espermatozoide (àrea del cap) durant la pre- i post- activació tant en aigua de mar, com en diferents condicions. Per primera vegada en una espècie d'aigua marina, s'ha demostrat un descens significatiu en l'àrea del cap després de l'activació amb aigua de mar. A més, els resultats d'aquesta tesi també van mostrar una important reducció en l'àrea del cap de l'espermatozoide quan Na^+ o K^+ van ser eliminats del plasma seminal, igual que una important reducció de la motilitat espermàtica. Per tant, els nostres resultats van demostrar que la presència de Na^+ i K^+ en el plasma seminal és important per a la preservació de la motilitat espermàtica en anguila europea, en part a causa del manteniment d'un volum cel•lular adequat en estat quiescent.

En el present treball, s'ha desenvolupat un mètode quantitatiu d'anàlisi de $[Na^+]$ ($[Na^+]_i$) i pH (pH_i) intracel•lulars per a l'esperma d'anguila europea, i per mitjà de l'ús d'aquesta metodologia s'han estudiat les variacions de $[Na^+]_i$ (per primera vegada en un peix marí) i pHi durant l'activació espermàtica en anguila europea. El fi en estat quiescent va ser 1.3 unitats menor que el pH del plasma seminal, demostrant l'existència d'un gradient de H^+ en estat quiescent major que la $[H^+]_i$ en el plasma seminal, sent aquesta diferència important per a la motilitat espermàtica. En canvi, es va observar un equilibri de Na^+ dins i fora de l'espermatozoide en estat quiescent.

Els nostres resultats van mostrar que per a una correcta activació de la motilitat, el pH_i deuria ser menor que el pH del plasma seminal i menor o igual que el pH del mig activador.

Després de l'activació espermàtica, la concentració de $[Na^+]_i$ en termes absoluts de l'espermatozoide d'un peix marí va ser 1.5 vegades major que en estat quiescent. Aquest

increment en Na^+ intracel•lular després de l'activació podria ser causat tant per un descens en el volum cel•lular com per un flux d'entrada de Na^+ de l'exterior.

Respecte a l'ió Ca^{2+}, els nostres resultats suggereixen de forma fefaent que l'increment de la concentració de Ca^{2+} intracel•lular ($[Ca^{2+}]i$) no és un prerequisit per a la inducció de la motilitat espermàtica en anguila europea, encara que les velocitats espermàtiques (VCL, VSL, VAP) van disminuir en absència de Ca^{2+}, per tant és possible que el Ca^{2+} tinguera un efecte modulador en la motilitat espermàtica. A més, l'ió Ca^{2+} té una funció en la motilitat espermàtica de l'anguila europea a causa de la presència de l'intercanviador Na^+/Ca^{2+}, el qual ha sigut demostrat per l'increment de $[Ca^{2+}]_i$ i el descens de $[Na^+]_i$ produït pel bepridil, el qual va inhibir de forma dràstica la motilitat espermàtica. L'ús d'inhibidors específics durant aquesta tesi ha demostrat que el canal de potassi sensible a 4-AP i controlat per voltatge, i el canal de sodi sensible a amiloride estan involucrats en l'activació de la motilitat espermàtica.

Finalment, estudis proteòmics s'han usat durant aquesta tesi per a realitzar una completa descripció de la composició proteica del plasma seminal, amb l'objectiu d'estudiar possibles correlacions entre els paràmetres de qualitat espermàtica i el perfil proteic del plasma seminal en anguila europea. Per a això, i per primera vegada, s'ha realitzat el perfil proteic en 2DE del plasma seminal d'anguila europea, mostrant 14 proteïnes diferents corresponents a 9 famílies majoritàries. Diferents perfils proteics han sigut observats depenent de les categories de motilitat espermàtica; en concret, les proteïnes relacionades amb el transport de lípids (apolipoproteïnes) i amb el sistema immune (complement C3). L'elevada presència de proteïnes transportadores de lípids (apolipoproteïnes) en mostres amb baixa motilitat, suggereix que aquesta família de proteïnes podria tindre algun paper en fases primerenques de la maduració espermàtica. En canvi, les proteïnes del complement C3 van mostrar elevada presència en mostres amb elevada motilitat, la qual cosa suggereix un paper immunològic contra infeccions bacterianes, especialment durant la fase final de maduració espermàtica, quan la cèl•lula espermàtica es troba prop de ser alliberada al medi extern. Per tant, aquestos resultats suggereixen que les proteïnes relacionades amb el transport de lípids (apolipoproteïnes) i amb el sistema immune (complement C3) podrien dur a terme les seues funcions durant diferents fases del procés de maduració espermàtica.

GENERAL INTRODUCTION

1. Life cycle and current situation

The European eel (*Anguilla anguilla*) is a peculiar teleost fish with an interesting physiology due to their unusual life cycle (Figure 1). The migration and reproduction of the European eel was a mystery until the 20th century, when the spawning area in the Sargasso Sea was discovered (Schmidt, 1923). The reproductive migration begins during the autumn months, before which the eels adapt their physiology in preparation for their migration in the deep oceanic waters. Changes in their physiology include eye enlargement, darkening of the dorsal skin and fins, and the silvering of the abdomen. As such, the eels acquire characteristics typical of mesopelagic fish.

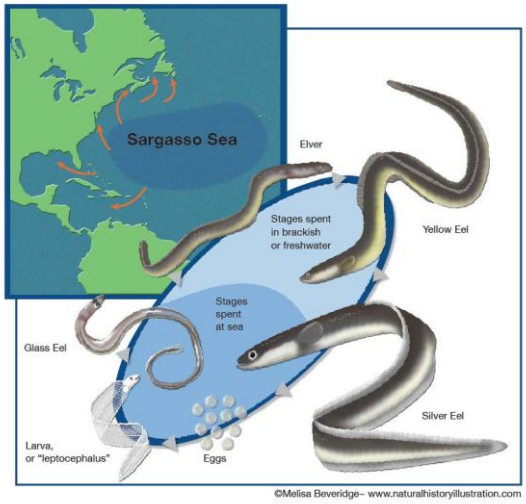

Figure 1. Pictures of European eel metamorphosis: a) eggs; b) leptocephali; c) glass eel; d) yellow eel; e) silver eel; f) mature eel (by Melisa Beveridge).

Apparently, the eels take approximately 6-7 months to travel from the European coasts to the Sargasso Sea (about 5000-7000 km), where spawning takes place during the spring or summer months. After the spawning, it would appear that the adults die, as none have been caught on their return trip (Usui, 1978). The tiny eel larvae (called leptocephali), similar in appearance to “olive leaves”, begin the return to Europe with the help of the Gulf Stream. When they arrive near the coasts, the leptocephali metamorphose into glass eels, with transparent cilindric bodies (Tesch, 1978; Van Ginneken and Maes, 2005).

These glass eels enter the rivers and swim upstream searching for freshwater ecosystems inland. Once the eels are established, a reactivation of growth occurs as a result of trophic activity. Nowadays it is known that, years later, mature adults return to the sea for the reproductive migration when the water temperatures decrease in autumn, and at specific times depending on the lunar phase and atmospheric conditions (Tesch, 2003).

The European eel population has declined dramatically since the 1980’s, with levels decreasing to only 1% of the population existing in the 1950’s. This species has listed as “critically endangered” in the Red List of the IUCN, and in 2007 the EU established a Law for the protection of the species (EC 1100/2007) through specific national management plans for the European eel. Several causes have contributed to the decline of the European eel:

overfishing, migration barriers, exotic parasites, pollution and habitat reduction. Because of this decline, and the importance of this species in the food market researchers have been focusing their efforts on understanding how to reproduce the eel in captivity.

2. The fish testis and spermatozoa

2.1. General structure of the teleost testis

In all vertebrates, from fish to mammals, the testis is composed of two main compartments, the intertubular (or interstitial) and the tubular compartment. The intertubular compartment contains steroidogenic Leydig cells, blood/lymphatic vessels, macrophages and mast cells, neural and connective tissue cells, with the latter surrounded by the tunica albuginea (Koulish et al., 2002). The tubular compartment is delineated by a basement membrane and peritubular myoid cells, and houses the germinal epithelium. The basic functional unit of the spermatogenic epithelium in fish is a spermatogenic cyst where a small number of spermatogonial stem cells produce a large number of highly differentiated spermatozoa (Figure 2).

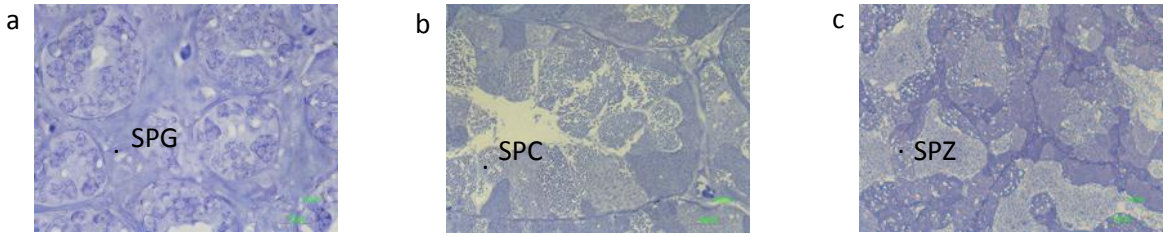

Figure 2. Photomicrographs of histological sections of the different stages observed during treatment in the testis of European eel. Testis at stage of spermatogonia proliferation (a), testis at stage of meiosis (b), and testis at stage of spermatozoa (c). SPG, spermatogonia; SPC, spermatocyte; SPD, spermatid; SPZ, spermatozoa. Scale bar, 100 lm (A–C).

2.2. General structure of fish spermatozoa

The usual structure of the spermatozoa is composed of a head, containing the nucleus (the acrosome is absent in most teleost species) and mitochondria; a middle piece formed by the centriole; and a flagellum, which provides motility. The internal flagellum cytoskeleton is the axoneme, which is essential for motility. The axonemes are comprised of 9 pairs of peripheral microtubules (A and B) and one center (9 + 1), although the flagellum from the eels lacks the central pair (9 + 0) (Billard and Ginsburg, 1973).

Sperm motility occurs by active sliding of the axonemal microtubules caused by the dynein arms. The sliding of a pair of microtubules respect to the adjacent pair of microtubules produces a wave-like movement which is transmitted along the flagellum. The ATP hydrolysis is necessary for this movement (Inaba, 2011).

3. Seminal plasma in fish

Seminal plasma is a multi-functional, heterogeneous and complex protein-rich fluid in which spermatozoa cells are diluted (Rodríguez-Martínez et al., 2011). The seminal plasma of teleosts is a product of the testes (Clemens and Grant, 1964) and of the sperm duct (Billard et al., 1971). In the lobular lumen and in the sperm duct, spermatozoa are suspended in

seminal fluid which inhibits sperm motility and provides the components needed for sperm metabolism (Loir et al., 1990). Variations in the composition of seminal plasma may impair spermatozoa storage and consequently lead to a reduction in sperm quality. As such, studies focusing on the composition of seminal plasma may play an important role in the improvement of reproductive methods. For example, the composition of the seminal plasma of the European eel was studied by Asturiano et al. (2005). In this case, both the physio-chemical characteristics and the ionic composition (concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+}) were evaluated. Their results determined the best conditions to desing a diluent media (P1; Peñaranda et al., 2009) for the handling and cryopreservation of European eel sperm. Indeed, correlations between the composition of the seminal fluid and motility provided further insight into the physiological mechanisms important for sperm function, such as motility and fertilizing ability.

Fish seminal plasma, in contrast to that of higher vertebrates, is principally comprised of mineral compounds (Na^+, K^+, Mg^{2+} and Ca^{2+}), and characterized by low concentrations of proteins as well as other organic substances, such as hormones and pheromones, cholesterol, glycerol, vitamins, free amino acids, sugars, citric acid and lipids (Linhart et al., 1991; Ciereszko et al., 2000; Cosson et al., 2004). However, protein is the main organic component present in the seminal plasma of teleost fish (Loir et al., 1990). In salmonids, the proteins present in the seminal fluid seem to play a role in gamete protection (Billard et al., 1983a). In turbot, (*Scophthalmus maximus*), sperm motility is depressed at high dilution rates (Suquet et al., 1992a) and is maintained by adding BSA (Bovine Serum Albumin, Fauvel et al., 1993a).

It is known that teleosts exhibit wide inter-species variations in sperm morphology, physiology and seminal plasma composition (Serrao et al., 2009). Although it is known that multiple families of proteins exist in the seminal plasma of teleosts, the exact role of these proteins remains poorly understood.

Scarce proteomic studies have been carried out, and those have focused on the identification of seminal plasma proteins and their physiological functions in a few model fish species. For example, Ciereszko et al. (2008) showed that transferrins and lipoproteins, together with proteinase inhibitors, participate in the protection of spermatozoa during their storage in fish seminal plasma in the spermatic duct.

4. Sperm motility in fish

Motility is perhaps the most easily observed function of sperm. In species which exhibit external fertilization, the spermatozoa are immotile in the testes prior to their release in the surrounding water. In teleosts, the spermatozoa are immotile in the seminal plasma as it is isosmotic with the sperm cells (Figure 3).

The spermatozoa become motile when they are released into a hypotonic, hypertonic or a low K^+ medium, depending on whether they are from a freshwater, marine or salmonid species, respectively (Morisawa and Suzuki, 1980). Therefore, no single model is able to explain sperm motility activation in all the fish species.

Sperm motility activation in fish is a process which involves activating stimuli (changes in osmolality, K^+ concentrations or activation by chemotaxis), followed by intracellular signaling

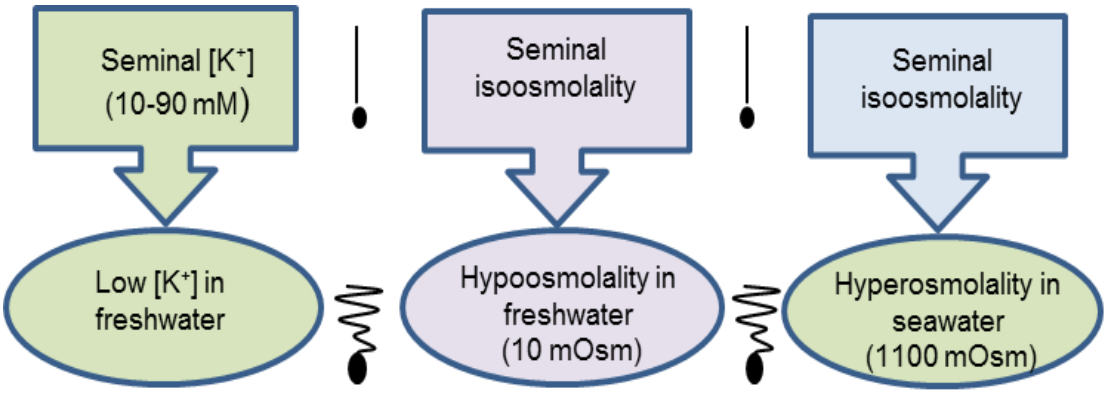

Figure 3. Schematic illustration showing how spermatozoa of salmonids (a), freshwater fish (b) and marine fish (c) initiate motility.

produced by second messengers such as cAMP or Ca^{2+} (Morisawa, 1994; Inaba, 2003; Cosson et al., 2008a; Chang and Suárez, 2010). Finally, sperm motility is caused by the activation of the dynein arm in the flagellum, after phosphorylation and dephosphorylation processes (Inaba et al., 1998; 1999; Nomura et al., 2000; Hozumi et al., 2008).

4.1. Model of the sperm activation process in marine fish

Studies of marine teleosts such as pufferfish (*Takifugu niphobles*; Oda and Morisawa, 1993), flounder (*Kareius bicoloratus*; Morisawa and Suzuki, 1980; Oda and Morisawa, 1993*),* Pacific cod (*Gadus macrocephalus;* Morisawa and Suzuki, 1980*)*, turbot (*Scophthalmus maximus*; Cosson et al., 1999) and Atlantic croaker (*Micropogonias undulatus*; Detweiler and Thomas, 1998) have shown that spermatozoa are quiescent in the seminal plasma (isosmotic medium) and become motile when the semen is diluted in hypertonic solutions. Therefore, the osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water across the spermatozoa membrane, activating cell motility (Figure 4, Morisawa, 2008). However, the exact mechanism through which this happens is poorly undertood.

A model to explain sperm motility in marine fishes was proposed by Morisawa (2008). After hyperosmotic shock, the sperm membrane is depolarized, by the opening of the ion channel, resulting in an increase in the intracellular concentration of K^+ and Ca^{2+}. The increase of $[Ca^{2+}]_i$ could be due to: i) the influx of the ion Ca^{2+} from the extracellular medium, or ii) a release of this ion from intracellular stores. Moreover, Zilli et al. (2008) proposed that the increase in Ca^{2+} and K^+ was due to a reduction in cell volume. This increase in intracellular Ca^{2+} is considered to be part of the intracellular signaling which leads to sperm motility activation.

The current model is based on a few studies of only two marine species, the herring (*Clupea pallasii*) and the pufferfish (*Takifugu niphobles*), but there are approximately 26,800 species of fish, of which 18,000 are marine species.

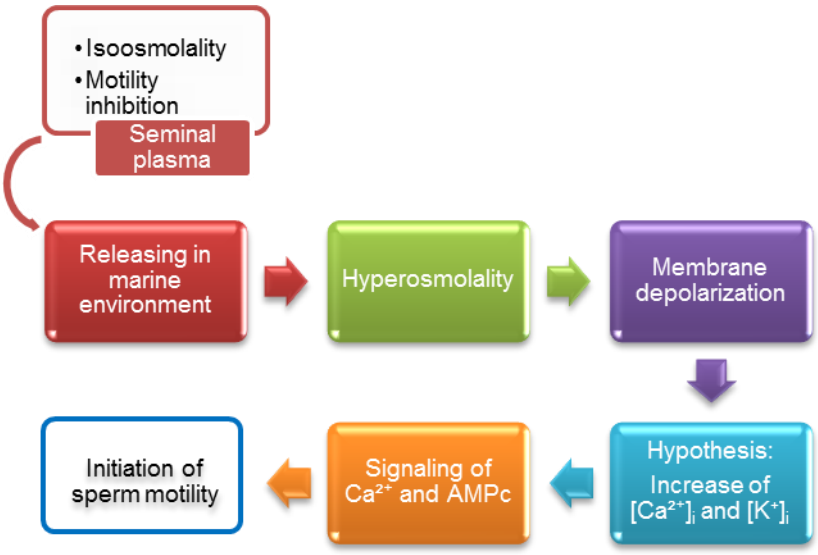

Figure 4. Activation process and signal transduction in marine spermatozoa: scheme of the interacting processes occurring during the motility period.

5. Factors affecting motility

Several factors are involved in the activation of sperm motility in fish: osmolality, pH, and the presence of extracellular ions and, in some species, chemoatractants released by the egg (Figure 5). Therefore, a cascade of motility activation can be distinguished according to two modes: osmotic or ionic.

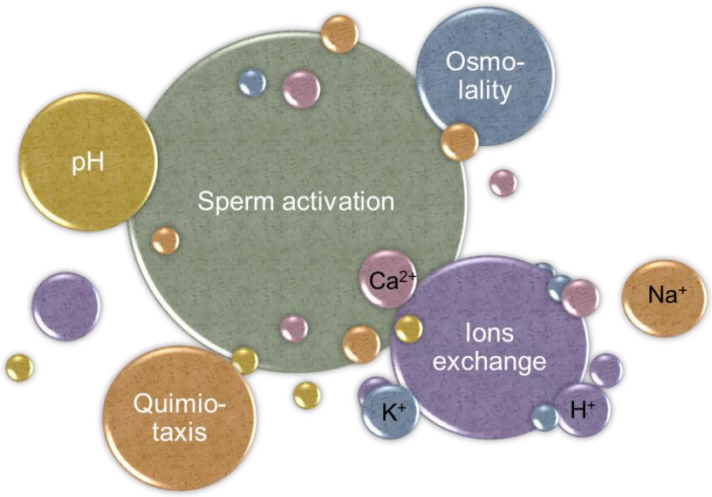

Figure 5. Factors involved in the activation of sperm motility in fish.

5.1. Osmolality

As previously explained/discussed, teleost spermatozoa are inmotile in the seminal plasma (generally around 300 mOsm kg^{-1}) or sperm duct (Morisawa and Suzuki, 1980; Groison et al., 2010) because the seminal plasma is isosmotic with the spermatozoa, thus inhibiting the motility activation of the sperm cells (Alavi and Cosson, 2006; reviewed by Morisawa, 2008).

Their release into the external environment activates spermatozoa movement, due to the hiposmotic shock in cyprinids (hypotonic water <300 mOsm kg^{-1}) or the hyperosmotic shock (hypertonic water >300 mOsm kg^{-1}) in marine fish. The osmotic shock leads to a rapid flux of ions and water across the spermatozoa membrane, which in turn activates cell motility. So, the direction and intensity of this movement of water is also dependent on the internal solute concentration in the cytoplasm.

Therefore, the different components of the surrounding fluids also control sperm motility in fishes. It is already known that for some fish species, a specific environmental ionic composition is needed for sperm motility activation (Alavi and Cosson, 2006). For these species, osmotic pressure might not be so crucial.

5.2. pH

The intracellular and extracellular pH is one of the limiting factors needed for achieving motility, thus the ability to fertilize. The reason being that HCO^{3-} and pH are involved in sperm maturation in the sperm duct. In salmonids and Japanese eel (*Anguilla japonica*), sperm extracted directly from the testis (without passing through the sperm duct) was immotile, unless the sperm had been incubated in an alkaline solution (Morisawa and Morisawa, 1988; Ohta et al., 1997a).

In 1993, Oda and Morisawa proposed that pH_i (intracellular pH) changes were the main trigger for sperm motility initiation in isosmotic media, because an increase in the pH_i by ammonium salt induces motility in the pufferfish (*Takifugu* spp, Oda and Morisawa, 1993).

In addition, the pH of the diluent media and activation solutions influence sperm motility (Cosson et al., 1999; Woolsey et al., 2006). In the case of the European eel, maintaining the sperm in an isosmotic medium with a low pH (pH 6.5) resulted in an inhibition of sperm motility (Peñaranda et al., 2010a).

5.3. Ions fluxes

5.3.1. Mechanism for studying the intracellular ions transported in sperm cells.

The development of methodologies based on the use of fluorescence molecules to selectively detect changes in the intracellular concentrations of Ca^{2+}, K^+, Na^+, etc., has made it possible to demonstrate the existence and the activity of the ions fluxes during sperm activation in fish species (Darszon et al., 2006; Krasznai et al., 2003a,b). Marian et al. (1997) was the first to use flow cytometry to quantify intracellular ions ($[H^+]_i$ and $[Na^+]_i$) in carp sperm, albeit indirectly, by measuring the fluorescence emitted by a pH-indicator dye. In 2005, the activity of individual ion channels in the sperm of sea urchin (*Strongylocentrotus purpuratus*) was identified for the first time by Darszon et al. using electrophysiological techniques called "patch-clamp". These techniques involve measuring tiny ion fluxes, such as fluxes through a single channel, or fluxes passing through a set of cellular channels.

Also, in several fish species, especially in freshwater species, the use of specific ion channel inhibitors has demonstrated the existence of ion fluxes during sperm activation (Table1; Cosson et al., 1986; Morisawa et al., 1990; Krasznai et al., 1995a,b).

Table 1. Ion channel inhibitors/modulators tested in marine fish species.

Study	Species	Inhibitor	Effect
Tanaka et al., 2004	Japanese eel	4AP	Inhibition
Detweiler and Thomas, 1998	Atlantic croaker	4AP	Inhibition
Detweiler and Thomas, 1998	Atlantic croaker	amiloride	Inhibition
Zilli et al., 2008	Gilthead seabream	$BaCl_2$	No inhibition
Vines et al., 2002	Herring	bepridil	Inhibition
Krasznai et al., 2003a	Puffer fish	W-7	Inhibition

5.3.2. Ion changes in sperm cells

In all cells, ion transport occurs via specific ion-permeable protein pores (ion channels) located in the lipid membrane. Ion channels are key elements in cell signaling (Hille, 1992), and sperm motility, maturation, and acrosome reaction are inhibited by certain ion channel blockers (Florman et al., 1998; Publicover and Barrat, 1999). Moreover, ion channels are incredible catalyzers of ion transport through the non-conducting lipid bilayer. Because each bilayer allows the flow of millions of ions per second, a few can induce substantial electric and concentration changes in a small cell, such as the spermatozoa, in milliseconds (Hille, 1992).

Therefore, ion transport can categorized as follows:

- Mono-transport. Transport of one ion or cation via a highly selective channel or pump. an example is the Cation channel in sperm (CatSper); this sperm-specific, slightly voltage-dependent, Ca^{2+} selective, and pH-sensitive ion channel controls the entry of positively charged calcium ions into the sperm cells, essential for sperm hyperactivation and male fertility (Kirichok et al., 2006; Lishko et al., 2011).

- Cotransport. Transport of at least two different ions or substances in one direction. an example is the Na^+/HCO_3^- cotransporter present in mouse sperm which is linked to the regulation of the capacitation process (Demarco et al., 2003).

- Ionic exchange. Transport of either at least two different ions or one ion in oposite directions. In the case of herring spermatozoa it has been suggested that, a sodium-calcium (Na^+/Ca^{2+}) exchange is present which could play a role in ligand-induced sperm motility initiation (Vines et al., 2002).

It has been proposed that Ca^{2+} and K^+ are the main ions involved in sperm motility activation in marine fish (see reviews of Cosson, 2008; Morisawa, 2008), but Na^+ and Cl^- have also been linked to motility initiation (Detweiler and Thomas, 1998; Thomas et al., 1998).

- <u>Potassium channels</u>

In freshwater salmonids, the motility of intact sperm cells is suppressed by high concentrations of external K^+, and the efflux of K^+ caused by the reduction of external K^+ concentrations hyperpolarizes the plasma membrane, leading a to cAMP-dependent initiation of spermatozoa motility (Morisawa and Suzuki, 1980). However, in cyprinids such as carp, sperm cell motility can be activated in hypotonic media with different Na^+, K^+ or Ca^{2+}

concentrations (Morisawa and Suzuki, 1980). In both cyprinids and salmonids an efflux of K^+ and thus a decrease in the $[K^+]_i$ levels ,occurs during sperm activation (Krasznai et al., 1995b, 2003).

An increase in $[K^+]_i$ after sperm activation has only been demonstrated in two marine species (pufferfish and European eel; Takai and Morisawa, 1995; Gallego et al., 2013b) in contrast to the findings observed in freshwater species.

It is strange that there aren´t more studies on the role of the ion K^+ because it seems that this ion could play an important role in the sperm motility of marine fish. Specifically, an increase in $[K^+]_i$ seems necessary for the initiation of sperm motility in pufferfish (Takai and Morisawa, 1995). Moreover, it has been demonstrated that the use of a K^+ channel inhibitor (4-aminopyridine) in Japanese eel significantly reduces sperm motility (Tanaka et al., 2004).

- Calcium increase

Ion Ca^{2+} signaling is considered essential for sperm motility, not only in fish, but in all organisms (Darszon et al., 2011). Many studies agree that the trigger for the initiation of sperm motility in some fish species is the increase in $[Ca^{2+}]_i$ (Cosson et al., 2008; Morisawa, 2008; Zilli et al., 2012). For example, in freshwater species like carp (*Cyprinus carpio*) and rainbow trout (*Onchorynchus mykiss*) sperm it has been demonstrated that for an increase in $[Ca^{2+}]_i$ an influx from the external medium is required, as sperm cells were immotile in a Ca-free activator (Cosson et al., 1989; Krasznai et al., 2000).

But, while there are abundant studies about this ion in sperm from freshwater fish species such as rainbow trout (Cosson et al., 1989; Boitano and Omoto, 1992; Tanimoto et al.,1994; Takei et al., 2012), carp (Krasznai et al., 2000, 2003b) and tilapia (*Oreochromis mossambicus*; Morita et al., 2003), the studies of marine fish species are scarcer, restricted to pufferfish (Oda and Morisawa, 1993; Gallego et al., 2013b) and pacific herring (Cherr et al., 2008).

In some species such as carp, the presence of Ca^{2+} in the hypotonic solution is necessary for the activation of sperm cell motility (Krasznai et al., 2000). It was observed that the use of Ca^{2+} channel blokers in carp reversibly inhibits sperm motility (Krasznai et al., 2000). The presence of this ion is essential in carp, because even in the absence of the sperm membrane (eliminated by detergents), a minimum content of Ca^{2+} in the solution has been seen to induce sperm motility. Therefore, Krasznai et al. (2000) proposed that the influx of the ion Ca^{2+} in carp spermatozoa is an essential requirement for sperm activation in this species.

The importance of Ca^{2+} for the sperm motility of marine fish has also been demonstrated. In pufferfish, Oda and Morisawa (1993) observed that treatment with a Ca^{2+} ionophore induces spermatozoa motility in isosmotic conditions. Moreover, in that study the levels of $[Ca^{2+}]_i$ from the pufferfish spermatozoa increased in hiposmotic medium even in absence of extracellular Ca^{2+}. Therefore, Oda and Morisawa (1993) proposed that the release of Ca^{2+} from intracellular stores could play an important role in the initiation of sperm motility.

- Sodium

Several mammalian studies have suggested that Na^+ is required for the initiation of the acrosome reaction (AR; Hyne et al., 1984; Fraser, 1993; Fraser et al., 1993). In capacitated human sperm the extracellular Na^+ reduction modulates the acrosomic reaction. Such reduction depends on the Na^+/H^+ exchangers (NHEs), which also affect the absorption of salt and water across various epithelia and then the regulation of cell volume (García et al., 1993; Martins et al., 2014).

Also, the importance of NHEs has been demonstrated in sea urchin sperm. NHEs are stimulated after the hyperpolarization of the spermatozoa membrane, and the folloween influx of Na^+ through the NHEs may control the rhythmic beating of the flagella (Beltrán et al., 2007).

In contrast to the abundant bibliography on the role of K^+ or Ca^{2+} in sperm motility, studies about the ion Na^+ in fish are very scarce. In 2003, Krasnai et al. demonstrated that both $[K^+]_i$ and $[Na^+]_i$ decreased after motility activation in carp sperm.

Also, in another marine species, the Atlantic croaker, the use of a Na^+ channel blocker (amiloride) was seen to inhibit sperm motility, suggesting that Na channels are involved in sperm motility (Detweiler and Thomas, 1998). However, in carp the same channel bloker, amiloride, has no inhibitory effect on sperm motility (Krasznai et al., 1995b).

Previous studies have shown that extracellular Na^+ and Ca^{2+} concentrations influence sperm motility and fertilization in salmonids and herring (Yanagimachi et al., 1992). In salmonids which spawn in freshwater, K^+ efflux and Ca^{2+} influx triggers the initiation of sperm movement (Morisawa, 1985; Morisawa et al., 1990). The herring is a curious marine species because the sperm cells of this fish are unique in that they are immotile upon spawning. Herring sperm have evolved to remain immotile for up to several days after spawning, yet they are still capable of fertilizing eggs. These spermatozoa are activated by glycoprotein (a sperm motility initiation factor, SMIF) but, the sperm motility also depends on a reduction in extracellular sodium (<350 mM). So, herring sperm can be activated in the absence of a SMIF if sodium concentrations in the seawater are very low (Vines et al., 2002).

5.4. Membrane potential

In sperm, as in most cells, the internal ion concentrations are markedly different from those in the extracellular medium. These differences result from the relative ion permeability of the plasma membrane to each of the ions found in the inner and outer media given by the specific ion channels and transporters present in the cell, from the gradients they establish and the metabolic state of the cell. In theresting stage, the balance of these fluxes, gradients and permeabilities produces an electrical potential, known as the resting Em (membrane potential; Visconti et al., 2011).

Em governs the rates and direction of ion flow through channels and exchangers, and its fluctuations modulate intracellular pH (pH_i), Ca^{2+} ($[Ca^{2+}]_i$) and other second messengers that are important in influencing sperm flagellar motility and acrosome reaction (Darszon et al., 2001).

In carp, the hypoosmotic induction of motility is accompanied by a reorganization of the

membrane structure (Márián et al., 1993) and the hyperpolarization of the sperm membrane. Also in carp, Krasznai et al. (2003b) demonstrated that the sperm cells were in a depolarized state in the seminal plasma (Em= –2.6 ± 3 mV) and they hyperpolarize after hypoosmotic activation (Em= –29 ± 4 mV).

Indeed, the importance of the membrane potential has been demonstrated in trout sperm, which can be activated by manipulating the membrane potential, even in the presence of the ionophore valinomycin (which can override the entrance of K^+; Boitano and Onamoto, 1991), thus regulating sperm activation irrespective of the [K^+] levels.

5.5. Chemotaxis

Chemotaxis was first described as the attraction of the spermatozoa to the egg (Dan, 1950). Since then, this mechanism has been widely described.

In sea urchin sperm, the egg factor that triggers the acrosome reaction, induces a 10- to 20-fold increase in $[Ca^{2+}]_i$, a 0.2–0.3 change in pH_i, and a Em depolarization (Darszon et al., 1999).

As previously discussed, an egg chorion ligand termed ‘‘sperm motility initiation factor’’ (SMIF) induces motility in herring sperm and is required for fertilization. Vines et al. (2002) showed that the SMIF induced a calcium influx, a sodium efflux, and a membrane depolarization in herring sperm.

5.6. cAMP

In some fish species, cAMP is a second messenger in spermatozoa activation, whereas in others sperm activation has been demonstrated to be independent of cAMP.

In salmonids, the increase of cAMP at sperm activation is related to the hyperpolarization of the sperm membrane, which is due to the opening of the K^+ channel (Morisawa, 2008). However, Krasznai et al. (2000) showed that the membrane-permeable cAMP does not initiate motility of carp sperm that is quiescent in isotonic solution, and that motility of the demembranated sperm cells could be reactivated without cAMP. Furthermore, the cAMP level does not change during the initiation of spermatozoa motility, and inhibitors of the protein kinase do not affect sperm cell motility, suggesting that no cAMP-dependent system is necessary for the regulation of sperm motility in carp.

6. Spermatozoa volume changes in fish

Adaptation to the changes in environmental osmolality is a physiological feature of spermatozoa from evolutionally distant species. In mammals, spermatozoa need to undergo volume regulation because the fluids along the male and female tract possess different osmolalities (Yeung et al., 2006). However, the reaction of sperm cells to osmotic shock in terms of cell volume alteration is species-specific.

Fish spermatozoa undergo osmotic stress when isotonic seminal fluid is diluted in the external water at spawning. In general, freshwater fish spermatozoa swell after hypoosmotic activation, due to the influx of water.

In cyprinids, for example, it is exactly this alteration of cell volume that activates the biochemical cascade that leads to spermatozoa motility (Krasznai et al., 2003b). It has been demonstrated that the volume of carp spermatozoa is dependent on the environmental osmolality (Perchec et al., 1995; Dzuba and Kopeika, 2002; Dzuba et al., 2001). Also, Cabrita et al. (1999) detected swelling in rainbow trout spermatozoa 0.5, 2, 5, 10, 20 and 30 min after incubation in a hypotonic non-activating solution.

Studies on the volume changes of the sperm of marine species are very scarce. In the case of seabass, *Dicentrarchus labrax*, swelling of the sperm head was observed after hyperosmotic activation (Dreanno et al., 1999a). Also, in turbot spermatozoa, an increase in the size of the midpiece was observed after sperm activation.

7. Projects, grants and companies involved in this Thesis

All the studies carried out in this Thesis have been funded by different national and international projects and grants.

My research degree has been supported by a 4-years predoctoral fellowship within the "Formación de Personal Investigador" UPV sub-programme 2 (FPI; 2011-s2-6521) granted by the Project PRO-EEL (*Reproduction of European eel towards a self-sustained aquaculture;* grant agreement n°245257) funded by the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology".

During my PhD studies, I have also been supported by three fellowships granted by the AQUAGAMETE COST Action to carry out studies in different research hosting centres. The first training period, which lasted 1 month in 2013, was carried out in the University of South Bohemia in Ceské Budejovice (Czech Republic) under the supervision of Dr. Martin Psenicka (grant reference: CZ.1.07/2.3.00/20.0024). During the same year, a second period, which lasted 1 week, was completed in the Tolmin Angling Association (Slovenia) under the supervision of Dr. Ákos Horváth (grant reference: COST-STSM-FA1205-14580). Finally, the last training period, which lasted 2 months in 2014, was carried out at the Universidade do Algarve, Campus de Gambelas (Faro, Portugal) under the supervision of Dra. Elsa Cabrita (grant reference: COST-STSM-FA1205-20666).

Moreover, National projects have helped in global terms with the costs to carry out the research for this thesis (animal facilities, equipment, consumables, management costs, staff employed in the lab, overheads, etc.). Regarding this, Chapters 1, 2 and 3 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 MICINN (AGL2010-16009), and Chapter 4 has been funded by the Project REPRO-TEMP (*La anguila europea como modelo para estudiar la temperatura como modulador de la maduración sexual en teleósteos. Potencial aplicación en acuicultura*) funded by MINECO (AGL2013-41646-R).

The fish farm Valenciana de Acuicultura, S.A. (Puzol, Spain) supplied all the male eels used in the experiments, and finally the Instituto de Biomedicina de Valencia (CSIC) contributed to the experimental design and to the protein analyses described in Chapter 4.

OBJECTIVES

The chapters presented in this thesis have a primary objective in common the contribution to the knowledge of the mechanism involved in the sperm motility activation of the European eel, through the study of the spermatozoa reaction under ionic environment changes. The specific objectives were:

- To test the importance of the presence of Ca^{2+}, Na^{+}, K^{+}, ions from the seminal plasma and the activation media on the sperm motility activation,

- To study the sperm volume changes before and after sperm motility activation, and the role of Na^{+}, K^{+} on this process.

- To understand the ion fluxes involved on sperm motility activation by measuring sperm intracellular concentrations of Ca^{2+}, Na^{+}, K^{+}, pH before and after motility activation, and by the use of specific inhibitors/ionophores,

- To develop a method for quantitative analysis of intracellular [Na^{+}] and pH in the spermatozoa of a marine fish and to measure the specific concentration of this ions before and after sperm activation.

- To check if some seminal plasma protein/s are related to the improvement of sperm motility usually observed during the hormonal treatment.

CHAPTER 1

Role of potassium and pH on the initiation of sperm motility in the European eel

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Abstract

The role of potassium from the seminal plasma and/or the activation media was examined by selectively removing K^+ from this media, and by testing the use of K^+ channel inhibitors and a K-ionophore. Sperm motility was measured using a CASA system, intracellular K^+ and pH were measured by flow cytometry, and sperm head area was measured by ASMA: Automated Sperm Morphometry Analyses. Sperm motility was notably inhibited by the removal of K^+ from the seminal plasma and by treatment with the K^+ ionophore valinomycin. This therefore indicates that a reduction of K^+ levels in the quiescent stage inhibits further motility. The normal decrease in sperm head area induced by seawater activation was altered by the removal of K^+ from the seminal plasma, and an increase in the pH_i in the quiescent stage was also induced. Intracellular pH (pH_i) was quantitatively measured for the first time in European eel spermatozoa, being 7.2 in the quiescent stage and 7.1 post-activation. Intracellular and external pH levels influenced sperm motility both in the quiescent stage and at activation. The alkalinization of the pH_i (by NH_4Cl) inhibited sperm motility activation, while acidification (by Na-acetate) did not have any effect. Our results indicate that a pH gradient between the sperm cell and the seminal plasma is necessary for sperm motility activation. The presence of the ion K^+ in the seminal plasma (or in the extender medium) is necessary in order to maintain sperm volume, intracellular pH and sperm motility.

1. Introduction

Motility activation in fish spermatozoa is controlled by specific ion concentrations, osmolality, and environmental pH (Alavi and Cosson, 2005, 2006; Morisawa, 2008). In cyprinids, motility is initiated in both electrolytic and non-electrolytic hypotonic solutions, suggesting that motility is suppressed by the seminal plasma osmolality, and exposure to the hypotonic environment of freshwater at spawning sites induces sperm motility (Morisawa and Suzuki, 1980; Morisawa et al., 1983). However, in salmonids, the regulatory role of osmolality on spermatozoa activation seems to be minor, with activation occuring in a wide range of environmental osmolalities (Morisawa and Suzuki, 1980; Alavi and Cosson, 2006). In these species, both the concentrations of certain ions as well as the pH level are critical for sperm activation under any osmotic condition (Morisawa and Suzuki, 1980; Boitano and Omoto, 1991). Spermatozoa motility in salmonids is suppressed by extracellular K^+ in the seminal plasma, and the decrease in K^+ concentrations in the freshwater at spawning initiates sperm motility (Morisawa and Suzuki, 1980; Baynes et al., 1981). Also, Krasznai et al. (2000) demonstrated that in the common carp (*Cyprinus carpio*), a voltage-gated potassium channel blocker (4-aminopyridine, 4-AP), eliminated the hyperpolarization of the sperm cells after hypoosmotic shock, and inhibited sperm motility. This finding suggests that an increase in potassium permeability (and an efflux of K^+_i) is responsible for the hyperpolarization observed in freshwater fish spermatozoa at sperm activation.

In nature, the sperm motility of marine teleosts is initiated when sperm is released into seawater, and it has been demonstrated that osmolarity (in this case, hyperosmolarity) is the main factor triggering sperm motility (Takai and Morisawa, 1995) as both ionic and non-ionic hyperosmotic solutions can activate motility in all the species tested: gilthead seabream (*Sparus* aurata; Zilli et al., 2008; Morisawa, 2008), pufferfish (*Takifugu niphobles*; Morisawa and Suzuki, 1980; Gallego et al., 2013b), halibut (*Hippoglossus hippoglossus*; Billard et al., 1993), European sea bass (*Dicentrarchus labrax*; Dreanno et al., 1999) and cod (*Gadus*

morhua; Cosson et al., 2008).

However, some ions from the seminal plasma and/or the activation media seem important for sperm motility in marine fish. In the puffer fish $[K^+]_i$ increased at sperm activation in hypertonic conditions, (Takai and Morisawa, 1995), and in demembranated sperm, motility was induced by increasing $[K^+]$ to levels higher than those of the seminal plasma $[K^+]$, and ended by decreasing levels back to those of the seminal plasma. In the same study it was shown that pH also regulated sperm motility in puffer fish; in demembranated sperm motility was initiated at a high pH, and terminated at a low pH (Takai and Morisawa, 1995). In the Atlantic croaker (*Micropogonias undulatus*) and the Japanese eel (*Anguilla japonica*), the K^+ channel inhibitor 4-AP inhibited sperm motility (Detweiler and Thomas, 1997; Tanaka et al. 2004). In the Japanese eel the inhibitory effect of 4-AP disappeared when the pH_i was decreased using Na-acetate, when CO_2 or $NaHCO_3^-$ was added, or when the pH from the activation media was lower than 8.2 (Tanaka et al., 2004). It was suggested that in the Japanese eel, an uptake of H^+ triggers the initiation of sperm motility with the participation of a K^+ transport through a K^+ channel sensitive to 4-AP. In the same species, it was observed that the elimination of K^+ (or HCO_3^-) from the artificial seminal plasma induced a rapid decrease in motility which was reversible (Ohta et al., 2001).

There is no consensus regarding how pH changes relate to sperm motility. Oda and Morisawa (1993) indicated that sperm motility was initiated by an increase in the pH_i even in isosmotic conditions, as observed when NH_4Cl was applied to the sperm. However, Tanaka et al. (2004) suggested the opposite, that a H^+ uptake (then a pH decrease) triggers sperm motility in Japanese eel, and Gallego et al. (2014) observed a decrease in post-activation pH levels in the European eel (*Anguilla anguilla*).

It has been suggested that the increases in $[K^+]_i$ and/or other ions during activation in seawater are caused by a decrease in the spermatozoa cell volume (Zilli et al. 2008, Cosson et al. 2008), but until very recently this reduction in size has not been demonstrated in a marine species (Vílchez et al., 2016; Chapter 1, in European eel). In contrast, in some freshwater fish like common carp, brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*), an increase in cell volume is observed after sperm activation in hypoosmotic media (Bondarenko et al., 2013; Takei et al., 2015). However, information about spermatozoa volume changes relating to extracellular ion concentrations in marine fish species is scarce. In a previous study on European eel, Vílchez et al. (2016) observed that the sperm head area reduced in size during normal motility activation in hyperosmotic seawater, and that a reduction was also observed in isosmotic conditions when the ion Na^+ was removed from the seminal plasma, an occurrence which also causes a marked reduction in sperm motility. Thus, it was concluded that the presence of the ion Na^+ in the seminal plasma (or in an extender medium) was necessary for the preservation of sperm motility in European eel, and that it is also involved in maintaining the sperm volume during the quiescent stage.

One approach for studying the ion exchanges related to sperm motility activation is to measure the intracellular ion levels before and after sperm activation. Gallego et al. (2014) demonstrated that the intracellular Ca^{2+} and K^+ sperm ion levels of European eel increased after hyperosmotic shock, while the intracellular pH gradually decreased post-activation. The

intracellular ion measurements performed in that study were relative measurements, without an absolute quantification. However, recently, and for the first time in a marine species, we analyzed (Vílchez et al., 2016; Chapter 2), intracellular sodium $[Na^+]_i$ quantitatively in European eel spermatozoa, and the $[Na^+]_i$ measurements were taken before and after motility activation, by Flow Cytometry. The same methodology has been used in the present study to quantify the pH_i before and after sperm motility activation for first time in the European eel.

Moreover, this study focuses on the importance of the K^+ and pH levels present in the seminal plasma and the intracellular changes in these ions during sperm activation. For this purpose, the removal of K^+ from the seminal plasma and/or from the activation media, as well as the effects of K^+ ionophore and K^+ channel inhibitors both on sperm motility and on the intracellular K^+ and pH have been evaluated. In addition, the effects of alkalinization and acidification of the pH_i on sperm motility were tested.

2. Material and methods

2.1. Chemicals and solutions

Bovine Serum Albumin (BSA), the ionophore valinomycin, and the potassium channel inhibitors 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), barium chloride (BaCl2), and amiloride hydrochloride hydrate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorochroms PBFI-AM (potassium sensitive dye) and SNARF-5F AM (pH indicator dye), as well as the reagents Pluronic® F-127 and TO-PRO-3 were purchased from Molecular Probes (Life Technologies, Madrid, Spain). Salts were of reagent grade. Stock solutions 100 mM 4-AP, 200 mM TEA, 1 M amiloride, and 1 mM of valinomycin were prepared in DMSO, aliquoted and kept at -20 °C until use. For treatments, an aliquot of the stock solution was thawed only once and mixed with the sperm to reach a final concentration of 1 mM 4-AP or $BaCl_2$, 10 mM TEA, 2 mM amiloride and 10 µM valinomycin. The final DMSO concentration in sperm was less than 0.05% in all the cases, and therefore a DMSO effect on motility could be discarded. Controls were treated with the same DMSO concentration as the experimental treatments.

Stock solutions 2 mM SNARF-AM, 1 mM CoroNa Green AM and PBFI were prepared in Pluronic 20% in DMSO (Pluronic® F-127, Molecular Probes™) and used as described in Section 2.7.

2.2. Fish maintenance and hormonal treatment

A total of 50 male eels (mean body weight 124±5 g) were transported to our facilities at the Universitat Politècnica de València (Spain) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain). The fish were distributed in two 90-L aquaria (approximately 20 male eels per aquarium) equipped with separate recirculation systems, thermostats, and coolers, and covered with black panels. The animals were gradually acclimatized to seawater (salinity 37±0.3 g/L) over the course of 1 week, and were then maintained in seawater at 20 ºC until the end of the experiment, as in previous experiments (Peñaranda et al., 2010a; Gallego et al., 2012a)

Once the fish were in seawater the hormonal treatment with hCGrec (recombinant hCG; Ovitrelle, Merck Serono, Madrid) was initiated. Once a week, the animals were anaesthetized with benzocaine (60 ppm) and weighed before receiving a intraperitoneal injection of hCGrec (diluted in NaCl 0.9%) at a dose of 1.5 IU/g fish.

The fish were fasted throughout the experiment, and handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.3. Sperm collection and sampling

Sperm samples were collected once a week, from the 6th week of hormonal treatment until the end of the experiment (with a total of 7 samplings over the course of the experiment). The samples were collected 24 h after the administration of the hormone to obtain maximum sperm quality (Pérez et al., 2000). The sperm was collected in Falcon tubes by applying gentle abdominal pressure, after fish anesthetization. The genital area was previously cleaned with distilled water, and dried, in order to avoid sample contamination by feces, urine and seawater. The sperm samples were kept refrigerated (4 °C) until the motility analyses, which took place within the first hour after collection.

2.4. Sperm motility evaluation

The standard sperm diluent used in this work was P1 (Peñaranda et al., 2009; Table 1), which is a non-activating media isosmotic and isoionic with the European eel seminal plasma (Asturiano et al., 2004). The sperm motility activation was carried out as per the method described by Gallego et al. (2013a), by mixing 1 µl of diluted sperm (dilution 1/25 in P1 extender, Table 1) with 4 µl of artificial seawater (ASW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was prepared in a SpermTrack-10® chamber, 10 µm depth (Proiser R+D, S.L.; Paterna, Spain) and observed in a microscope Nikon Eclipse 80i, with a 10x lens (Nikon phase contrast 10x 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after mixing the sperm with ASW, using a high-sensitivity video camera HAS-220 and the ISAS software (Proiser R+D, S.L.; Paterna, Spain). The frame rate used was 60 fps. For each motility test, samples were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 ºC in a water bath until the sperm motility evaluation. The best samples (>50% total motility) were selected for the studies.

Table 1: Composition (mM), osmolality (mOsm) and pH of the media used. Extender media; control (with potassium) and K-free (without potassium). Activation media: ASW (control, with potassium) and K-free activator (without potassium).

	NaCl	**$MgCl_2$**	**$CaCl_2$**	**KCl**	**$NaHCO_3$**	**Na_2SO_4**	**TAPS**	**Osm**	**pH**
Extender:									
P1 (control)	125	2.5	1	30	20	-	-	325	8.5
K-free	155	2.5	1		20	-	20	325	8.5
Activator:									
ASW	354.7	52.4	9.9	9.4	20	28.2		1100	8.2
K-free	550	-	-	-	-	-		1100	8.2

The sperm motility parameters considered in this study were: total motility (MOT, %); progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast spermatozoa (FA; average path velocity, VAP>100 µm/s); 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; ALH, amplitude of the lateral movement of the sperm head and cross beating frequency (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was <10 µm/s (Martínez-Pastor et al., 2008).

2.5. Composition of extenders and activation media

Table 1 shows the composition of the extenders and activation media. To examine the effects of the ion potassium on the initiation of sperm motility in the European eel, two kinds of diluents and activators media were prepared, with or without potassium. Extender P1 was used as an artificial seminal plasma in this study; its composition mimics the seminal plasma of European eel (Asturiano et al., 2004; Peñaranda et al., 2010a). The K-free extender was prepared by replacing KCl present in P1 with NaCl in the same molarity, and 20 mM TAPS was added as a buffer. Another extender with a high potassium concentration was used as an artificial seminal plasma (extender 150 mM K^+). In all the isosmotic media the osmolality was 325 mOsm/kg and the pH was adjusted to 8.5. The pH of the extenders and activators was adjusted the same day of use.

The hyperosmotic activators were: artificial seawater (ASW, prepared as indicated in section 2.4) and a K-free activator (550 mM of sodium chloride). In both hyperosmotic activation media the osmolality was 1100 mOsm/kg, the pH was adjusted to 8.2 and 2% BSA (w/v) was added. The K-free extender and K-free activation media were prepared with ultra-pure water and with autoclaved material.

2.6. Removal of extracellular potassium from the seminal plasma

After the initial evaluation of the sperm motility, individual samples showing >50% of total motility were selected, and the next step was removing the potassium ion from the seminal plasma. Each sample was first diluted 1:25 in: a) control extender (P1, with potassium) or b) K-free extender (without potassium) and then washed three times as described by Pérez et al. (2016), at 500 g, for 4 min at 4 ºC. It was already proved that this treatment did not reduce the sperm motility (Pérez et al., 2016). Between centrifugations, the supernatant was carefully removed, and the sperm pellet was resuspended in P1 or the K-free extender (at 1:25 v:v) by gentle shacking. This process was repeated three times and then samples were finally resuspended in P1 or the K-free extender, and maintained at 4 ºC until analysis.

2.7. Relative intracellular K^+ and pH measurements

The relative amount of $[K^+]_i$ was determined by flow cytometry using a CyAn ADP Flow Cytometer (Beckman Coulter, Brea,CA) equipped with three lasers, including a violet laser (405 nm). The potassium-sensitive indicator PBFI cell-permeant (AM) was used as the

selective potassium indicator as it exhibits an increase in fluorescence emission intensity upon the binding of K^+. A stock solution of 1 mM PBFI AM (potassium-sensitive dye) in DMSO was kept at −20 °C until use. Samples of 100 µl diluted sperm (1/25 in P1, v/v) were loaded with PBFI AM to a final concentration of 5 µM, by incubating in darkness for 90 min at 4 °C.

The relative pH_i was determined by flow cytometry using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser and a red laser. SNARF-5F AM was used as the pH fluorescence indicator. A stock solution of 1mM SNARF-5F AM in DMSO was kept at −20 °C until use. Each sample of 100 µl of diluted sperm (1/25 in P1, v/v) was incubated with 0.5 µl SNARF-5F AM (final concentration 5 µM) at 4 °C for 45 min, in darkness.

Both Flow Cytometers were equipped with an argon ion laser and a red laser. In both cases slightly angled scattered front light was used for the electronic gating of data collection, allowing the exclusion of dead cells from the analyses. To exclude them, the spermatozoa were also incubated with TO-PRO-3 to reach a final concentration of 5 µM. The final DMSO concentration in sperm was less than 0.05% in all the cases, and therefore a DMSO effect on motility could be discarded. Sperm motility was not reduced by incubation with PBFI AM plus TO-PRO-3, or with SNARF-5F AM plus TO-PRO-3.

After incubation with PBFI AM or SNARF-5F AM, 5 µl of each diluted sperm sample was added to a tube containing 500 µl of the isoosmotic extender medium (see section 2.9, experiments 3 and 4), to measure the fluorescence emitted by K^+ or the pH in the quiescent stage, before sperm activation. Later, 5 µl of each diluted sperm sample was added to another tube containing 500 µl of hyperosmotic activation medium (see section 2.9, experiments 3 and 4) and the fluorescence emitted by sensitive indicators in sperm cells was recorded at 30 s after the motility activation. This is the time that lapses between creating the mixture of sperm-activator and obtaining the final fluorescence measurement. The final sperm dilution used for measurements in the flow cytometer was 1/2500 (v:v), with approximately 400 cells/µl.

The pH_i was determined by using a ratio method (Balkay et al., 1992). For this purpose, SNARF-5F AM was excited at one wavelength by the blue laser (488 nm) and the fluorescence emission was read at two wavelengths by the FL2 (575/25BP filter) and FL4 (680/30BP filter) photodetectors. The pH-dependent spectral shifts exhibited by carboxy SNARF-5 allow calibration of the pH response in terms of the ratio (FL2/FL4) of fluorescence intensities measured at two different wavelengths.

PBFI AM, the K^+ indicator, was excited by the ultraviolet laser (340 nm) and its fluorescence was read by the FL6 photodetector (450/50BP filter). TO-PRO-3 was excited by the red laser (635 nm), and its red fluorescence was read by the FL8 photodetector (665/20BP filter).

For both SNARF-5F AM and PBFI AM fluorescence data were displayed in logarithmic mode. Five thousand events were collected per sample, with a flow rate of 500 cells/s. A gate in forward and side scatter was used to exclude debris and aggregates from the analysis. Flow cytometry data were processed using WEASEL software (v 3.1, Walter 288 and Eliza Hall Institute).

2.8. Quantification of intracellular pH: calibration curve.

A pool of sperm made from 6 individual sperm samples showing >50% of sperm motility (see section 2.4.), was diluted and washed in P1 as described in section 2.6. Then the sperm pellet was resuspended in the calibration solutions, which were isosmotic high K^+ extenders (100 mM K^+) with known pH concentrations (from pH 6.6 to 8.5). The samples were incubated with the pH indicator SNARF-5F AM in the presence of nigericin (4 µM), which permeabilizes the sperm membrane to the ions H^+, and thus, equals pH_i=$pH_{e,}$ (see section 2.7). This method was based on Balkay et al. (1997) and the technical specifications of SNARF-5 (Molecular Probes). The Fluorescence intensity of the cell suspensions was measured by flow cytometry (see section 2.7), and the calibration equation was obtained as described in the technical specifications of SNARF-5F AM, with the following equation:

$$pH = pK_A - \log\left[\frac{R - R_B}{R_A - R} X \frac{F_B(\lambda_2)}{F_A(\lambda_2)}\right]$$

Where R is the ratio $F(\lambda_1)/F(\lambda_2)$ of fluorescence intensities (*F*) measured at two wavelengths λ_1 and λ_2 and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration respectively.

2.9. Relationship between $[K^+]_i$ *and* pH_i *changes and sperm motility in different conditions*

Experiment 1: Effect of the removal of extracellular potassium on sperm motility.

Twelve sperm samples (one sample/male) were selected and washed with/without K^+ (P1/K-free extender; see section 2.6). Then, the sperm motility of each sample was measured in triplicate, after activation in ASW and a K-free activation medium.

In order to study the recovery of the sperm motility in samples previously washed in K-free extender, the sperm motility of eight samples washed in P1 or the K-free extender, and activated with ASW, were measured in triplicate. Later, the samples that had been washed with the K-free extender were incubated at 4 ºC in the control extender (P1, containing 30 mM K^+), or in a medium with high $[K^+]$ levels (150 mM K^+). The motility was checked in triplicate after 15, 30 and 60 min of incubation.

Experiment 2: Changes in the sperm head area before and after activation

A total of 7 sperm samples (one sample/male) with >50% of total motility were selected to study the changes in sperm head area in relation to activation. The spermatozoa were fixed with glutaraldehyde at 2.5% (v:v, Gallego et al., 2012b) before and after washing the sperm in P1 and the K-free extender (quiescent stage) and after activation in ASW.

The fixed sperm samples were examined using a phase contrast microscope with a 100x contrast phase lens. Microphotographs of the spermatozoa were taken using an ISAS 782M camera (Proiser R+D, S.L.; Paterna, Spain), and the morphometric analyses of sperm samples were performed using the morphometry module of the ISAS software. The spermatozoa head area (*A*) was calculated automatically by capturing 110 digitized spermatozoa from each sample.

Experiment 3: Effect of ionophore and inhibitors on the sperm motility and $[K^+]_i$

The effect of several K^+ channel inhibitors and the ionophore valinomycin on sperm motility was assessed in two sessions, in 7-10 samples. The effect of valinomycin and 4-AP inhibitor was tested in 10 sperm samples, and TEA and $BaCl_2$ effect was evaluated in 7 samples.

Each sperm sample was washed with P1 (see section 2.6) and divided in aliquots: one of which was used as the control, and the others incubated with K^+ channel inhibitors (4-AP 1 mM, TEA 10 mM, $BaCl_2$ 1 mM) or K^+ ionophore valinomycin (10 µM) at 4 ºC, for 30 min. The sperm motility of each sub-sample was measured in triplicate after activation with ASW or the K-free activation medium.

The fluorescence emitted by $[K^+]_i$ sensitive indicator was measured in 9 sperm samples. Each sample was subdivided into 5 aliquots and washed in the P1 extender (see section 2.6.). After washing, each sub-sample was incubated for 60 min with PBFI AM, and for 30 min with 1 mM 4-AP, 10 mM TEA, 1 mM $BaCl_2$ or 10 µM valinomycin. The incubations were performed at 4 ºC in the dark. The fluorescence emitted by $[K^+]_i$ sensitive indicator was measured in the quiescent stage and after motility activation in ASW or the K-free activation medium.

Experiment 4: Effect of valinomycin and 4-AP on the pH_i

Sperm samples (n= 5-10, according to the session) were used in order to examine the effect of the K^+ ionophore (valinomycin) and the K^+ channel inhibitor (4-AP) on the pH_i changes during sperm activation. The fluorescence emitted by the pH_i sensitive indicator was measured in samples washed in P1 (see section 2.6). After washing, each sample was divided into three aliquots: one of which was used as the control, and the other two were treated with 1 mM 4-AP or 10 µM valinomycin for 30 min at 4 ºC. All the aliquots were also incubated with SNARF-5 AM (see section 2.7). The fluorescence emitted by the pH_i sensitive indicator was measured in the quiescent stage and after sperm motility activation with ASW or the K-free activation medium at 60 s and 120 s post-activation. The same pH_i measurements were repeated in the 5 sperm samples previously washed in K-free extender instead of P1 extender.

Experiment 5: Effect of the pH_e and pH_i on sperm motility

To examine the effect of pH_i on sperm motility, sperm samples (n=8) were diluted in P1 and activated (mixed) with solutions with different concentrations (25-100 mM) of sodium acetate ($NaC_2H_3O_2$, acidifying the pH_i) and ammonium chloride (NH_4Cl, alkalinizing pH_i) in hyperosmotic (ASW) media (Oda and Morisawa, 1993; Tanaka et al., 2004). The sperm motility of each sample was measured in triplicate.

2.10. *Statistical analysis*

Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by flow cytometry. After the removal of dead spermatozoa (TO-PRO-3) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) of each sample was obtained. All the statistical procedures were run using Statgraphics Plus 5.1. 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.

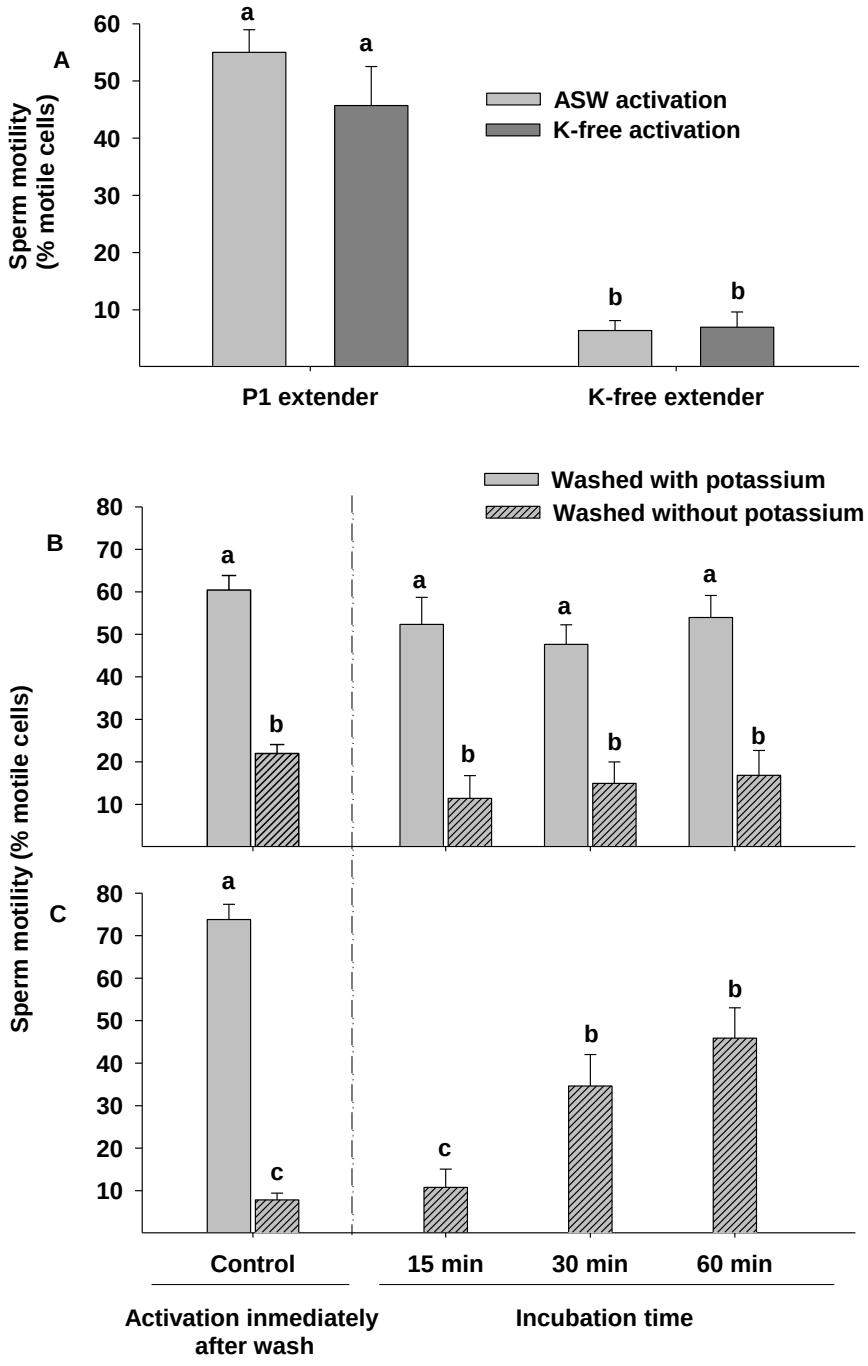

Figure 1. A) Percentage of motile spermatozoa after washing in control extender (P1) or K-free extender and activated with or without potassium (n=12). B) Percentage of motile spermatozoa after 15, 30 and 60 minutes of re-incubation in control extender, in samples previously washed in control extender (P1) or K-free extender. Samples were activated with ASW (n=10). C) Percentage of motile spermatozoa after 15, 30 and 60 minutes of re-incubation in 150 mM K^+ extender, in samples previously washed in control extender (P1) or K-free extender. Samples were activated with ASW (n=8). Data are expressed as mean ± SEM. Different letters indicate significant differences (P<0.01) between treatments.

3. Results

3.1. Effect of potassium removal from the extender and the activation media

Figure 1A shows that the elimination of K^+ from the extender by washing resulted in a reduction of 94% of the sperm motility in relation to the control (washed in P1). There was also a notable reduction in the rest of the kinetic parameters (data not shown). On the other hand, the activation media (ASW or K-free activator) did not result in any differences in motility.

A different batch of samples was used to study if the loss of motility due to the removal of external K^+ could be reversed by incubation in a control extender (P1, 30 mM K^+, Figure 1B), or in a medium with high [K^+] (150 mM K^+, Figure 1C), during 15, 30 or 60 minutes. Like in the previous experiment, the elimination of extracellular K^+ by washing resulted in a strong reduction in sperm motility, to only 11% of the controls. Re-incubation in the P1 extender (Figure 1B) did not recover motility, even after 60 min of incubation. However, re-incubation in a medium with high [K^+], produced a significant recovery in sperm motility after 30 min, reaching 46% of total motile cells after 60 min (Figure 1C).

3.2. Effect of K+ on sperm head area after sperm activation

Figure 2 shows the changes in spermatozoa head area under the different conditions. After motility activation in standard conditions (samples diluted or washed with P1, activated in ASW), there was a significant reduction in sperm head area ($p<0.01$), which was 94.3 % of quiescent sperm.

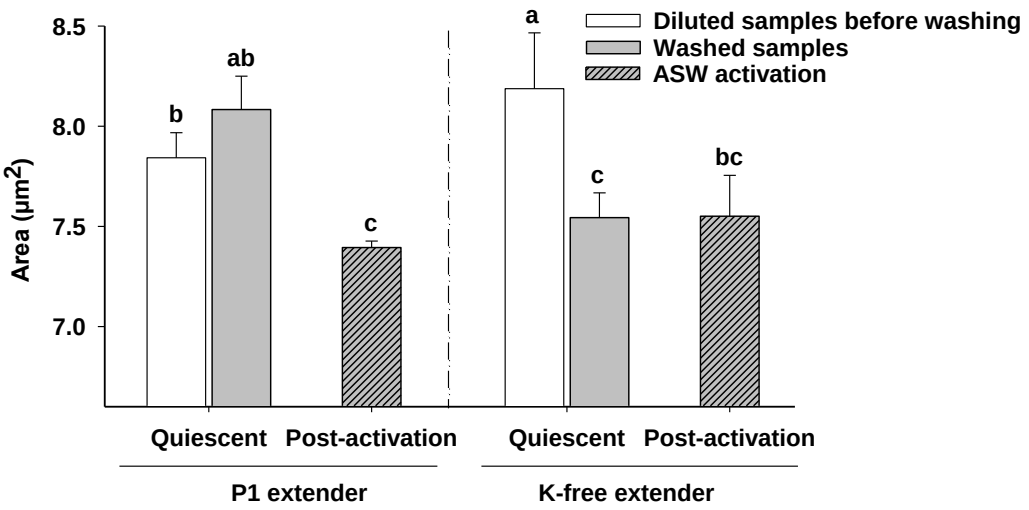

Figure 2. Spermatozoa head area of samples in different conditions: samples diluted in P1 or K-free extender. Before washing (white bars), washed samples (grey bars) and after ASW activation (stripped bars). Data are expressed at the mean ±SEM (n=7). Different letters indicate significant differences ($P<0.01$) between treatments.

Washing the sperm in P1 extender did not significantly alter the head size, while washing it in K-free extender caused a marked reduction in sperm head area, which was 92.2% of controls (diluted samples before washing in K-free extender). While sperm activation induced a significant decrease in sperm head area (in the controls), no further decrease was

observed in the sperm washed in K-free extender and then activated. It was also observed that only the dilution in the K-free extender resulted in a significant increase in sperm head area, which was 104.3% of controls (Figure 2).

3.3. Effect of the ionophore valinomycin and several K^+ inhibitors on the sperm motility and $[K^+]_i$

Treatment with the ionophore valinomycin or the inhibitor 4-AP resulted in a significant reduction in sperm motility (Figure 3A) both after activation with or without potassium (ASW or K-free activation media).

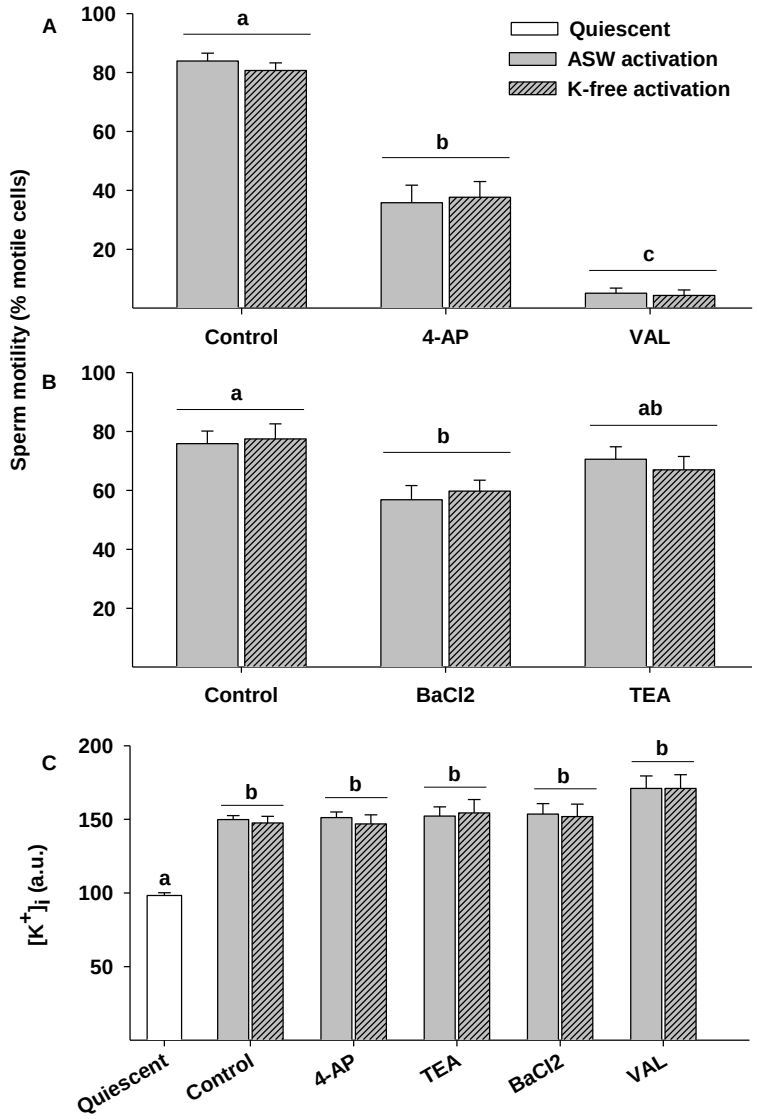

Figure 3. Effect of the inhibitors or modulators on sperm motility and [K+]i. A) Percentage of motile spermatozoa after incubation with or without inhibitor 4-AP or ionophore valinomycin (VAL), and activated with or without potassium (n= 10). B) Percentage of motile spermatozoa after incubation with or without inhibitors: BaCl2 or TEA, and activated with or without potassium (n=7). C) Emitted fluorescence by intracellular K+ sensitive indicator (a.u.: arbitrary fluorescence units), in quiescent sperm and activated with or without potassium after 30 min of incubation with or without inhibitors/ionophores (n=9). Final concentrations: 1 mM 4-AP or BaCl2, 10 mM TEA, 10 µM valinomycin (VAL). Data are expressed as mean ± SEM (n= 10). Different letters indicate significant differences (P<0.01) between treatments

Valinomycin caused the strongest inhibition (to 7.7% of control), while 4-AP resulted in a reduction to 36 % of control

Supplementary Table 1 shows the effect of 4-AP on the other sperm kinetic parameters. A significant reduction was seen in all the parameters of the samples treated with 4-AP. The activation media did not appear to have a significant effect media, except for BFC and STR, which were inhibited by 4-AP after activation in ASW ($p<0.01$), but not after activation in the K-free media.

Treatment with valinomycin also resulted in reductions of varying amounts in most of the kinetic parameters (Supplementary Table 2), MP and FA reduced to 4-5% of the control values, velocities (VCL, VSL, VAP) and ALH reduced to 40-60% of the control values, and lower but significant reductions were observed in STR and BFC (90%, and 64% of control values, respectively).

Barium chloride slightly decreased sperm motility to 75-77% of the control values ($p<0.01$, Figure 3B) and also significantly reduced FA (to 72% of control value, Supplementary Table 3).

TEA did not significantly reduce sperm motility in relation to the controls (Figure 3B). Of the rest of the kinetic parameters, only STR was slightly affected, reaching values 5.5 higher than those of the controls, and LIN, which was 6.5% higher than in the controls (data not shown.

The fluorescence emitted by intracellular [K^+] sensitive indicator increased 1.5-fold after activation in both ASW and the K-free activator (Figure 3C) in relation to the levels of quiescent sperm. Sperm treated with the inhibitors: 4-AP, TEA or $BaCl_2$ and ionophore valinomycin showed a similar increase in [K+]i post-hyperosmotic activation to the controls. The activation media (ASW or K-free activator) did not affect the increase in [K+]i observed post-activation in samples treated with inhibitors/ionophore (Figure 3C).

3.4. Quantification of intracellular pH in quiescent stage

The fluorescence intensity emitted by SNARF-5F in sperm dilutions with different pHs (Figure 4) was used in the calibration equation, which was obtained as described in the technical specifications of SNARF-5F. The linear plot calculated, showed a slope of 1.18 and pKa =7.16, with a high correlation and significance ($R^2 = 0.99$ and $P<0.05$, respectively). The pH_i was calculated using the following equation:

$$pH_i = -8.53 + 1.18F$$

(Where F denotes the fluorescence intensity from SNARF-5F). The calculated pH_i was 7.2 in quiescent stage and 7.1 post-activation in ASW. Thus, a decrease of 0.1 pH units has been demonstrated at activation.

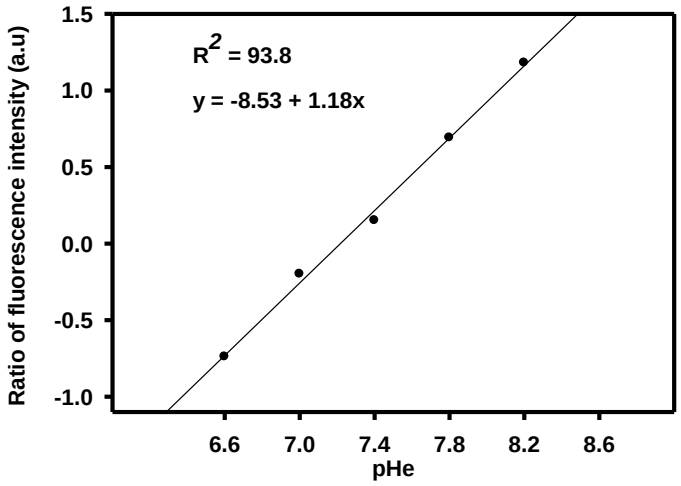

Figure 4. Calibration Plot of intracellular pH. Ratio of intracellular fluorescence emission of SNARF-5 AM (pH indicator) in solutions with different pHs. Cells were loaded with 5 µM SNARF-5 AM for 45 min at 4 ºC in darkness. Calibration was achieved by incubation with 4 µM of nigericin**.**

3.5. Effect of pH_i modifiers NH_4Cl and Na-acetate on sperm motility

Sperm samples activated in ASW with 25-100 mM NH_4Cl (alkalinizing pH_i) showed a strong decrease in motility (Figure 5) in comparison with the controls. In contrast, sperm samples activated in ASW containing 25-100 mM Na-acetate (acidifying the pH_i), showed the same motility as the controls.

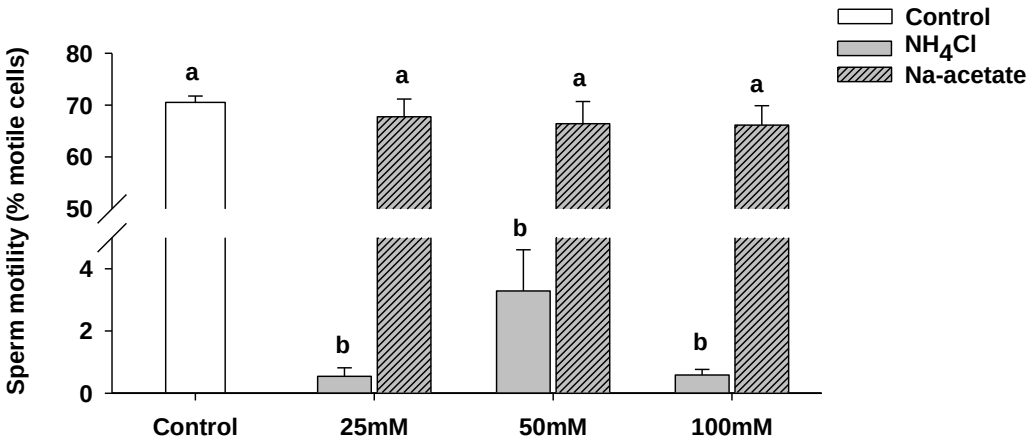

Figure 5. Percentage of motile spermatozoa in samples with modified pH_i by adding Na-acetate and ammonium chloride in hyperosmotic medium (ASW). Data are expressed as mean ± SEM (n= 8). Different letters indicate significant differences (P<0.01) between treatments.

3.6. Effect of K^+ removal, K^+ channel inhibitors and ionophore valinomycin on the pH_i

The changes in the fluorescence emitted by the pH_i sensitive indicator in samples washed in a K-free extender before and after ASW activation are shown in Figure 6. In the controls, the pH remained constant after 30s post-activation. Washing in K-free extender (with or without inhibitor 4-AP) significantly increased the pH_i ($p< 0.01$) in the quiescent stage.**Figure 6.** Emitted fluorescence by intracellular pH sensitive indicator (a.u.: arbitrary fluorescence units), in

quiescent sperm after washing in control extender or K-free extender, and activated with or without potassium after incubation with or without 4-AP (n=5). Data are expressed as mean ± SEM (n= 10). Different letters indicate significant differences (P<0.01) between treatments.

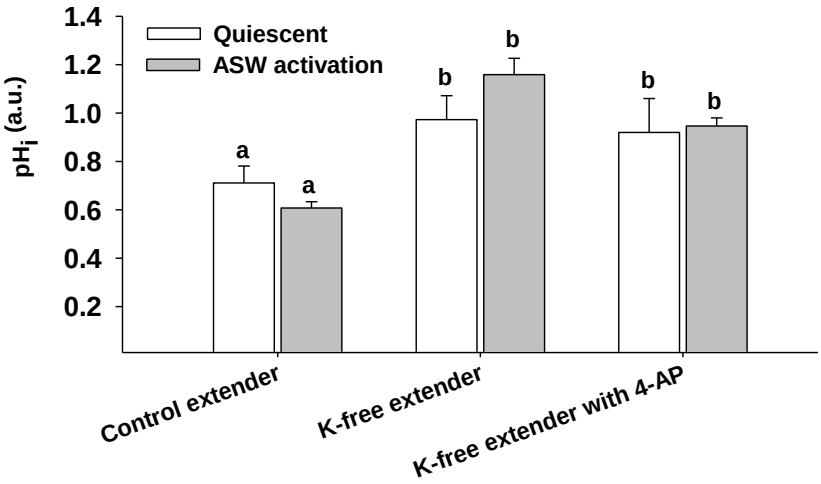

Fig.6. Emitted fluorescence by intracellular pH sensitive indicator (a.u.: arbitrary fluorescence units), in quiescent sperm after washing in control extender or K-free extender, and activated with or without potassium after incubation with or without 4-AP (n=5). Data are expressed as mean ± SEM (n= 10). Different letters indicate significant differences (P<0.01) between treatments

The potassium from activation media had a significant effect on the pHi post-activation (Figure 7A). When sperm was activated in a K-free media, the pHi remained unchanged in relation to quiescent stage, instead of decreasing like in the control activation (with ASW).

When the sperm was treated with valinomycin, the reduction in pHi post-activation was more marked than in their respective controls (activated in ASW or K-free activation media). Similar results were observed at 120 seconds post-activation. Also, it was observed that 4-AP did not alter the pHi neither after activation in ASW nor the K-free media (Figure 7B).

3. Discussion

Effect of removing extracellular potassium from the seminal plasma

In this study we have shown that the removal of extracellular potassium from the seminal plasma resulted in a marked reduction in total motility, even when activation was carried out in the presence of potassium (with ASW, Fig. 1A). This agrees with a previous study on Japanese eel (Ohta et al., 2001) where a similar decrease in sperm motility was observed after 30 min of incubation in a K-free extender.

There are several possible explanations for the reduction of sperm motility in the sperm washed in the K-free extender: it could be linked to the reduction in cell volume observed after washing in the K-free extender, to the pHi increase observed in the same conditions, and to a change in the resting membrane potential induced by the reduction in external K^+.

The present study confirms that the spermatozoa head area of this species decreases after hyperosmotic activation (Fig. 2), as was recently described by Vílchez et al., (2016). This

reduction seems to be necessary for sperm activation, as when the sperm head area was reduced by the removal of sodium from the seminal plasma (Vílchez et al., 2016) or by the removal of K^+ (this study), sperm motility was highly reduced. Thus, both ions seem to be involved in sperm volume regulation in the quiescent stage.

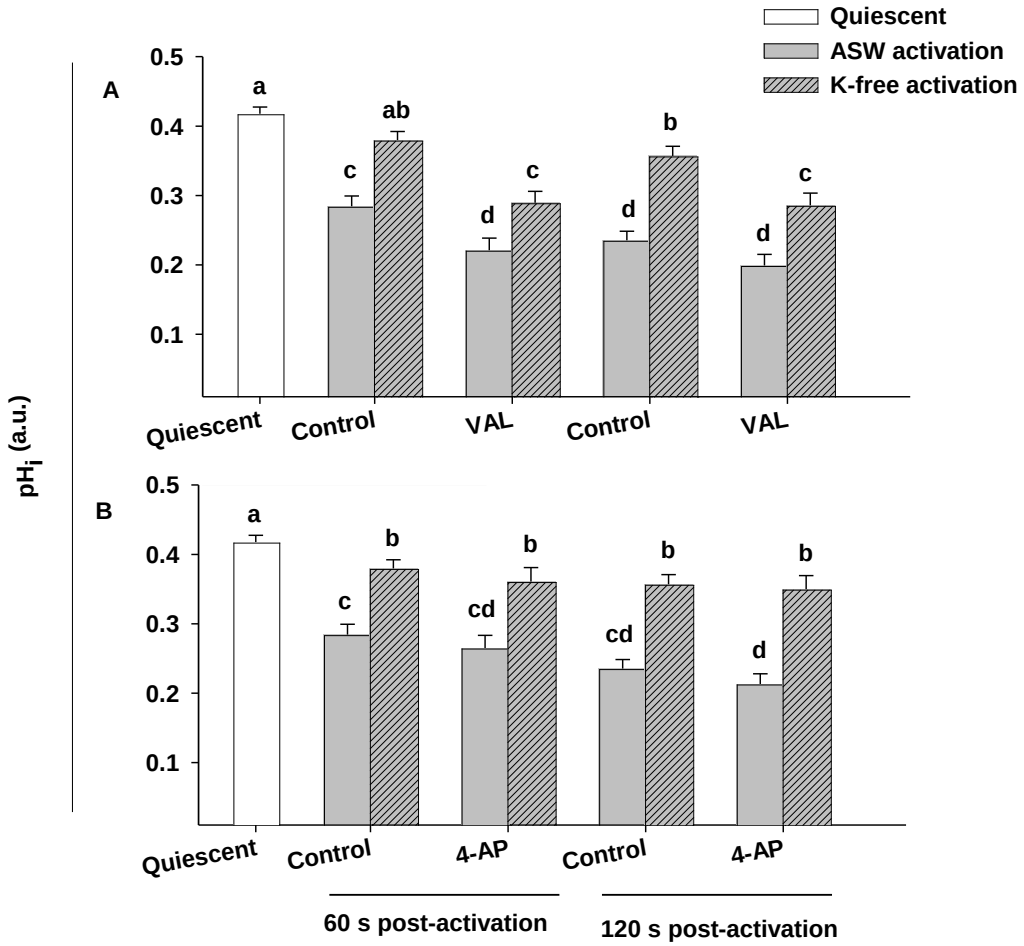

Figure 7. A and B) Emitted fluorescence by intracellular pH sensitive indicator (a.u.: arbitrary fluorescence units), in samples incubated with/without valinomycin and 4-AP respectively and in quiescent sperm and activated with or without potassium (n=11). Final concentrations: 1 mM 4-AP and 10 µM valinomycin (VAL). Different letters indicate significant differences (P<0.01) between treatments.

Regarding the pH, it was observed that aside from a motility inhibition, the removal of K^+ from the seminal plasma induced an increase in pH_i compared to the controls (Figure 6), by an efflux of H^+ from the sperm cells. This suggests that spermatozoa need a low pH_i in order to maintain their capacity to be activated.

Therefore, as reported by Vílchez et al. (2016; Chapter 2), our results indicate a close relationship between spermatozoa volume changes and sperm motility in the European eel. These results also indicate that the presence of these ions (K^+ and Na^+) in the seminal plasma (isosmotic medium) is essential for sperm motility, at least in the European eel.

Sperm volume changes in relation to the osmotic environment have been studied in sperm from a few fish species. Environmental osmolality reduction has been demonstrated to lead

to sperm head swelling in common carp or rainbow trout (Perchec et al., 1997; Takei et al., 2015); however, sterlet (*Acipenser ruthenus*) and brook trout (*Salvelinus fontinalis*) sperm did not changed their cell volume in response to hypoosmotic motility activation (Bondarenko et al., 2013). Thus, sperm volume changes as a response to environmental osmolality seem to be species-specific.

It has previously been noted that aquaporins (AQP) are involved in the motility of sea bream and trout sperm (Zilli et al., 2009). The cell size reduction observed in eel spermatozoa after ASW activation suggests a water efflux after sperm activation, which agree with the role of aquaporins in sperm motility.

In most animal cells, including sea urchin and mammals sperm, the resting membrane potential is primarily set by K^+ permeability (Schackmann et al., 1984; Navarro et al., 2007), and the same fact was observed in freshwater fish sperm. Although in common carp and salmonids the first trigger of sperm motility is considered to be different, (hypoosmolarity in carp, decrease in K^+ concentration in salmonids), in both cases this first signal causes a hyperpolarization of the sperm membrane by the K^+ efflux through K^+ channels (Tanimoto and Morisawa, 1988; Gatti et al., 1990; Boitano and Omoto, 1991; Tanimoto et al., 1994; Krasznai et al., 1995b, 2000; Morisawa, 2008).

Thus, considering K^+ as the main ion involved in sperm membrane potential (Em), and based on the Nerst equation, it could be inferred that, eel sperm will become hyperpolarized after contact with seawater, as $[K^+]$ is higher in the seminal plasma than in seawater. (Em=-34 mV if $[K^+]_i$ =105.5 mM, based on puffer fish (Takai and Morisawa, 1995) and $[K^+]_{out}$=30 mM, based on eel seminal plasma (Asturiano et al., 2004); Em= -60 mV if $[K^+]_i$ =105.5 mM, and $[K^+]_{out}$=11 mM K^+). However, if external K^+ is removed from the seminal plasma (as it was done by washing the sperm in K-free extender), Em would become even more hyperpolarized than after SW activation (to -186 mV if we consider ($[K^+]_i$ =105.5 mM, and $[K^+]_{out}$=0.1 mM). So, in theory K^+ removal would cause a hyperpolarization of the sperm membrane that could be related with sperm motility inhibition. This hypothesis should be further confirmed. Thus, further studies could begin by looking at the relationship between potassium and the membrane potential in European eel sperm motility.

After the removal of potassium from the seminal plasma, the reduction in sperm motility was partially recovered after re-incubation in a medium with high K^+ concentrations (Figure1), but not in a medium with the same K^+ as the seminal plasma. These results partially agree with those obtained by Ohta et al. (2001) in Japanese eel, who showed how sperm can recover motility several times after re-incubation with 10 to 30 mM K^+ (Japanese eel has 15 mM K^+ in seminal plasma). Therefore, the results of the present study corroborate those of Ohta et al. (2001) that suggested that the motility of Japanese eel spermatozoa can be regulated by altering the extracellular concentrations of K^+.

Effect of removing extracellular potassium from the activation media on motility, pH_i and $[K^+]_i$

The sperm activated in K-free media showed similar motility to the ASW controls (Figs. 1, 3). Therefore, the presence of the ion K^+ in the activator media is not necessary for sperm activation. It has been also proven that the presence of other ions, such as Ca^{2+} and Na^+ is not necessary for sperm activation in this species (Pérez et al., 2016, Chapter 3; Vílchez et

al., 2016, Chapter 2). This supports the fact that European eel sperm can be activated in hyperosmotic non-ionic solutions (data not shown), just like many other marine fish species as pufferfish (Morisawa and Suzuki, 1980; Gallego et al., 2013b), halibut (Billard et al., 1993), European sea bass (Dreanno et al., 1999) and cod (Cosson et al., 2008). Thus, K^+, Na^+ and Ca^{2+} in the activation media does not affect sperm motility, but pH and osmolality do, as in other marine species (Morisawa, 2008).

A clear reduction in the pH_i was observed after activation in ASW (Figure 7), whereas only a slight decrease was seen after activation in the K-free activator. This suggests that the post-activation pH_i reduction is not important for sperm motility, although an absence of K^+ does alter this response.

In this study, an increase in $[K^+]_i$ was observed after sperm activation (Figure 4C), corroborating results from previous studies of ours (Gallego et al., 2014). Surprisingly, when the sperm was activated in the K-free activator, the $[K^+]_i$ increase was similar to that of the control. This increase could be due to an influx from the extender media (30 mM K^+) or to the decrease in cell volume observed at activation. Further studies would be needed in order to understand if this increase in $[K^+]_i$ still occurs when the sperm is washed in a K-free extender.

Effect of the ionophore valinomycin

It was observed that valinomycin inhibited sperm motility, with a reduction of 4.76% of control (Figure 3A). In addition, valinomycin also caused a reduction in pH_i (Figure 7). The permeation of the sperm membrane to K^+ ions in quiescent sperm inhibited further sperm motility. It is not known whether this permeation by valinomycin induced an influx or a efflux of K^+ in the sperm cell. Our hypothesis is that, in the quiescent stage, both treating with valinomycin and removing external K^+ caused an efflux of K^+ outside the cell. This in turn reduces the activation capability, due to the modification of the pH_i, and the decrease in the cell volume.

Regarding the relationship between K^+ and pH, our results suggest a co-transport of both K^+ and H^+ at activation, rather than a K^+/H^+exchange. When valinomycin was added, and sperm was activated, a slight (non-significant) increase in $[K^+]_i$ was observed at the same time as a significant increase in $[H^+]_i$ (or a decrease in the pH_i, Figure 7 and 3C). In addition, when external K^+ was removed (prior to activation, Figure 6) a significant increase in the pH (thus a significant decrease in $[H^+]_i$) was observed. These facts suggest a co-transport of K^+ and H^+ at activation, but confirmation of this hypothesis is needed.

Effect of potassium channel inhibitors

Three K^+ channel blockers were tested in the European eel sperm; TEA did not reduce sperm motility (Figure 3), whereas $BaCl_2$ induced a moderate inhibition, and 4-AP induced a stronger motility inhibition. 4-AP is a voltage-gated K^+ channel inhibitor, while Barium and TEA are non-specific K^+ channel inhibitors. 4-AP also inhibited sperm motility in Japanese eel (Tanaka et al., 2004), Atlantic croacker (*Micropogonias undulatus*; Detweiler and Thomas, 1998), and carp (*Cyprinus carpio*; Krasznai et al., 1995b).
Despite their effects on sperm motility neither $BaCl_2$ nor 4-AP caused changes to the $[K^+]_i$ levels after activation, which remained similar to the controls. The K^+ current inhibited by 4-

AP is likely to be of low intensity, as it was not detected by flow cytometry, or perhaps transient, occurring only in the first few seconds after activation, and thus not detected.
In conclusion, the results of this study indicate that a potassium channel, probably a voltage-gated K^+ channel, is involved in European eel sperm motility activation.

Role of pHi in sperm motility and the direct relationship with the presence of K^+

A direct quantitative analysis has been used for first time to measure the pH_i in eel sperm cells by flow cytometry. The pH_i was 7.2 in the quiescent stage and reduced to 7.1 after sperm activation. This confirms previous results of ours, where a decrease in pH_i was observed in European eel sperm after activation (Gallego et al., 2014). The decrease in the pH_i does not appear to be linked to motility, as a decrease in the pH_i was also observed when the sperm was activated but remained immotile, as was the case of the sperm incubated with valynomicin or 4-AP.

Importance of pH_i in the quiescent stage

The pH_i measured in the quiescent stage (pH_i = 7.2) was 1.3 units lower than the pH measured in the seminal plasma (8.5) (Asturiano et al., 2005). Thus a H^+ gradient exists in the quiescent stage, with higher $[H^+]_i$ levels than in the surrounding media.

This pH gradient seems to be important in maintaining the sperm functionality of the quiescent sperm, as when the sperm were incubated in P1 at 6.5 (0.6 units lower than the pH_i) sperm motility was suppressed after activation (Peñaranda et al., 2009). Also, the gradient between the pH_i and the pH of the activation media is important for sperm motility. When the pH of the ASW was lower (6.2) than the pH_i, a strong reduction in sperm motility was observed (data not shown). Similarly, acidification of the pH_i by CH_3COONa resulted in no effects to sperm motility, whereas alkalinization of the pH_i by NH_4Cl, inhibited motility (Figure 5). Indeed, the alkalinization of the pH_i in the quiescent stage caused a reduction in motility activation. In contrast, it has been suggested that internal alkalinization in pufferfish sperm (*Takifugu niphobles*) is responsible for the initiation of sperm motility (Oda and Morisawa, 1993).

All these facts indicate that for successful sperm motility activation the pH_i should be lower than the seminal plasma pH (7.2 and 8.5 respectively, in normal conditions), and lower or equal to the activation medium pH (as sperm can be activated in ASW at pH 7.2, data not shown). Indeed, in this study the removal of K^+ from the seminal plasma not only resulted in a significant increase in the pH_i but also in a strong reduction in the sperm motility.

4. Conclusions

Our results demonstrate that the presence of K^+ in the extender medium (and in the seminal plasma) is important for the preservation of sperm motility in the European eel, at least in part by maintaining the right sperm cell volume. Eel extenders should therefore contain the right amount of K^+. The intracellular K^+ increase observed after sperm activation does not seem to be related to sperm motility, as it also happens in low sperm motility conditions. A voltage-gated K^+ channel inhibited by 4-AP seems to be involved in sperm motility, but its effect was not detected in the intracellular K^+ measurements.

As a result of this study, the absolute pH_i before and after sperm activation (7.2 and 7.1, respectively) in the European eel have been determined. The intracellular pH in the quiescent stage is 1.3 units lower than the seminal plasma pH, and this difference is important for sperm motility. Indeed for successful sperm motility activation, the pH_i should be lower than the seminal plasma pH and lower or equal to the activation media pH. A relationship between potassium and pH has been observed, and a co-transport of both ions related to sperm motility has been suggested.

Acknowledgments

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SUPLEMENTARY DATA

Tables

Table 1. Effect of 4-AP on sperm kinetics after activation in ASW or K-free activation medium. **A**ctivation media had 1100 mOsm, pH= 8.2, and 2 % (w/v) BSA. Data are expressed as mean ± SEM (n = 10). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC, beat frequency, STR, ALH. SE: standard error. Different letters indicate significant differences (P<0.01) between treatments.

Activator	ASW control	K-free control	ASW 4-AP	K-free 4-AP
MP %	42.10 b	41.43 b	5.50 a	10.19 a
FA %	71.96 b	71.08 b	8.36 a	12.45 a
VCL (µm/s)	168.01 b	165.66 b	72.78 a	81.48 a
VSL (µm/s)	89.02 b	87.54 b	31.70 a	38.14 a
VAP (µm/s)	116.99 b	115.11 b	46.93 a	52.79 a
STR	76.06 b	76.02 b	65.80 a	70.69 b
ALH	2.96 b	2.89 b	1.91 a	1.95 a
BFC beats/s	30.66 b	30.48 b	23.04 a	27.47 b

Table 2. Effect of valinomycin on sperm kinetics after activation in ASW or K-free activation medium. Activation media had 1100 mOsm, pH= 8.2, and 2 % (w/v) BSA. Data are expressed as mean ± SEM (n = 10). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC, beat frequency. SE: standard error. Different letters indicate significant differences (P<0.01) between treatments.

Activator	ASW control	K-free control	ASW Valinomycin	K-free Valinomycin
MP %	42.09 a	41.43 a	1.91 b	1.81 b
FA %	71.95 a	71.08 a	3.11 b	3.13 b
VCL (µm/s)	168.01 a	165.66 a	76.38 b	94.75 b
VSL (µm/s)	89.02 a	87.54 a	35.05 b	39.45 b
VAP (µm/s)	116.99 a	115.11 a	49.3 b	56.62 b
BFC beats/s	30.66 a	30.48 a	19.91 b	19.02 b

Table 3. Effect of $BaCl_2$ on sperm kinetics after activation in ASW or K-free activation medium. Activation media had 1100 mOsm, pH= 8.2, and 2 % (w/v) BSA. Data are expressed as mean ± SEM (n = 7). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC, beat frequency. SE: standard error. Different letters indicate significant differences (P<0.01) between treatments.

Activator	ASW control	K-free control	ASW $BaCl_2$	K-free $BaCl_2$
MP %	34.36	38.18	31.11	37.35
FA %	61.47 bc	67.10 c	43.54 a	48.97 ab
VCL (µm/s)	157.87 a	175.86 c	147.27 a	162.31 ab
VSL (µm/s)	80.67 a	92.41 b	81.06 a	94.13 b
VAP (µm/s)	108.19 ab	122.61 c	101.52 bc	115.53 a
BFC beats/s	30.19 a	32.93 bc	31.69 ab	33.67 c

CHAPTER 2

Sodium affects the sperm motility in the European eel

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Abstract

The role of seminal plasma sodium and activation media sodium on sperm motility was examined by selectively removing the element from these two media, in European eel sperm. Sperm size (sperm head area) was also measured using an ASMA (Automated Sperm Morphometry Analyses) system, in the different conditions. Intracellular sodium $[Na^+]_i$ was quantitatively analyzed by first time in the spermatozoa from a marine fish species. Measurement of $[Na^+]_i$ was done before and after motility activation, by Flow Cytometry, using CoroNa Green AM as a dye. Sperm motility activation induced an increase in $[Na^+]_i$, from 96.72 mM in quiescent stage to 152.21 mM post-activation in seawater. A significant decrease in sperm head area was observed post-activation in seawater. There was a notable reduction in sperm motility when sodium was removed from the seminal plasma, but not when it was removed from the activation media. Sodium removal was also linked to a significant reduction in sperm head area in comparison to the controls. Our results indicate that the presence of the ion Na^+ in the seminal plasma (or in the extender medium) is necessary for the preservation of sperm motility in European eel, probably because it plays a role in maintaining an appropriate sperm cell volume in the quiescent stage of the spermatozoa.

1. Introduction

The spermatozoa of species which exhibit external fertilization are generally immotile in the seminal plasma and initiate their motility immediately upon the dilution in freshwater or seawater at spawning (Morisawa, 1985). The mechanism for sperm activation has been widely studied in mammals and in sea urchin (*Strongylocentrotus purpuratus*, see review of Espinal et al., 2011). However, little is known about this mechanism in fish sperm. In marine teleosts, spermatozoa are immotile due to the iso-osmolality with the seminal plasma (Morisawa, 1980; Stoss, 1983; Alavi and Cosson, 2006). Then, the hyperosmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water across the spermatozoa membrane which activates the cells´ motility (Morisawa, 2008). However, few studies have been conducted in marine fish species about the changes in the concentrations of ions inside the sperm cells before and after motility activation.

The main ions present in marine fish seminal plasma are: Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- (Suquet et al., 1993; Asturiano et al., 2004). In a previous study on European eel the ionic composition of seminal plasma was linked to sperm motility. It was observed that the concentration of some seminal plasma ions (K^+, Ca^{2+}, Mg^{2+}) changed in a progressive way from the low motility sperm samples to the high motility sperm samples. For instance, seminal plasma K^+ concentrations increased during the improvement in sperm quality, while Ca^{2+} and Mg^{2+} concentrations showed a progressive reduction in correlation with the improvement in sperm quality (Asturiano et al., 2004). Na^+ was also present in European eel seminal plasma, but the concentrations of this ion were almost constant, between 110-120 mM, irrespective of the motility category. The selective elimination, one by one, of the main ions (Na^+, K^+, Ca^{2+}, H^+) present in the seminal plasma could be key to determining the role they play in the further sperm activation process due to hyperosmotic shock, or, in other words, in maintaining, in quiescent stage, the capability for further sperm activation.

In a preliminary study on European eel, Gallego et al. (2014) observed that intracellular concentrations of Ca^{2+} and K^+ in sperm increased after hyperosmotic sperm activation, with a progressive decrease in intracellular pH suggesting a flux of these ions through the spermatozoa membrane during sperm activation. Also, it has been demonstrated the presence of a Na^+/Ca^{2+} exchanger involved in the sperm motility of European eel. The inhibition of this exchanger by bepridil induced a suppression of the increase in $[Na^+]_i$ and was linked to a notable reduction in sperm motility (Pérez et al., 2016). Although Na^+ is one of the main constituents of fish seminal plasma (Suquet et al., 1993; Asturiano et al., 2004), Na^+ fluxes during sperm activation in marine fish sperm have been poorly studied.

Regarding the involvement of Na^+ in sperm motility, several studies have demonstrated the importance of this ion in the sperm cells from mammals and sea urchin (Escoffier et al., 2012; Espinal et al., 2011). In mammalian sperm, the hyperpolarization associated with capacitation involves a decrease in $[Na^+]_i$ mediated by an inhibition of epithelial Na^+ channels (ENaC; Escoffier et al., 2012). Na^+/H^+ exchangers (NHEs) are also present in the sperm membranes of mammals and sea urchin, and are known not only to participate in the regulation of intracellular pH, but also in water absorption across epithelia, and cell volume regulation (Martins et al., 2014; Nomura et al., 2006). However, information about sodium channels in marine fish sperm is restricted to the use of a sodium channel inhibitor, amiloride, which inhibits sperm motility in Atlantic croaker (*Micropogonias undulatus*; Detweiler and Thomas, 1998). However, in other marine species, the Pacific herring (*Clupea pallasi*; Vines et al., 2002), it is the decrease in external sodium (not the increase) that appears to be involved in sperm activation. Therefore, the ion sodium could play species-specific roles in sperm motility in marine species. For this reason, the present study tries to analyse and understand the role of the ion Na^+ in the sperm motility of a marine species, the European eel.

There are several methods that can be used to measure the intracellular ion concentrations in sperm. In sea urchin, (*S. purpuratus;* Rodriguez and Darszon, 2003) the intracellular concentrations of Na^+, Ca^{2+} and pH_i were measured by spectrophotometry, while Marian et al. (1997) was the first to use flow cytometry to quantify intracellular ions in sperm cells from a freshwater fish. However, Marian et al. (1997) and her group quantified some ions, including $[Na^+]_i$ by indirect methods, through the measurement of the fluorescence emitted by a pH-indicator dye in the presence of nigericin, which equals the $[H^+]_i=[Na^+]_i$. The present work shows for the first time the intracellular sodium concentrations measured by a direct method (through the intensity of the fluorescence emitted by ion sodium) and by flow cytometry (through the intensity of fluorescence emitted cell by cell) in the sperm of a teleost species.

In the present study, the European eel was used as the experimental organism, as it is easy to produce high quantities of good quality sperm after a 6-7 weeks of treatment with weekly injections of hCG, and to maintain spermiation for several weeks (Gallego et al., 2012a). The present study focuses on the importance of the Na^+ present in the seminal plasma and in the activation media in the sperm motility activation. The involvement of cell volume changes in sperm motility has been also studied by measuring the sperm head area. Moreover, this study looks at the $[Na]_i$ before and after sperm activation of the European eel to determine whether Na^+ fluxes participate in the sperm motility of this species.

2. Material and methods

2.1. Chemicals and solutions

The Na ionophore Monensin (M5273), amiloride inhibitor (A7410), EDTA, and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich Co. (St. Louis, MO). CoroNa Green AM (C36676), and Propidium Iodide (R37108) fluorochroms were purchased from Invitrogen and Molecular Probes respectively (Life Technologies, Madrid-Spain). Salts were of reagent grade.

A stock solution of 20 mM of monensin was diluted in DMSO, aliquoted and kept at -20 °C until use. An aliquot of the stock solution to be used with the sperm, was thawed only once and mixed with the sperm to reach a final concentration of 20 µM.

In the same day, a stock solution of 50 mg/ml of amiloride was diluted in ultrapure hot water and mixed with the sperm to a final concentration of 2 mM. DMSO stock 1 mM CoroNa Green AM was prepared and used as described in Section 2.7.

2.2. Fish maintenance and hormonal treatment

A total of 40 adult male eels (mean body weight 115±8 g) were transferred to our facilities at the Universitat Politècnica de València (Spain) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain). The fish were distributed into two 90-L aquaria (approximately 20 male eels per aquarium) equipped with separated recirculation systems, thermostats, and coolers, and covered with black panels to maintain constant darkness. The animals were gradually acclimatized to seawater (salinity 37.0±0.3 g/L) over the course of 1 week, and were then maintained in seawater at 20 ºC until the end of the experiment, as in previous works (Peñaranda et al., 2010a, Gallego et al., 2012a). Water renewal was 1/3 of the volume of each aquarium per week.

Once the fish were in seawater the hormonal treatment with hCGrec (recombinant hCG; Ovitrelle, Merck Serono, Madrid) was initiated. Once a week, the animals were anaesthetized with benzocaine (60 ppm) and weighed before receiving the intraperitoneal injection of hCGrec (diluted in NaCl 0.9%) at a dose of 1.5 IU/g fish.

During the experiment the fish were starved, and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.3. Sperm collection and sampling

Sperm samples were collected once a week, 24 h after the administration of the hormone, to obtain maximum sperm quality (Pérez et al., 2000). Sperm was collected in Falcon tubes by gentle abdominal pressure, after fish anesthetization. The genital area had been previously cleaned with distilled water, and dried, in order to avoid sample contamination with feces, urine and seawater. The sperm samples were kept refrigerated (4 °C) until the motility analyses, which took place within the first hour after collection.

2.4. Sperm motility evaluation

The sperm motility activation was carried out as per the method described by Gallego et al. (2013a), by mixing 1 µl of diluted sperm (dilution 1/25 in control extender, Table 1, Peñaranda et al., 2009) with 4 µl of artificial seawater (ASW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was made in a SpermTrack-10® chamber, 10 µm depth (Proiser R+D, S.L.; Paterna, Spain) and observed in a microscope Nikon Eclipse 80i, with a 10x lens (Nikon phase contrast 10x 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after the sperm was mixed with ASW, using a high-sensitivity video camera HAS-220 and the ISAS software (Proiser R+D, S.L.; Paterna, Spain). The frame rate used was 60 fps. For each motility test, samples were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 ºC in a water bath during the sperm motility evaluation. The best samples (>50% total motility) were selected for the studies.

The sperm motility parameters considered in this study were: total motility (MOT, %); progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast (FA; average path velocity, VAP>100 µm/s); 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 average spatial path, VSL/VAP; ALH, amplitude of the lateral movement of the sperm head and cross beating frequency (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was <10 µm/s (Martínez-Pastor et al., 2008).

Table 1. Composition (mM), osmolality (mOsm) and pH of the media used. Extender media; control (with sodium) and Na-free (without sodium). Activation media: ASW (control, with sodium) and Na-free activator (without sodium).

	NaCl	Choline chloride	$MgCl_2$	$CaCl_2$	KCl	$NaHCO_3$	Na_2SO_4	TAPS	Osm	pH
Extender:										
Control	125	-	2.5	1	30	20	-	-	325	8.5
Na-Free	-	125	5.3	1.3	30	-	-	20	325	8.5
Activator:										
ASW	354.7	-	52.4	9.9	9.4	20	28.2		1100	8.2
Na-Free		550	-	-	-	-	-	20	1100	8.2

2.5. Composition of extenders and activation media

Table 1 shows the composition of the extenders and activation media. To examine the effects of ion sodium on the initiation of sperm motility in the European eel, two kinds of diluents and activators media were prepared, with or without sodium. Control extender was used as the artificial seminal plasma in this work; its composition mimics the seminal plasma of European eel (Peñaranda et al., 2010a). The Na-free extender was prepared by replacing in the control extender, NaCl with choline chloride in the same molarity, and 20 mM TAPS

was added as a buffer. In both isosmotic media the osmolality was 325 mOsm and the pH was adjusted to 8.5.

Hyperosmotic activators were: artificial seawater (ASW) and Na-free activator (550 mM of choline chloride). In both hyperosmotic activation media the osmolality was 1100 mOsm, the pH was adjusted to 8.2 and 2% BSA (w/v) was added.

The Na-free extender and Na-free activation media were prepared with ultra-pure water and with autoclaved material. The pH of the Na-free media was adjusted with 1M KOH or HCl, while in control extender and ASW it was adjusted with 1 M NaOH or HCl.

2.6. Removal of extracellular sodium from the seminal plasma

After the initial evaluation of sperm motility, individual samples showing >50% of total motility were selected, and the next step was removing the sodium ion from the seminal plasma. Each sample was first diluted 1:25 in: a) control extender (with sodium) or b) Na-free extender (without sodium) and then washed three times as described by Pérez et al. (2016), at 500 g, for 4 min at 4 ºC. Between centrifugations, the supernatant was carefully removed, and the sperm pellet was re-suspended in control or the Na-free extender (at 1:25 v:v) by gentle shacking. This process was repeated three times and then the samples were finally re-suspended in control or the Na-free extender and maintained at 4 ºC until analysis.

2.7. Relative intracellular Na^+ measurements

The relative amount of $[Na^+]_i$ was determined by flow cytometry using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser and a red laser. Slightly angled scattered front light was used for the electronic gating of data collection, allowing us to exclude dead cells from the analyses. The CoroNa Green dye was used like a green-fluorescent sodium (Na^+) indicator that exhibits an increase in fluorescence emission intensity upon binding Na^+. A work solution 0.5 µM CoroNa Green-AM was prepared from a DMSO stock solution (1 mM, by diluting 1:1 in ultrapure water). The spermatozoa were loaded with CoroNa Green-AM indicator to a final concentration of 10 µM, and 2 µM IP (Propidium Iodide) was added to exclude dead cells from the analyses. Sperm incubation was performed in darkness, for 45 min at 4 ºC. The incubation media were control or Na-free extender. The final DMSO concentrations in the sperm were less than 0.05% in all the cases, and therefore a DMSO effect on motility could be discarded.

After incubation, 5 µl of each sperm sample (diluted in 1/25 of control or Na-free extender) was added to a tube containing 500 µl of the same extender medium (control or Na-free extender), to measure the fluorescence emitted by Na_i^+ in the quiescent stage, before activation. Later, 5 µl of each diluted sperm sample was added to another tube containing an activation medium (500 µl ASW or Na-free activator) and the fluorescence emitted by the sperm cells was recorded 30 s after the hyperosmotic activation, which is the time that lapses between creating the mixture of sperm-activator and obtaining the final F1 measurement from the Flow Cytometer. The final sperm dilution used for measurements in the flow cytometer was 1/2500 (v:v), with approximately 400 cells/µl.

CoroNa Green and IP dyes were excited by the blue laser (488 nm), and their fluorescence was read by the FL1 (530/40BP filter) and FL3 (665/20BP 284 filter) photodetector,

respectively. The fluorescence data was displayed in logarithmic mode. Five thousand events per sample were collected, with a flow rate of 500cells/s. A gate in forward and side scatter was used to exclude debris and aggregates from the analysis. The flow cytometry data was processed using WEASEL software (v3.1, Walter 288 and Eliza Hall Institute).

2.8. Quantification of intracellular sodium: calibration curve

A pool of sperm made from 6 individual sperm samples showing >50% of sperm motility (see section 2.4.), was diluted and washed in control extender as described in section 2.6. Then the sperm pellet was re-suspended in the calibration solutions. The calibration solutions were prepared by mixing Na^+ and K^+ solutions (see Table 2). The solution with the highest concentration of Na^+ contained 150 mM NaCl, 11 mM $MgCl_2$*$6H_2O$, 3.5 mM $CaCl_2$*$6H_2O$, and 10 mM HEPES. The solution with the highest concentration of K^+ solution was similar, but the NaCl was replaced on a molar basis by KCl. The pH of the solutions was increased to pH 8.5 using either NaOH (for 150 mM Na^+ solution) or KOH (for 150 mM K^+ solution).

Ionophore monensin was added in order to equilibrate $[Na^+]_e$ and $[Na^+]_i$. The sperm diluted in the different $[Na^+]$ calibration solutions and the control sample (washed and diluted in control extender) were incubated in the presence of 20 µM of ionophore monensin, and 10 µM of CoroNa Green indicator. All the samples were incubated for 30 minutes at 4 ºC in darkness. The response calibration was obtained by measuring the fluorescence intensity of the CoroNa Green indicator in the solutions with precise Na^+ free concentrations (see Table 2). The dissociation constant (K_d) was determined using a modified version of the Grynkiewicz equation (Amorino et al., 1995) for a single wavelength indicator:

$$[Na^+] = K_d\,(F - F_{min})/(F_{max} - F)$$

Table 2. Composition (mM) of the extender media used in the calibration curve. Solutions were brought to pH 8.5 using either NaOH (for 150 mM Na^+ solution) or KOH (for 150 mM K^+ solution).

	0 mM Na^+	**15 mM Na^+**	**30 mM Na^+**	**50 mM Na^+**	**100 mM Na^+**	**150 mM Na^+**
NaCl	0	15	30	50	100	150
$MgCl_2$* $6H_2O$	11	11	11	11	11	11
$CaCl_2$* $2H_2O$	3.5	3.5	3.5	3.5	3.5	3.5
KCl	150	135	120	100	50	0
HEPES	10	10	10	10	10	10

Where F denotes fluorescence intensity and *min* and *max* were the data points corresponding to the minimum and maximum Na^+ concentrations (0 and 150 mM).

Once the linear plot was obtained, the same equation was used to calculate the $[Na^+]$ values corresponding to the measured fluorescence intensities (F) of the experimental samples in the quiescent stage (control extender) and post-activation (with ASW).

2.9. Relationship between intracellular $[Na^+]$ changes and sperm motility in different conditions

Experiment 1: Effect of the removal of extracellular sodium on sperm motility and $[Na^+]_i$

Nineteen sperm samples (one sample/male) with >50% of total motility were selected and used for motility analyses and for $[Na^+]_i$ measurements, which were performed on the same day. Each sample was subdivided and washed with/without Na^+ (control/Na-free extender; see section 2.6.). Then, the sperm motility was measured in each sub-sample in triplicate, by activating the diluted sub-samples with ASW and Na-free activation medium. The fluorescence emitted by $[Na^+]_i$ was measured in the samples in the quiescent stage (pre-activation) and post-activation with both activators.

Experiment 2: Recovery of motility in samples washed in a Na-free extender

In order to study the recovery of sperm motility in samples previously washed in a Na-free extender, the sperm motility of eight samples washed with control or Na-free extender and activated in ASW was measured in triplicate. Later, the samples that had been washed with the Na-free extender were incubated at 4 ºC in a control extender (containing 155 mM Na^+), and the motility was checked in triplicate after 30 and 60 min of incubation.

Experiment 3: Changes in the sperm head area before and after activation

A total of 9 sperm samples with >50% of total motility were selected to study the changes in sperm head area in relation to activation. The spermatozoa were fixed with glutaraldehyde at 5% (v:v) before and after washing the sperm in control and Na-free extender (quiescent stage). Thus, in order to study the changes in the morphometry after activation in ASW (post-activation), the sperm washed in control and the Na-free extender were also fixed with glutaraldehyde at 5% (v:v) after activation in ASW.

The fixed sperm samples were examined using a phase contrast microscope with a 100X contrast phase lens. Microphotographs of the spermatozoa were taken using an ISAS 782M camera (Proiser R+D, S.L.; Paterna, Spain), and the morphometric analyses of the sperm samples were performed using the morphometry module of the ISAS software. The spermatozoa head measurements, including size variables such as area (*A*) and perimeter (*P*), were calculated automatically by capturing 110 digitized spermatozoa for each sample.

Experiment 4: Effect of monensin on sperm motility and $[Na^+]_i$

To examine the effect of the ionophore monensin on sperm activation, 10 sperm samples were used for motility analyses and for $[Na^+]_i$ measurements, which were performed on the same day. One aliquot from each sample was diluted and washed in control extender, and another aliquot was diluted and washed in a Na-free extender. Later, each sub-sample was divided into two aliquots; one of which was incubated with 20 µM of monensin (at room temperature, for 20 min, in the dark), and the second aliquot was incubated, with the same concentration of DMSO (0.001%), as a control. The sperm motility was measured in triplicate in each sub-sample (washed with control or a Na-free extender and incubated with or without monensin), by activating the diluted samples with ASW or a Na-free activation medium.

The fluorescence emitted by $[Na^+]_i$ was measured in the same samples (control and Na-free samples) incubated with or without monensin, in the quiescent stage and after ASW activation.

Experiment 5: Effect of amiloride in the sperm motility

To examine the effect of the inhibitor amiloride on sperm motility, 9 sperm samples were used for motility analyses and $[Na^+]_i$ measurements, which were performed on the same day. All the samples were first diluted 1:25 (v:v) and washed in control extender. Later the samples were incubated with or without 2 mM of amiloride at 4 ºC for 20 min. Sperm motility was measured after activating the samples with ASW or a Na-free activator.

Using the same samples (washed in control extender) incubated with or without amiloride the fluorescence emitted by $[Na^+]_i$ was measured in the quiescent stage and after ASW activation.

2.10. Statistical analysis

Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by flow cytometry. After removing dead spermatozoa (PI) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) was obtained for each sample. All the statistical procedures were run using Statgraphics Plus 5.1. 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. Quantification of intracellular sodium pre- and post-activation

The fluorescence intensity emitted by CoroNa Green AM in sperm dilutions with different sodium concentrations (Figure 1) increased as a linear function of $[Na^+]_e$.

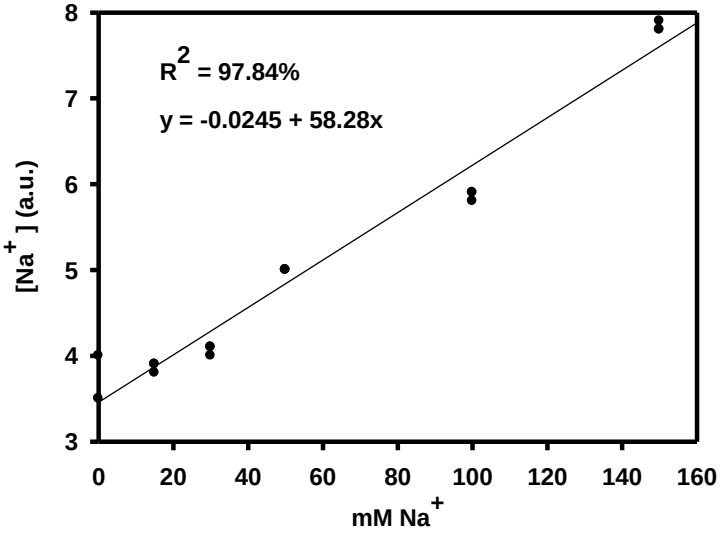

Figure 1. Calibration Plot of intracellular Na^+. Intracellular fluorescence emission of CoroNa Green (Na^+ indicator) in solutions with different Na^+ concentrations. Cells were loaded with 10 µM CoroNa Green for 45 min at 4 ºC in darkness. Calibration was achieved by incubation with 20 µM of monensin.

The linear plot calculated showed a high correlation and significance (R^2=97.84% and $P<0.05$, respectively).

The dissociation constant Kd calculated from this data (58.25 mM) was used to calculate the $[Na^+]_i$ in the sperm cells; 96.72 mM in quiescent sperm, and 152.21 mM post-activation with ASW. Thus, the $[Na^+]_i$ levels increased roughly 50% after activation.

3.2. Effect of the removal of sodium from the extender and the activation media

Figure 2A shows that the control samples (washed with sodium) showed similar motility after activation with or without sodium (with ASW or the Na-free activator, respectively). However, the elimination of Na^+ from the extender by washing resulted in a notable reduction in sperm motility irrespective of whether sodium was present at the time of activation (Figure 2A). Regarding other sperm kinetic parameters (see Suppl. Table 1), the removal of extracellular Na^+ by washing (Na-free samples) resulted in a marked reduction in all the sperm kinetic parameters, whether activation occurred in the presence or absence of sodium. Removing the extracellular Na+ by washing reduced MP and FA by up to -90% (Suppl. Table 1).

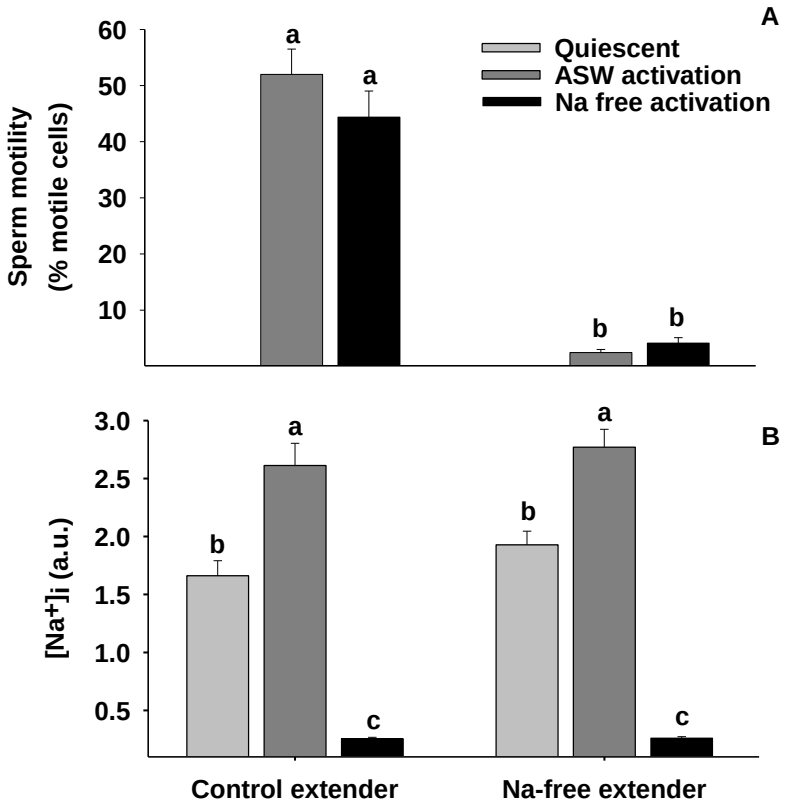

Figure 2. A) Percentage of motile spermatozoa after washing in control extender or Na-free extender and activated with or without sodium. B) Emitted fluorescence by intracellular Na^+ (a.u.: arbitrary fluorescence units), on quiescent sperm after washing in control or Na-free extender and activated with or without sodium. Data are expressed as mean ± SEM (n= 19) and different letters indicate significant differences ($P<0.05$) between treatments.

The changes in the fluorescence emitted by [Na+]i were studied (Figure 2B). There were no significant differences in $[Na^+]_i$ after washing with/without Na^+, in the samples in the quiescent stage. After sperm activation with sodium (ASW), the $[Na^+]_i$ increased significantly compared to $[Na^+]_i$ from quiescent sperm in all the samples, whether they had previously been washed with control or the Na free extender. On the contrary, activation without Na^+ (Na-free

activation medium), resulted in a marked decrease in $[Na^+]_i$ compared to quiescent stage in all the cases.

3.3. Recovery of motility in Na-free samples after incubation with a control extender

As a reduction in sperm motility caused by Na-free washing (Figure 2A) was observed, a test was carried out to see if this process could be reversed by re-incubating the Na-free samples, in a control extender (with sodium). In this experiment (Figure 3), the elimination of extracellular Na^+ by washing caused a marked reduction in sperm motility compared to samples washed in control extender, as in the previous experiment. When Na-free samples were re-incubated in the control extender, there was no recovery of the motility, even after 60 min. Regarding the sperm kinetic parameters (Suppl. Table 2), the samples washed without extracellular sodium showed much lower kinetic values than the control samples (washed with sodium), with reductions of up to -90% in MP and FA. The kinetic parameters of the Na-free samples were similarly low after 30 min and 1 h of re-incubation in control extender at 4 ºC. However, the control samples (washed and re-incubated with sodium) showed a significant increase in VSL after 1 h of incubation at 4 ºC, compared with previous activations at 15 s and 30 min.

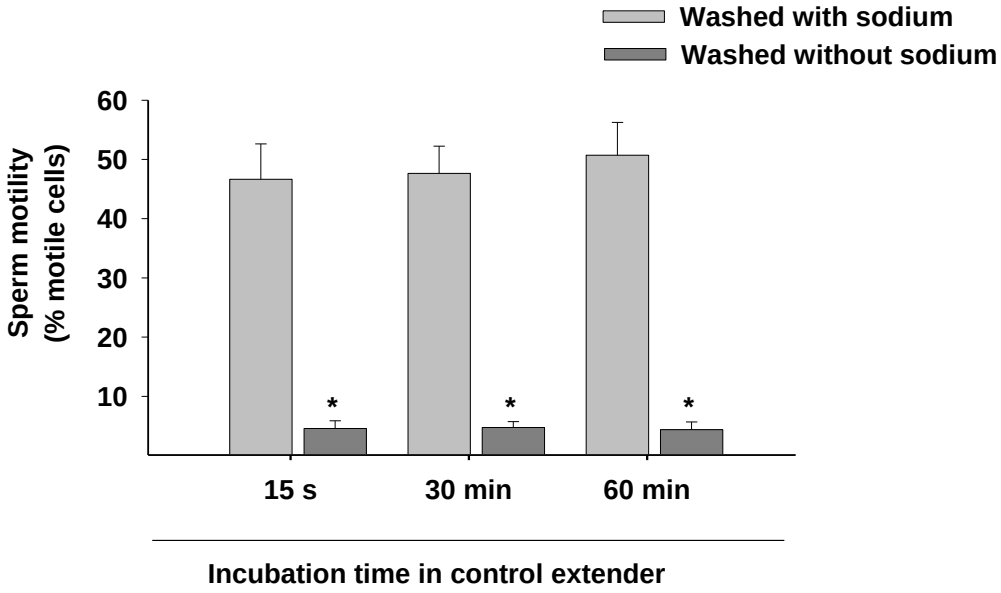

Figure 3. Percentage of motile spermatozoa at different times of incubation in control extender, in samples washed with and without sodium. Data are expressed as mean ± SEM (n= 8) and asterisks indicate significant differences (P<0.05) between washed samples with and without sodium.

3.4. Effect of Na^+ on the sperm head area after sperm activation

Figure 4 shows the changes in spermatozoa head area in different conditions. Sperm head area was reduced ($p<0.05$) after motility activation in the standard conditions of the control samples (diluted or washed with sodium, activated in ASW). Interestingly, dilution or washing in a Na-free extender caused, without activation (in quiescent stage), a significant head area reduction ($p<0.01$) compared to control samples (diluted or washed with sodium).

When these samples (washed in Na-free extender) were activated with ASW, the sperm head area did not change, in contrast to the observed change in the control samples

(washed in with sodium).

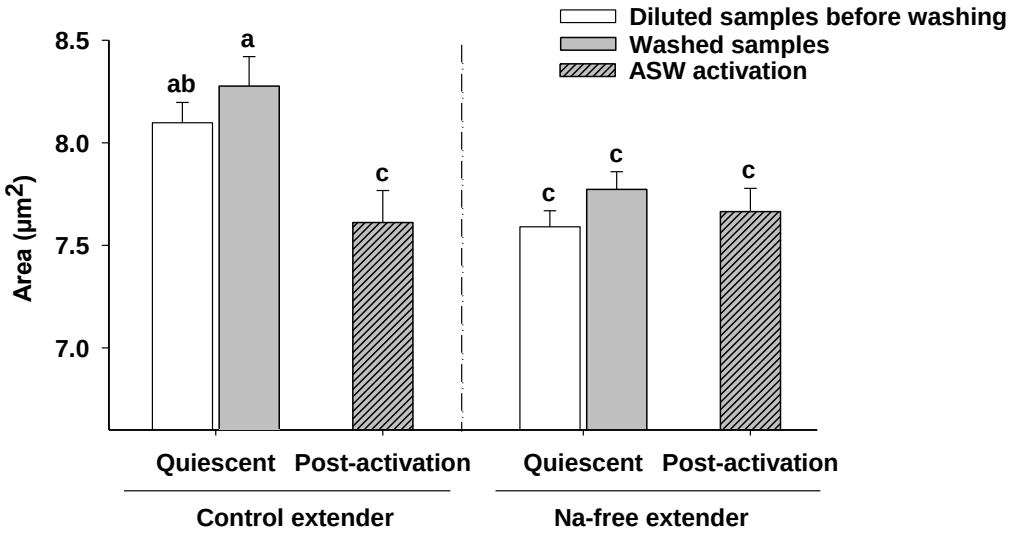

Figure 4. Spermatozoa head area of control and Na free samples (with or without extracellular Na^+ respectively), in different conditions: diluted samples before washing (white bars), washed samples (grey bars) and after ASW activation (stripped bars). Data are expressed at the mean ± SEM (n=9). Different letters mean significant differences ($p<0.05$) between the different stages.

3.5. Effect of ionophore monensin on sperm motility and $[Na^+]_i$

As seen in the previous experiments, the elimination of extracellular Na^+ by washing induced a marked reduction in sperm motility, irrespective of the activation medium (Figure 5A).

Moreover, the incubation with the ionophore monensin resulted in a significant decrease in sperm motility in the control samples (washed with sodium) after activation without sodium (in a Na-free activation media) compared to the control activation (with sodium, in ASW).

Regarding the fluorescence emitted by $[Na^+]_i$ (Figure 5B), all the samples (control or Na-free) showed a significant increase in $[Na^+]_i$ fluorescence after ASW sperm activation, as in Experiment 1 (see section 3.2.). Treatment with monensin caused higher increases in $[Na^+]_i$ after activation in ASW ($p<0.01$). No differences in the fluorescence emitted by $[Na^+]_i$ with/without monensin were observed inside each group (whether washed in the control or the Na-free extender). As per the previous experiment, the response of $[Na^+]_i$ to treatments was similar whether the samples had been washed in control or in the Na-free extender (Figure 5B), whereas sperm motility was very low in the Na-free extender group (Figure 5A).

3.6. Effect of the inhibitor amiloride on sperm motility and $[Na^+]_i$

Incubation with amiloride resulted in a significant reduction in sperm motility (Figure 6A) both after activation with ASW (reduction of 25.4%) and after activation with the Na-free activator (reduction of 45.8%).

All the samples showed a significant increase in $[Na^+]_i$ levels after activation in ASW compared to the samples in the quiescent stage. There were however no significant differences between the samples treated with and those treated without amiloride (Figure

6B).

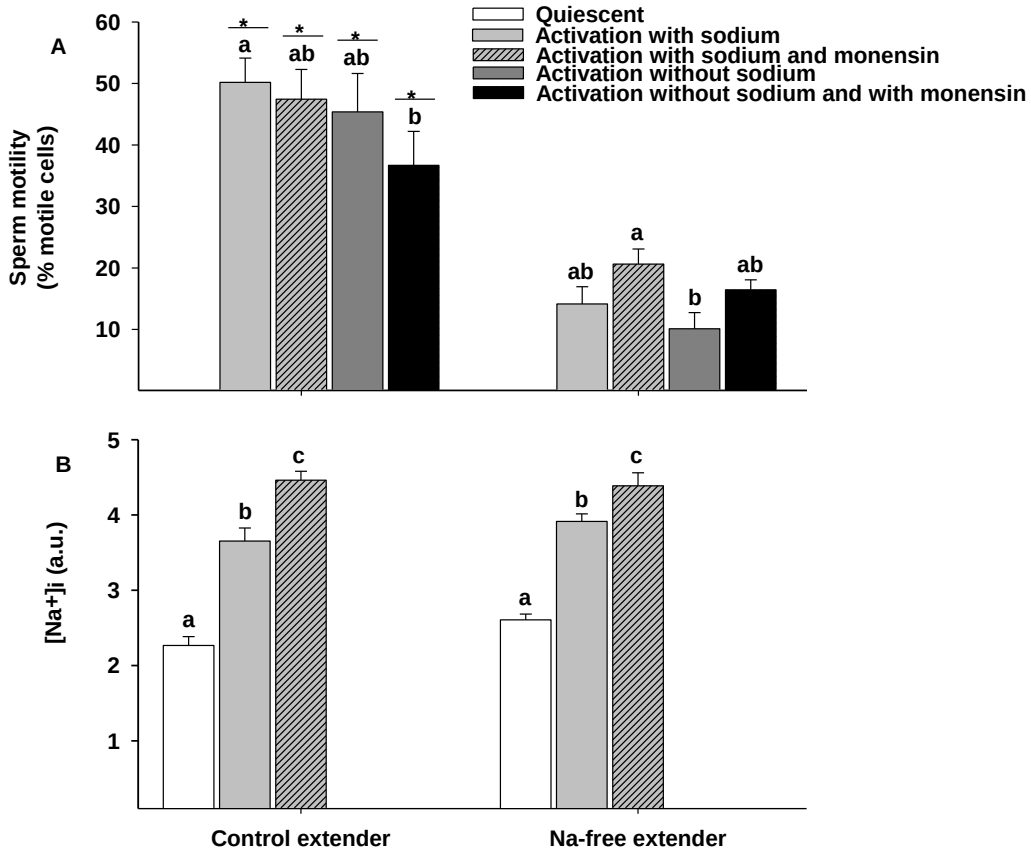

Figure 5. A) Percentage of motile spermatozoa after washing in control or Na-free extender and activated with or without sodium after incubation with or without monensin. B) Emitted fluorescence by intracellular Na^+ (a.u.: arbitrary fluorescence units), in quiescent sperm after washing in control extender or Na-free extender, and activated with orwithout sodium after incubation with or without monensin. Data are expressed as mean ± SEM (n= 10). Different letters indicate significant differences (P<0.05) between treatments (for samples washed in the same extender). Asterisks indicate significant differences (P<0.05) between extenders (for samples in the same conditions of quiescent or post-activation stage).

4. Discussion

4.1. Role of the ion Na^+ in standard conditions

A direct quantitative analysis has been used for the first time to measure the $[Na^+]_i$ concentrations in fish sperm cells by flow cytometry. The Na^+ dye used in this study was CoroNa Green AM. In sea urchin (*S. purpuratus*, Rodríguez and Darszon, 2003), the $[Na^+]_i$ from the sperm cells was directly quantified by measuring the fluorescence from $[Na^+]_i$, labelled with SBFI-AM, by spectrofluorometry. The only other preexisting study of $[Na^+]_i$ in fish sperm (Krasznai et al., 2003b), determined $[Na^+]_i$ concentrations by flow cytometry, but by measuring the fluorescence emitted by intracellular pH (pH_i; Balkay et al., 1997) and then calculating the $[Na^+]_i$ levels using the following equation: $[H^+]_i/[H^+]_e = [Na^+]_i/[Na^+]_e$.

The methodology that we have used in this experiment combines the direct method used by Rodríguez and Darszon (2003) to measure $[Na^+]_i$, with the flow cytometry method used by

Krasznai et al. (2003b). Therefore, the quantification obtained is more accurate than that of previous studies, for two reasons; a) flow cytometry is more sensitive than the measurements obtained by spectrofluorometry (Kalbácová et al., 2003) and b) the fluorescence measured is emitted directly from the intracellular ion sodium and not indirectly through pH_i.

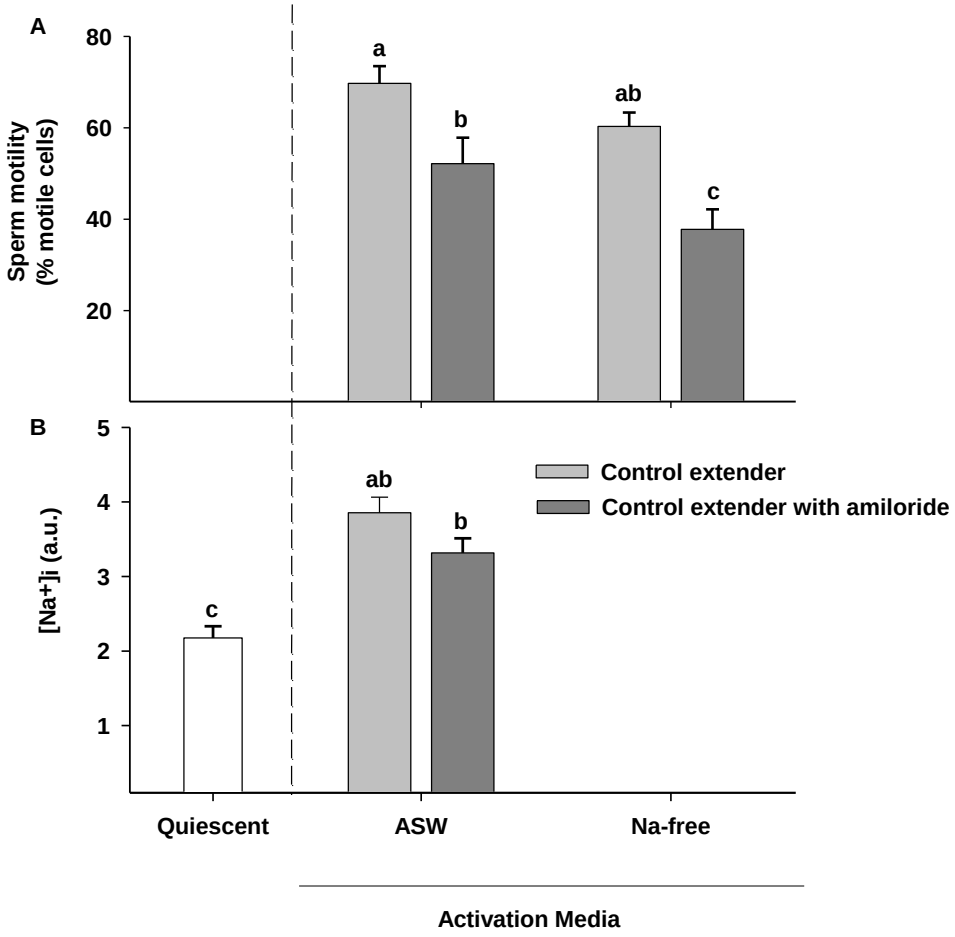

Figure 6. Variations in A) Total sperm motility and B) $[Na^+]i$ before and after sperm activation in ASW or Na-free activator in control washed samples incubated with or without amiloride (2 mM, 4 ºC, 20 min). a.u.: arbitrary fluorescence units. Data are expressed as mean ± SEM (n= 10) and different letters indicate significant differences ($P<0.05$) between activation samples.

The $[Na^+]_i$ concentrations measured from samples in the quiescent stage (96.72 mM) is very similar to the $[Na^+]$ levels found in the seminal plasma of the European eel (109 mM) reported by Asturiano et al. (2004), indicating that, in the quiescent stage, there is a Na^+ equilibrium outside/inside the spermatozoa. In other words, a Na^+ gradient across the sperm membrane does not exist in quiescent sperm. The concentration of Na^+ in the control extender, commonly used by our group to dilute eel sperm, is 145 mM (Peñaranda et al., 2009; Table 1), 1.5-fold higher than $[Na^+]_i$, and therefore, it may be too high. This media could perhaps be improved by reducing the $[Na^+]$ to 98-109 mM, in order to maintain the equilibrium between the intracellular and extracellular sodium.

When the cells were washed in control extender (containing 145 mM Na^+) the $[Na^+]_i$ levels were measured at 96.7 mM, suggesting that the sperm cells could maintain $[Na^+]_i$ at lower

levels than Na^+ from external media. In any case, it has been demonstrated that the control extender (Peñaranda et al., 2009) is able to keep sperm motility intact for up to 24 h at 4º C (Peñaranda et al., 2010a).

The intracellular $[Na^+]_i$ concentrations (96.72 mM) of the quiescent European eel spermatozoa is slightly higher than those found in carp sperm (78 mM; Krasznai et al., 2003b) and much higher than the $[Na^+]_i$ levels of sea urchin sperm (20 mM; Rodriguez and Darszon, 2003). In this paper, a marked increase in $[Na^+]_i$ levels was observed post-activation (Figs. 1B and 4B). This is in correlation with previous results from sea urchin (Rodriguez et al., 2003), but in contrast to what was observed in a freshwater fish species, the common carp (Krasznai et al., 2003b). Thus, it seems that during sperm motility activation, the flux of the ion sodium in the spermatozoa is different in freshwater and marine species.

The increase in $[Na^+]_i$ levels that we have observed after activation could be due to: a) a reduction in sperm cell volume after activation, b) an influx of Na^+ from the external media, or c) a combination of both processes. The first hypothesis has been corroborated by the results obtained in this study (Figure 4), as the spermatozoa head area decreased after hyperosmotic activation. An influx of Na^+ from the external medium through Na^+ channels has been also demonstrated, by the fact that when the sperm is activated in the ASW activator (with sodium), $[Na^+]_i$ levels increase, but this increase does not happen when the sperm is activated in a Na-free activator. This Na^+ influx may be at least partially due to the influx through Na^+/Ca^{2+} channels, which we recently demonstrated in another study of the sperm of this species (Pérez et al., 2015). Therefore, a combination of an influx of Na^+ with a decrease in cell size may be what is responsible for the $[Na^+]_i$ increase at activation.

It can be concluded from this study that the presence of the ion Na^+ in the activation media is not essential to sperm motility, as the total sperm motility is similar whether the sperm is activated in seawater or in a Na-free activator (Figure 2A). This agrees with the fact that sperm activation can be induced by hypertonic sugar (non-electrolyte) solutions in this species (data not shown) as with many other marine fish species (pufferfish (*T. niphobles*; Morisawa and Suzuki, 1980; Gallego et al., 2013b), halibut (*Hippoglossus hippoglossus*; Billard et al., 1993), European sea bass (*Dicentrarchus labrax*; Dreanno et al., 1999) and cod (*Gadus morhua*; Cosson et al., 2008).

4.2. Effect of removing extracellular Na^+

In this study we have shown for the first time that, in quiescent sperm, the removal of extracellular sodium from the seminal plasma causes a marked reduction in total motility, even up to -90% (Figs. 1A, 4A and 5). This marked reduction happened even when activation was carried out in the presence of sodium (with ASW). Therefore, our results indicate that the $[Na^+]$ present in the seminal plasma is important for preserving further motility in this species. This reduction was not reversible, as the re-incubation with sodium (in a medium with 145 mM of Na^+ (control extender, Table 1) during 1 h at 4 ºC (Figure 3) did not recover the sperm motility of the controls. This reduction in motility was not due to cell death, as the percentage of dead cells measured by flow cytometry was very low (≤7%). Thus, perhaps this “immotile stage” can be reversed by incubation in a media with a higher Na concentration and/or increasing the incubation time. The reasons for the reduction in the sperm motility washed in Na-free extender are unknown. However, the elimination of Na^+

from the seminal plasma produced in itself a marked decrease in the spermatozoa head area. In fact, even simply diluting the sperm in a Na-free extender (before washing) resulted in a significant decrease in the spermatozoa head area. This reduction could be linked to the efflux of water mediated by molecular water channels or aquaporins (AQPs), whose presence in fish sperm was demonstrated by Zilli et al. (2009). Thus, if the spermatozoa head area (and therefore the cell volume) is reduced before sperm activation, then motility cannot be activated. This would suggest a close relationship between sperm volume changes and sperm motility in the European eel, like in some freshwater species, as carp or rainbow trout (Bondarenko et al., 2013).

Although the efflux of water during sperm motility in hyperosmotic media has been previously hypothesized (Cosson, 2008), this is the first time that such sperm size reduction has been demonstrated in a marine fish species. Dreanno et al. (1999) reported the opposite in a marine fish species, the seabass; it was observed that the sperm head swollen after hyperosmotic activation. Such differences could be related to species-specific differences in the activation process, like those observed between salmonids and cyprinids. For instance, in some freshwater fish species, like carp, brook trout (*Salvelinus fontinalis*), and rainbow trout, an increase in cell volume is observed after sperm activation in hypoosmotic media (Bondarenko et al., 2013; Takei et al., 2015), while in others, as the sterlet (*Acipenser ruthenus*) the sperm volume does not change.

4.3. Effect of the Na-ionophore monensin

Monensin is a sodium ionophore which transports the ion across the sperm membrane. It was observed that monensin increased the influx of Na^+ after sperm activation in ASW, but that fact was not related to sperm motility (Figure 5A and B).

Monensin only reduced sperm motility when sperm was activated in a Na-free activation medium. This partial reduction could have been caused by an efflux of intracellular Na^+ at the moment of activation, forced by the ionophore in the absence of external Na^+. This is supported by the fact that, when sperm is activated in the absence of Na, there is a decrease in $[Na^+]_i$. In this case, the reduction in $[Na^+]_i$ would be higher due to the presence of the ionophore. This hypothesis should be tested by measuring $[Na^+]_i$ levels after Na-free activation. On other hand, monensin did not change the sperm motility of the Na-free samples

4.4. Effect of Na-inhibitor amiloride

Amiloride is an epithelial sodium channel blocker. In the present study treating the samples with a high concentration of amiloride (2 mM) resulted in a moderate decrease in sperm motility, thus indicating that a sodium channel is also involved in eel sperm motility activation. This agrees with a previous study of Atlantic croaker, where the sperm motility was also reduced when treated with 2 mM of amiloride (Detweiler et al., 1998). In contrast, amiloride up to a concentration of 0.2 mM was ineffective in inhibiting motility of carp sperm (Krasznai et al., 1995b). Therefore, the results of the present study suggest that a sodium channel sensitive to amiloride inhibition is involved in European eel sperm motility activation.

5. Conclusions

This work determines for the first time the absolute $[Na^+]_i$ concentration before and after sperm activation in the European eel, which is in equilibrium with $[Na^+]_e$ in the quiescent stage. Our results demonstrate that the presence of Na^+ in the extender medium (and in the seminal plasma) is important for the preservation of sperm motility in the European eel, at least in part by maintaining the right sperm cell volume. It has been proven that extracellular Na^+ is linked to sperm cell volume, which decreases during the normal sperm activation process.

Although an increase in intracellular sodium occurs after sperm activation, this increase is not related to sperm motility. This increase in intracellular Na^+ after activation is caused both by a cell volume decrease and by an influx of external Na^+, and could be a consequence of sperm activation, rather than a trigger for sperm motility. The presence of an amiloride-sensitive sodium channel seems to be involved in European eel sperm-motility activation.

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CHAPTER 3

Role of calcium on the initiation of sperm motility in the European eel

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Abstract

Sperm from European eel males treated with hCG_{rec} was washed in a calcium free extender, and sperm motility was activated both in the presence (seawater, SW) and in the absence of calcium (NaCl+EDTA), and treated with calcium inhibitors or modulators. The sperm motility parameters were evaluated by a computer-assisted sperm analysis (CASA) system, and changes in the $[Ca^{2+}]_i$ fluorescence (and in $[Na^+]_i$ in some cases) were evaluated by flow cytometry.

After sperm motility was activated in a medium containing Ca^{2+} (seawater, SW) the intracellular fluorescence emitted by Ca^{2+} increased 4-6-fold compared to the levels in quiescent sperm. However, whilst sperm activation in a Ca-free media (NaCl+EDTA) resulted in a percentage of motility similar to seawater, the $[Ca^{2+}]_i$ levels did not increase at all. This result strongly suggests that increasing $[Ca^{2+}]_i$ is not a pre-requisite for the induction of sperm motility in European eel sperm. Several sperm velocities (VCL, VSL, VAP) decreased when sperm was activated in the Ca-free activator, thus supporting the theory that Ca^{2+} has a modulatory effect on sperm motility. The results indicate that a calcium/sodium exchanger (NCX) which is inhibited by bepridil and a calcium calmodulin kinase (inhibited by W-7), are involved in the sperm motility of the European eel. Our results indicate that the increase in $[Ca^{2+}]_i$ concentrations during sperm activation is due to an influx from the external medium, but, unlike in most other species, it does not appear to be necessary for the activation of motility in European eel sperm.

1. Introduction

In teleost, spermatozoa are immotile in the testis and sperm duct, and in general they become motile when released into the surrounding water. Hyperosmotic sea water induces sperm motility in marine fish sperm, while hypo-osmotic freshwater induces sperm motility in freshwater fish species. Apart from the hyper- or hypo-osmotic shock, there are other factors involved in sperm motility acquisition, including the ion Ca^{2+} (Morisawa. 2008). Studies on the effect of this ion on sperm motility, including measurements of $[Ca^{2+}]_i$ levels, have primarily focused on freshwater fish species, such as rainbow trout (*Onchorynchus mykiss;* Cosson et al. 1989, Boitano and Omoto, 1992; Tanimoto et al., 1994; Takei et al., 2012) carp (*Cyprinus carpio*; Krasznai et al., 2000, 2003b) and tilapia (*Oreochromis mossambicus*, Morita et al., 2003). Studies of sperm $[Ca^{2+}]_i$ in marine fish species are even more scarce, restricted to pufferfish (*Takifugu niphobles*; Oda and Morisawa, 1992; Gallego et al., 2013b) and pacific herring (*Clupea pallasi*; Cherr et al., 2008), the latter of which is an unusual case, as sperm activation is triggered by two egg molecules, one of which induces an influx of Ca^{2+} into the sperm cell. Recently it was demonstrated that, similarly to these marine species, European eel (*Anguilla anguilla*) sperm experienced an increase in $[Ca^{2+}]_i$ during sperm activation in seawater (Gallego et al., 2014). However, it is not known if this increase in $[Ca^{2+}]_i$ is the trigger for sperm motility in this species.

Cosson et al. (1989) demonstrated that there is an increase in $[Ca^{2+}]_i$ in rainbow trout sperm when sperm cells start to move, whereas in conditions where the spermatozoa are immotile, for example after being washed in a Ca-free extender and activating in a Ca-free activator, the $[Ca^{2+}]_i$ levels did not increase. In other studies, an increase in $[Ca^{2+}]_i$ post-activation has been observed even in the absence of external Ca^{2+}, indicating that the increase in $[Ca^{2+}]_i$ is due to it being released from intracellular stores (rainbow trout, Boitano and Omoto, 1992;

puffer fish, Oda and Morisawa, 1993; tilapia, Morita et al., 2003). In rainbow trout and carp sperm (Cosson et al., 1989; Krasznai et al., 2000) the increase in $[Ca^{2+}]_i$ required an influx from the external medium, as sperm cells were immotile in the Ca-free activator. In some cases, Ca-free extenders or activators had not been used, like in the study carried out by Tanimoto et al. (1994) on salmonids. In this case, the external or internal origin of the increase in $[Ca^{2+}]_i$ could not be discovered.

Indirect evidence of the importance of Ca^{2+} fluxes on fish sperm motility comes from studies with calcium channel inhibitors. In some marine species inhibitors of voltage-gated calcium channels reduced or suppressed sperm motility (Atlantic croaker *Micropogonias undulatus*, Detweiler and Thomas 1998; Pacific herring, *Clupea pallasi*, Vines et al., 2002). In addition, inhibitors of voltage-gated calcium channels inhibited sperm motility in other freshwater species, including the bluegill (*Lepomis macrochirus*; Zuccarelli and Ingermann, 2007) and sterlet (*Acipenser ruthenus*; Alavi et al., 2011), and reduced sperm curvilinear velocity (VCL) in redside dace (*Clinostomus elongatus*; Butts et al., 2013).

Calcium has been linked to the flagellar beating pattern, inducing asymmetric beating, or circular motility, in several freshwater fish species, including rainbow trout (Cosson et al., 1989; Boitano and Omoto, 1992), and sterlet (Alavi et al., 2008); and in marine fish species, such as European sea bass (*Dicentrarchus labrax*; Cosson et al., 2008) and hake (*Merluccius merluccius*; Cosson et al., 2010). At the same time, sperm motility activation in a Ca^{2+}-free medium reduced VCL in gilthead seabream (*Sparus aurata)* and stripped seabream (*Lithognathus mormyrus*; Zilli et al., 2008). Likewise, in marine invertebrates, such as sea urchins or ascidians (Brokaw et al., 1974; Shiba et al., 2006) the degree of flagellar beating asymmetry is linked to Ca^{2+} concentrations, and in mammals, hyperactivated sperm motility, characterized by high amplitude and asymmetrical flagellar waveform, is Ca^{2+}-dependent. In mammals, sperm activation occurs in two stages: firstly, straight motility (*activated* stage) occurs during ejaculation, and later, *hyperactive,* more circular motility occurs as part of the capacitation process, in the female tract. Both *active* and *hyperactive* motility are calcium-dependent (Wade et al., 2003; Darszon et al., 2011), with hyperactive motility being mediated by an influx of Ca^{2+} through a sperm specific calcium channel (CatSper) which is pH-dependent (Carlson et al., 2003).

In the present study the European eel was used as the experimental organism. They could be considered a marine species, as their spawning grounds are in the sea, presumably in the Sargasso Sea (Tesch, 1977; Van Ginneken and Maes, 2005). This species has a particular life cycle and this, coupled with its phylogenetic position as an ancient teleost, makes it an interesting model for the investigation of the regulatory mechanisms of reproductive physiology, and for providing insights into ancestral regulatory functions in teleost. Eel species do not mature spontaneously in captivity, but spermatogenesis and spermiation can be obtained in males after long-term treatment with human chorionic gonadotropin (hCG) (Pérez et al., 2000; Peñaranda et al., 2010a). Our research group recently demonstrated (Gallego et al., 2012a) that treatment with recombinant hCG (hCG_{rec}) gave better results in terms of milt quality and production levels than the traditional urine-purified hCG. Using this treatment it is possible to obtain good sperm quality (motility >60%) for at least 6 weeks from the 8^{th} week of hormonal treatment, if the eels are injected weekly. This feature makes it a useful model for the study of sperm physiology.

In this paper, the role of calcium ions on European eel sperm motility has been studied by testing the effect of several calcium channel inhibitors or modulators on sperm motility and kinetics. Flow cytometry has been used to measure variations in $[Ca^{2+}]_i$ levels in different conditions (with or without external calcium).

2. Material and methods

2.1. Chemicals and solutions

Bepridil hydrochloride, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), A-23187, EDTA, and Bovine Serum Albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Fluo-4 AM, CoroNa Green AM, Pluronic F-127, and propidium iodide (PI) were purchased from Life Technologies (Madrid, Spain). Salts were of reagent grade.

DMSO stocks 100 mM bepridil, 100 mM W-7, 10 mM A-23187 were prepared, diluted in ultrapure water at a ratio of 1:10 (v/v). Each product was aliquoted and frozen (-20 ºC). Each aliquot was used only once after being thawed. For use with the sperm, each product was thawed, and mixed with the sperm to final concentrations of 100 µM (bepridil, W-7) or 10 µM (A-23187). DMSO stocks 1 mM Fluo-4 AM, 1 mM CoroNa Green AM, were prepared and used as described in sections 2.7 and 2.8.

2.2. Preparation of Ca-free solutions

Two Ca-free solutions were prepared: A Ca-free extender (125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2*6H_2O$, 30 mM KCl, 5 mM EDTA, 20 mM TAPS, pH adjusted to 8.5), and a Ca-free activation media (550 mM NaCl, 5 mM EDTA, 20 mM TAPS, pH adjusted to 8.2).

To avoid Ca^{2+} contamination of these solutions the glass materials were autoclaved, and then rinsed in a solution of ultrapure milliq water plus 5 mM EDTA (Yoshida et al., pers. com.). The rest of the laboratory materials were also cleaned and rinsed in ultrapure milliq water plus 5 mM EDTA. The osmolality of these solutions was checked with an Osmomat050 (Gonotec, Germany), being 325±10 and 1100±20 mOsm, for Ca-free extender and Ca-free activation media respectively.

2.3. Fish and hormone treatment

Eighty adult male European eels 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 four 200-L aquaria (approximately 20 male eels per aquarium) equipped with separated recirculation systems, thermostats, and coolers, and covered with black panels 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 being administered with hCGrec (Ovitrelle®, Merck Serono; 1.5 IU per g of fish body weight) by intraperitoneal injection (as described by Gallego et al., 2012a).

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). In addition, this project received the approval of the Ethics Committee of the Polytechnic University of Valencia (Spain).

2.4. Sperm collection and sampling

The sperm samples were collected 24 hours after the administration of hCG because previous studies (Pérez et al., 2000) have demonstrated that this is the moment when the best sperm quality is found. Before sperm collection, the fish were anesthetized, and the genital area was cleaned with freshwater, and carefully dried to avoid contamination with faeces, urine, or sea water. The sperm was then collected in plastic tubes, by exerting abdominal massage, and refrigerated (4 ºC) until the motility analyses, which took place within the first hour after collection.

2.5. Sperm motility evaluation

Sperm motility activation was carried out as per the method described by Gallego et al. (2013a); by mixing 1 µl of diluted sperm (dilution 1/25 in Ca-free extender; 125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$*$6H_20$, 30 mM KCl, 5 mM EDTA; based on P1 extender, Peñaranda et al., 2009) with 4 µl of artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was made in a SpermTrack-10® chamber, 10 µm depth (Proiser, Paterna, Spain) and observed using a Nikon Eclipse 80i microscope, with a 10x objective lens (Nikon phase contrast 10x 0.25, Ph1 BM WD 7.0). Motility was recorded 15 seconds after the sperm was mixed with SW, using a high-sensitivity video camera (HAS-220) and ISAS software (Proiser, Paterna, Spain). For each motility test, samples were evaluated in triplicate. Both the sperm and the SW were maintained at 4 ºC in a water bath during the sperm motility evaluation. In some cases sperm motility was activated with a Ca-free activator (550 mM NaCl, 5 mM EDTA), but SW activation was always used as a control.

The sperm motility parameters considered in this study were: total motility (MOT, %); progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast (FA; average path velocity [VAP] >100 µm/s); 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; 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; WOB, wobble (velocity according to the smoothed path (VAP/VCL); ALH, defined as the amplitude of the lateral movement of the spermatozoa head; and cross beating 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.

For the CASA motility analyses in Ca-free activation media (550 mM NaCl, 5 mM EDTA), on the day of the test, 2% BSA (w/v) was added to the Ca-free activator, and the pH was later adjusted to 8.2. The counting chamber used (SpermTrack-10® chamber) was cleaned with milliq water and 5 mM EDTA before each analysis to avoid Ca contamination. For the flow cytometry analyses the pH of the Ca-free activation media was also adjusted to 8.2 on the

day of the analyses, but BSA was not added to the solution to avoid spermatozoa aggregation during the analyses. Sperm samples with >50% of motile cells (>40% in Trial 1) were selected for study.

2.6. Sperm washing protocol

A washing protocol was established for the European eel sperm (described in Supplementary Material). Sperm samples were washed in a Ca-free extender (Ca-free extender; 125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$ *$6H_20$, 30 mM KCl, 5 mM EDTA; 325 ±10 mOsm, pH=8.5, based on P1 extender (Peñaranda et al., 2010), at 4 ºC for 5 minutes, by centrifugation at 500 g, and washing was repeated three times. Later, the sperm pellet was re-suspended in a Ca-free extender and maintained at 4 ºC until analysis. Washing in the Ca-free extender (550 mM NaCl + 5 mM EDTA) does not affected sperm motility and kinetics in comparison to the control extender (P1 extender; Figure S1 Supplementary Material).

2.7. Intracellular Ca^{2+} measurement

Fresh sperm samples were first diluted and washed (1:25) three times in a Ca-free extender, as described in section 2.6. The relative amounts of the different ions were determined by flow cytometry using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Brea, CA). In order to determine the levels of $[Ca^{2+}]_i$ present, the spermatozoa were loaded with Fluo-4 AM indicator (Invitrogen) to a final concentration of 5 µM, adding of the non-ionic detergent Pluronic® F-127 (Invitrogen) to a final concentration of 0.02% (w/v). The sperm cells were also incubated with/in IP 2 µM, a nucleic acid stain used as cell dead indicator, in order to exclude dead cells from the analysis. Sperm incubation was carried out at room temperature (20 ºC) for 30 minutes.

$[Ca^{2+}]_i$ levels in the sperm were measured during the quiescent stage (after washing in a Ca-free extender, and diluting in an extender), and 30 seconds after hyperosmotic activation in SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA).

Fluo-4 AM and IP were both excited by the blue laser (488 nm), and their fluorescence was read using the FL1 (530/40BP filter) and FL3 (665/20BP filter) photodetector, respectively. The fluorescence data was displayed in logarithmic mode. Ten thousand events per sample were collected, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis. The flow cytometry data was processed using WEASEL software (v. 3.1, Walter and Eliza Hall Institute).

2.8. Intracellular Na^{2+} measurement

To determine the levels of $[Na^{2+}]_i$ present, the spermatozoa were loaded with CoroNa Green indicator (Invitrogen) up to a final concentration of 10 µM, adding of the non-ionic detergent Pluronic® F-127 (Invitrogen) making a final concentration of 0.02% (w/v). The sperm cells were also incubated with 2 µM propidium iodide (IP); a nucleic acid stain used as a dead cell indicator, in order to exclude any dead cells from the analysis. Sperm incubation was carried out at room temperature (20 ºC) for 30 minutes.

$[Na^{2+}]_i$ levels in the sperm were measured during the quiescent stage (after washing in a Ca-free extender, and diluting in a Ca-free extender), and 30 seconds after hyperosmotic activation in SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA).

CoroNa Green Fluo-4 AM and IP were both excited by the blue laser (488 nm), and their fluorescence was read using the FL1 (530/40BP filter) and FL3 (665/20BP filter) photodetector, respectively. The fluorescence data was displayed in logarithmic mode. Ten thousand events per sample were collected, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis. The flow cytometry data was processed using WEASEL software (v3.1, Walter and Eliza Hall Institute).

2.9. The relationship between intracellular [Ca2+] changes and sperm motility under different conditions

2.9.1. Trial 1. Sperm motility and intracellular [Ca^{2+}] with/without bepridil

Five sperm samples (one sample/male) each with a total motility of over 40% were selected The same samples were used for the motility analyses and for the $[Ca^{2+}]_i$ measurements, both of which were performed on the same day. All the samples were first diluted 1:25 (v/v) and washed in a Ca-free extender as described in Section 2.6. Intracellular Ca^{2+} levels were measured under different conditions:

- Diluted and washed in a Ca-free extender: quiescent stage
- After SW activation or Ca-free activation (550 mM NaCl, 5 mM EDTA): used as controls
- After pre-incubation with bepridil and activation with SW or a Ca-free activator

Incubation with bepridil was done by mixing the sperm with this product up to final concentrations of 100 µM in a Ca-free extender, and incubated for 30 minutes at 4 ºC. Samples without bepridil (controls) were treated with DMSO of the same concentration (0.1%).

2.9.2. Trial 2. Sperm motility and intracellular [Ca^{2+}] with/without bepridil or A-23187

Ten sperm samples (one sample/male) were selected, with total motility over 85%. All the samples were first diluted and washed in Ca-free extender as described above. Intracellular $[Ca^{2+}]$ was measured in different conditions:

- Diluted and washed in Ca-free extender: quiescent stage
- After SW activation or Ca-free activation (550 mM NaCl, 5 mM EDTA): used as controls
- After pre-incubation with bepridil (100 µM) or A-23187, (10 µM) and activation with SW or Ca-free activator

Incubation with bepridil or A-23187 was done by mixing the sperm with these products up to final concentrations of 100 µM and 10 µM, respectively, and incubating for 30 minutes at 4 ºC. The control samples were treated with DMSO of the same concentration (0.1 % v:v). In this case, sperm motility was measured using a different batch of samples to those used for the $[Ca^{2+}]$ experiment, albeit obtained from the same animals.

2.9.3. Trial 3. Sperm motility and intracellular [Ca^{2+}] with/without W-7

Ten sperm samples (one sample/male) with a total motility of over 85% were first washed in a Ca-free extender (1/25 v:v) to remove calcium from the seminal plasma. The samples were then treated with W-7 at a final concentration of 100 µM, or with 1/1000 (v:v) DMSO for the control. The non-chlorinated, ineffective form of W-7, W-5, which has been used as the control in several studies, was not used, as it has been found to be ineffective in blocking sperm motility in other fish species, including carp, pufferfish, tilapia and rainbow trout (Krasznai et al., 2000, 2003a; Koh et al., 2004; Morita et al., 2006).

The same samples were used for the motility analyses and for the $[Ca^{2+}]_i$ measurements, both of which were performed on the same day. Intracellular $[Ca^{2+}]$ levels and sperm motility were measured under the following conditions:

- Diluted and washed in a Ca-free extender: quiescent stage
- After activation with SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA) used as controls
- After pre-incubation with W-7, and activation with SW or a Ca-free activator.

Incubation with W-7 was done by mixing the sperm with these products up to final concentrations of 100 µM, and incubating for 30 minutes at 4 ºC.

2.9.4. Trial 4. The effect of ionic Ca-free activation media on sperm motility

An experiment (n=10 males) was performed to determine the effect of Ca-free activation media on sperm motility parameters. Ten sperm samples (one sample/male) showing >50% motility were washed in a Ca-free extender and then activated in SW or a Ca-free activator. CASA motility was then registered as described in Section 2.5.

Data from this experiment was analyzed together with data from Trial 3 (as there was not a significant effect of the trial), to analyze the differences in the sperm motility parameters in relation to the presence/absence of calcium in the extracellular medium.

2.9.5. Statistics

WEASEL software (v3.1, Walter and Eliza Hall Institute, 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) of each sample was obtained. Statistical analyses were performed using the statistical package Statgraphics Centurion software (Statistical Graphics Corp., Rockville, MO, USA). Kurtosis and Assimetry Standard coefficients were used to check the normality of data distribution. The variables that did not have a normal distribution were log-transformed and their normality was checked again. A two-way ANOVA was then performed to discover whether each variable was affected by the activation media and/or inhibitor. One-way ANOVA analyses were then performed to check the combined effect of activation media and inhibitor. Variance homogeneity was checked using the Bartlett test. The one-way ANOVA analyses were followed by a Duncan post-hoc test. If normality failed after the log transformation a non-parametric test was carried out (Kruskal-Wallis), followed by a Dunn's test.

3. Results

3.1. Efect of bepridil

Incubation with bepridil resulted in a strong inhibitory effect on sperm motility (Figures 1A, 2A) both after activation in Ca-free (reductions of 71 and 54% in Trials 1 and 2, respectively) or SW activators (reduction of 54 and 64% in Trials 1 and 2, respectively).

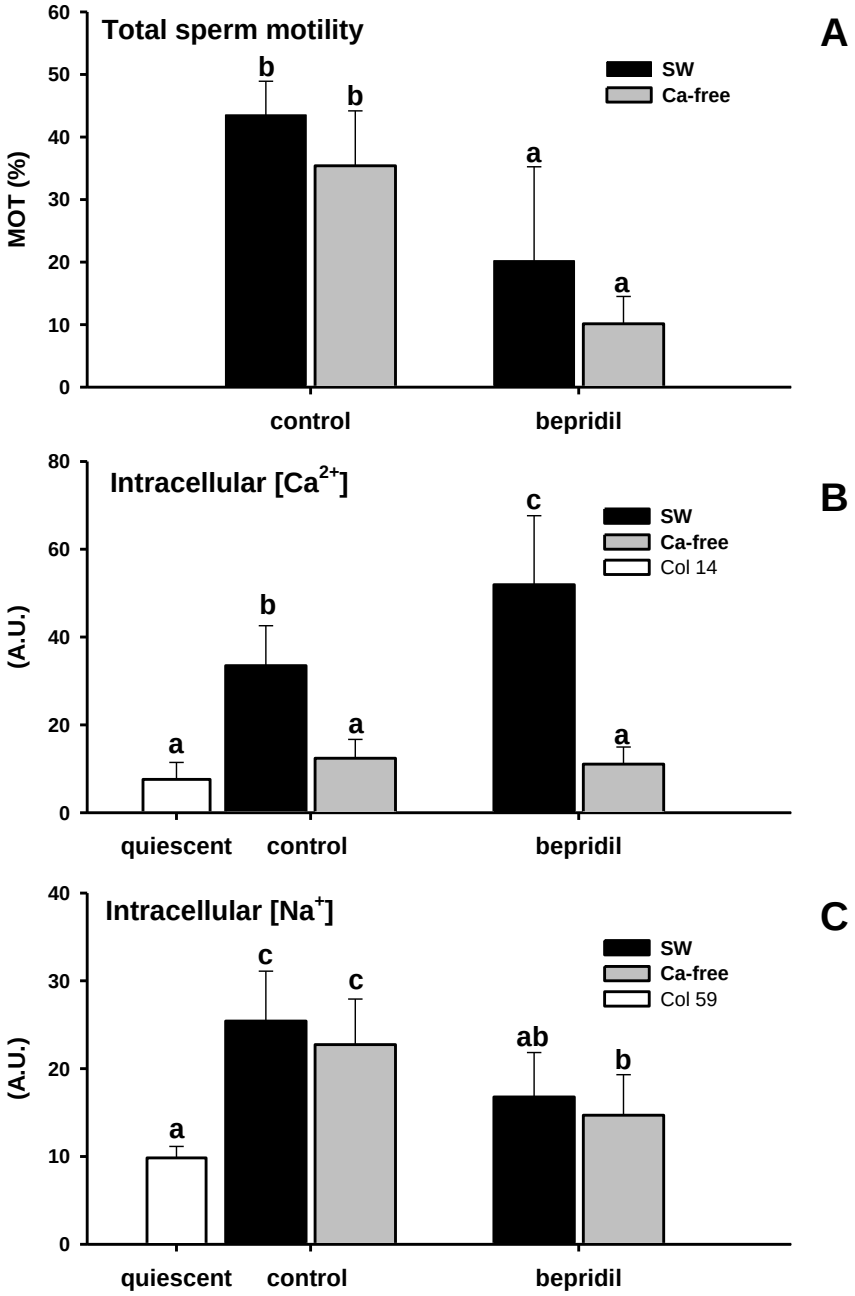

Figure 1. Trial 1.Variations in A) total sperm motility, B) $[Ca^{2+}]_i$, and C) $[Na^{+}]_i$ before and after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 µM bepridil hydrochloride, 30 min, 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. A.U.: arbitrary fluorescence units. Data are expressed as mean ± SEM (n=5). Different letters mean significant differences ($p<0.05$).

Regarding the other sperm parameters (Table 1), in Trial 1 FA (fast spermatozoa), VCL (curvilinear velocity) and ALH (lateral head displacement) were significantly reduced by bepridil in relation to their respective controls. In Trial 2 (Table 2) bepridil not only reduced FA, VCL, and ALH, but also reduced progressive motility (MP) (by 50 and 41% in SW and Ca-free activators, respectively), VSL, and VAP, both after activation in SW and in Ca-free media.

Table 1. Variations in sperm kinetics after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 µM bepridil hydrochloride, 30 min, 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n=5). Different letters mean significant differences ($p<0.05$). Abreviations: MP progressive motility; FA percentage of fast spermatozoa; VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BEP bepridil. p: ANOVA p-value.

	SW control	Ca-free control	SW BEP	Ca-free BEP	p
MP (%)	16.04±3.05	12.13±3.41	9.07±3.41	4.05±3.41	n.s.
FA (%)	22.80±3.13 c	18.40± 3.49 bc	8.97±3.49 ab	3.48±3.49 a	0.005
VCL (µm/s)	110.52±6.38 b	107.5± 6.37 b	92.08± 6.37 ab	86.68± 6.37a	0.026
VSL (µm/s)	56.62±5.17	49.80±5.78	44.53±5.78	41.2±5.78	n.s.
VAP (µm/s)	75.12±5.31	67.73±5.93	59.72±5.93	54.30±5.93	n.s.
ALH (µm)	2.46±0.13 b	2.38±0.14 b	1.80±0.14 a	1.48± 0.14 a	0.001

Samples pre-incubated with bepridil showed a higher increase in $[Ca^{2+}]_i$ levels after SW activation than the controls (Figures 1B, 2B). The effect of bepridil on $[Na^+]_i$ levels was also measured, and it was shown to inhibit ($p<0.01$) the increase in the $[Na^+]_i$ levels observed in the controls after activation with SW and the Ca-free activator (Figures 1C and 2C).

3.2. Effect of ionophore A-23187

Pre-incubation with A-23187 did not affect sperm motility (Figure 2A) neither after activation with SW nor after Ca-free activation. However, A-23187 resulted in a higher increase in $[Ca^{2+}]_i$ levels after SW activation compared to the controls (Figure 2B). However, the $[Ca^{2+}]_i$ levels measured after Ca-free activation were just as low as those found in the controls or quiescent sperm.

In terms of the other sperm motility parameters (Table 2), A-23187 had a positive effect on the percentage of fast spermatozoa (FA) after activation with the Ca-free medium, with values higher ($p<0.001$) than those seen in the controls.

3.3 Effect of W-7

Pre-incubation with W-7 (Figure 3A, Table 3) induced different effects on sperm motility when Ca^{2+} was present (SW activation) or absent (Ca-free activation) in the activation media. When the external media contained Ca^{2+} (SW activation), W-7 induced significant ($p<0.01$)

yet moderate reductions (≤20%) in MOT, VCL, VAP and ALH (reduction of 29%) in relation to the SW controls (Figure 3A, Table 3). However, when Ca^{2+} was absent in the extracellular media (Ca-free activation), W-7 caused important reductions compared to the Ca-free control in the majority of the sperm kinetic parameters: with reductions of 40-55% seen in MOT, MP, FA, 30-40% in velocities (VCL, VSL, VAP) and ALH, and 18% in BFC (Figure 3A, Table 3).

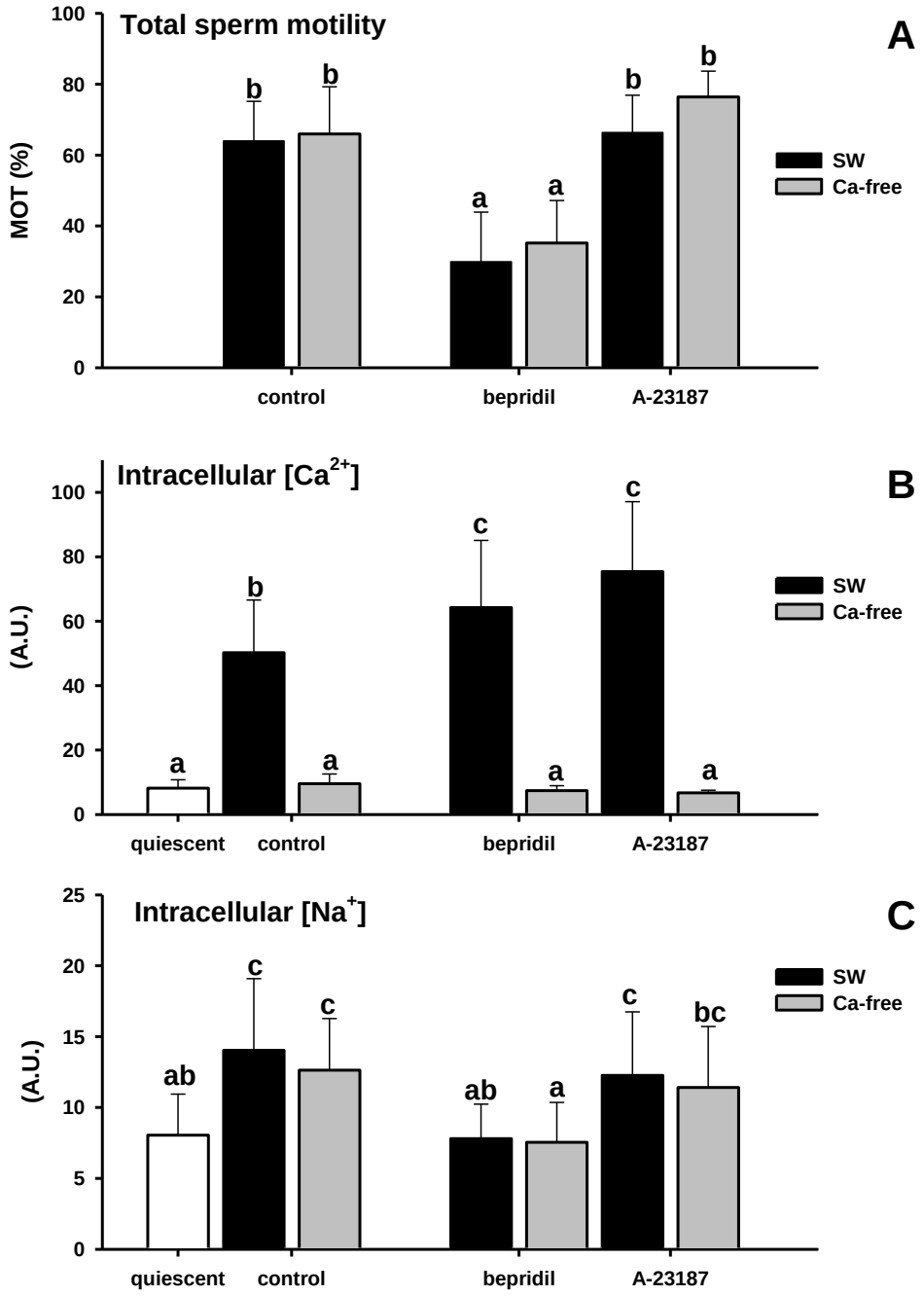

Figure 2. Trial 2. Variations in A) total sperm motility, B) $[Ca^{2+}]_i$, and C) $[Na^+]_i$ before and after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 µM, 30 min., 4 ºC) or calcium ionophore A-23187 (10 µM, 30 min, 4 ºC). Activation

media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. A.U.: arbitrary fluorescence units. Data are expressed as mean ± SEM (n=10). Different letters mean significant differences ($p<0.05$).

Table 2. Trial 2. Variations in sperm kinetics after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 µM, 30 min., 4 ºC) or calcium ionophore A-23187 (10 µM, 30 min., 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n=10). Different letters mean significant differences ($p<0.05$). Abreviations: MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat frequency; BEP bepridil; P: ANOVA p-value.

	SW control	Ca-free control	SW A-23187	Ca-free A-23187	SW BEP	Ca-free BEP	p
MP (%)	29.61± 2.75b	31.31± 2.75b	35.96± 2.75b	35.51 ± 2.75b	13.40± 2.75a	18.40± 2.75a	0.000
FA (%)	49.00±3.51b	51.07±3.51b	54.58±3.51bc	62.13±3.51c	19.06±3.51a	22.65±3.51a	0.000
VCL (µm/s)	152.31±4.83b	150.10±4.83b	161.23±4.8b	163.1±4.83b	108.64±4.83a	113.51±4.83a	0.000
VSL (µm/s)	85.14±4.35b	81.75±4.35b	94.92±4.35b	88.3±4.35b	58.92±4.35a	67.36±4.35a	0.000
VAP (µm/s)	109.28 ±4.21b	106.48±4.21b	117.74±4.21b	115.38±4.21b	76.25±4.21a	83.15±4.21a	0.000
ALH (µm)	2.42±0.07b	2.65±0.07c	2.39±0.07b	2.69±0.07c	1.93±0.07a	1.84±0.07a	0.000
BFC (beats/s)	31.76 ±0.83bc	30.47±0.83ab	33.30±0.83c	31.75±0.83bc	28.52 ±0.83a	30.41±0.83ab	0.004

Table 3. Trial 3.Variations in sperm kinetics after sperm activation in SW (seawater activator) or Ca-free activator (550 mM NaCl, 5mM EDTA) in samples incubated with W-7 (100 µM, 30 min. 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n=10). Abreviations: MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat frequency; P: ANOVA p-value.

	SW control	Ca-free control	SW W-7	Ca-free W-7	p
MP (%)	24.97±3.32 b	19.72±3.32 ab	26.79±3.32 b	10.69±3.32 a	0.012
FA (%)	75.09±6.28 b	64.04±6.28 b	57.46±6.28 b	30.04±6.28 a	0.000
VCL (µm/s)	179.73±7.91 c	171.52±7.91 c	147.53±7.91 b	108.31±7.91 a	0.000
VSL (µm/s)	77.24±3.97 b	70.09±3.97 b	70.11±3.97 b	45.84±3.97 a	0.000
VAP (µm/s)	118.1±4.55 c	108.9±4.55 bc	99.2±4.55 b	69.4±4.55 a	0.000
ALH (µm)	3.12±0.11 c	3.15±0.11 c	2.23±0.11 a	2.13±0.11 a	0.000
BFC (beats/s)	30.52 ±1.10 b	29.14±1.10 b	28.98 .±1.10 b	24,27 ±1.10 a	0.001

Regarding $[Ca^{2+}]_i$, when this ion was present in the activation media W-7 induced a higher increase in $[Ca^{2+}]_i$ than that seen in the controls. However, following activation in a Ca-free media the levels of $[Ca^{2+}]_i$ were similar to those found in the quiescent sperm. This was also found to be the case with the control samples activated in Ca-free media (Figure 3B).

3.4 Effect of activation in Ca-free media vs seawater activation on sperm kinetics

In the three trials, (Figures 1B, 2B and 3B) levels of intracellular Ca^{2+} increased after activation in SW, but did not increase when the sperm was activated in Ca-free hyperosmotic media (550 NaCl, 5 mM EDTA). However, the sperm motility (Figures 1A, 2A, 3A) after Ca-free activation was similar to the sperm activated in SW. Regarding other sperm kinetic parameters (Table 4), Ca-free activation resulted in a moderate yet significant reduction in all the velocities: VCL, VSL and VAP ($p<0.05$) as well as BFC ($p<0.05$).

4. Discussion

4.1. Motility can start without a (sustained) increase in intracellular $[Ca^{2+}]$

In this study, three trials have proven that total sperm motility is similar when sperm is activated in seawater to when the activation occurs in a Ca-free activator (containing EDTA). However, the fluorescence (FI) emitted by $[Ca^{2+}]_i$ did not increase when sperm was activated in a Ca-free medium, in any of the cases. The FI emitted by $[Ca^{2+}]_i$ was measured by Flow Cytometry at 30 sec. post-activation, which is the time we have estimated to be necessary in order to obtain the final measurement of FI. Thus, it was not possible with this method to measure any changes in $[Ca^{2+}]_i$ that could've taken place before the first 30 seconds post-activation. It is therefore not known if a transient increase in $[Ca^{2+}]_i$ happens in the first

seconds after activation, as is in the case in rainbow trout (Boitano and Omoto, 1992; Tanimoto et al.. 1994), and puffer fish sperm (Oda and Morisawa, 1993).

Table 4. Effect of Ca-free activation media on sperm motility. Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n = 20). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head, BFC, beat frequency. P: ANOVA p-value.

Activator	SW	Ca-free (NaCl+EDTA)	P-value
MOT (%)	69.33±2.75	66.03±2.75	n.s.
MP (%)	31.02±2.81	23.25±2.81	0.050
FA (%)	53.42±3.53	43.35±3.53	0.051
VCL (µm/s)	151.51±5.20 a	132.53±5.20 b	0.016
VSL (µm/s)	82.60±3.93 a	67.41±3.93 b	0.001
VAP (µm/s)	107.61±3.92 a	96.30±3.92 b	0.009
ALH (µm)	2.52±0.06	2.65±0.06	n.s.
BFC (beats/s)	30.98±0.88 a	28.17±0.88 b	0.018

However, a transient increase in $[Ca^{2+}]_i$ post-activation has not been observed in other studies, where the $[Ca^{2+}]_i$ increase was sustained with no decrease reported over time. In trout sperm (Cosson et al. 1989), the rapid increase in $[Ca^{2+}]_i$ fluorescence post-activation was followed by "a *plateau of the fluorescence level that lasted for several minutes*". Also in trout sperm, Cosson et al. (1990) observed an increase in $[Ca^{2+}]_i$ levels, which reached a peak approximately 45 s after sperm activation. In carp sperm, Krasznai et al. (2000) used both spectrofluorometry and flow cytometry to measure $[Ca^{2+}]_i$, and observed an increase in $[Ca^{2+}]_i$ fluorescence within 10-15 s after sperm activation, and this increase was maintained until at least 200 s In other papers, Krasznai et al. (2003b), when studying carp sperm, found that after an increase at activation, $[Ca^{2+}]_i$ levels remained similarly high at 20, 60 and 300 s post-activation. In tilapia sperm, Morita et al. (2003) measured the $[Ca^{2+}]_i$ fluorescence by confocal microscopy after sperm activation, and they found an increase in $[Ca^{2+}]_i$ fluorescence in the activated sperm, even after the cessation of sperm motility, 30 min after sperm activation. In a marine species, the herring, the $[Ca^{2+}]_i$ measured after sperm activation (with either SMIF or HSAP proteins) increased after a few seconds but remained stable until 55 s post-activation (Cherr et al., 2008). Also, our group (Gallego et al., 2013b) measured $[Ca^{2+}]_i$ in fugu sperm by fluorescence spectrophotometry at pre-activation, and at 5, 30 and 60 s post-activation. The observed increase in FI due to $[Ca^{2+}]_i$ remained equally high at 5, 30 and 60 s post activation. All these papers (Cosson et al., 1989; Krasznai et al., 2000; Morita et al., 2003; Gallego et al., 2013b, Cherr et al., 2008) support the validity of our $[Ca^{2+}]_i$ measurements at 30 s post-activation.

Nevertheless the studies on fish sperm that have reported a transient increase in $[Ca^{2+}]_i$ after sperm activation, have observed that after a peak in $[Ca^{2+}]_i$ levels 5-10 s post-activation (Boitano and Omoto, 1992; Oda and Morisawa, 1993, Tanimoto et al., 1994), FI decreased to a level which was still 1.8-2 fold higher than that of quiescent sperm (Boitano and Omoto, 1992; Tanimoto et al., 1994). This is quite different to our results, where the FI levels of Ca-free activated sperm were never higher than those of quiescent sperm. Thus, our results strongly support the theory that a transient increase in $[Ca^{2+}]_i$ does not occur when the motility of the eel sperm is activated in Ca-free conditions.

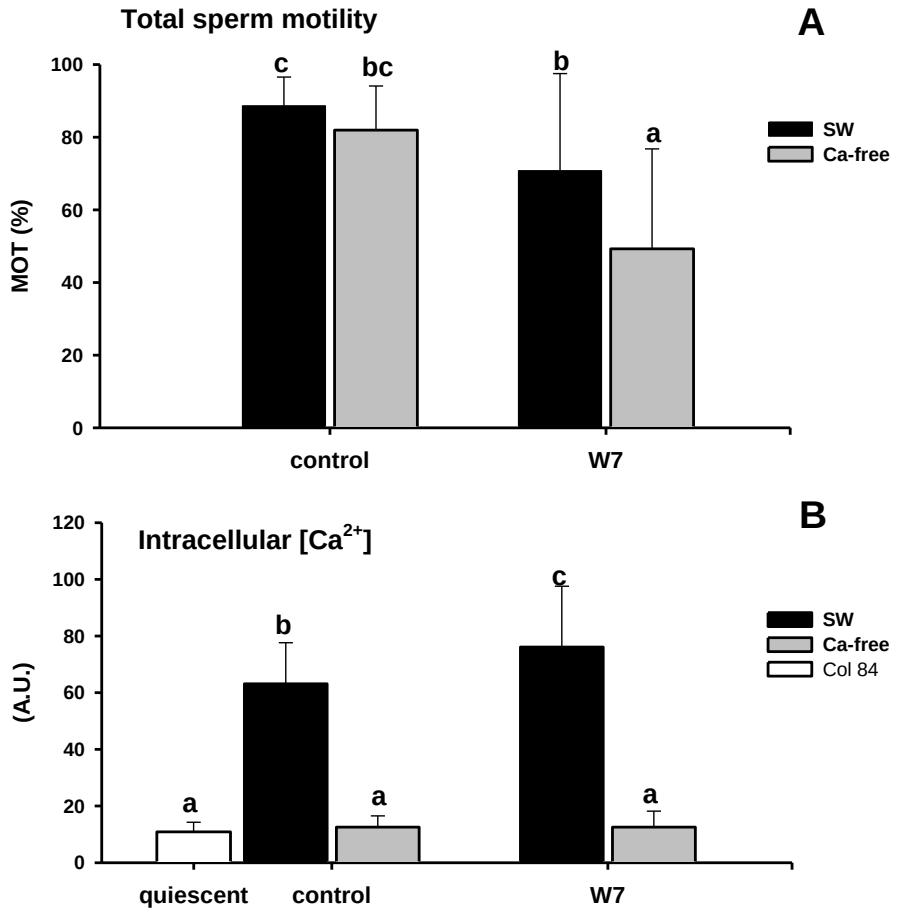

Figure 3. Trial 3.Variations in A) total sperm motility and B) $[Ca^{2+}]_i$, before and after sperm activation in SW (seawater activator) or Ca-free activator (550 mM NaCl, 5mM EDTA) in samples incubated with W-7 (100 µM, 30 min, 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. A.U.: arbitrary units. Data are expressed as mean ± SEM (n=10).

If we discard the possibility of a transient increase in $[Ca^{2+}]_i$ which has completely disappeared at 30 seconds, we can therefore say that the trigger of the initiation of sperm motility in this species is not an increase in $[Ca^{2+}]_i$ This opposes the common belief regarding fish sperm motility acquisition (Zilli et al., 2012; Morisawa, 2008; Cosson et al., 2008). However, this is not the first time that this fact has been observed in fish. A study from our group which was carried out in collaboration with Dr. Yoshida's group, showed that pufferfish (*Takifugu niphobles*) sperm washed and activated in Ca-free conditions (washed with a Ca-free extender: 300 mM glucose, 5 mM EGTA; activated with 1100 mM glucose, 5 mM EDTA) had normal sperm motility, while the $[Ca^{2+}]_i$ did not increase at 5, 30 or 60 seconds. post-

activation (Gallego et al., 2013b). Both that paper, as well as the present study contradict previous research on pufferfish: Oda and Morisawa (1993) found an increase in $[Ca^{2+}]_i$ after sperm activation, even in the absence of Ca^{2+} in the extracellular medium. However, in their study a Ca^{2+} chelator was not used in the activation medium, thus trace amounts of this ion could be masking the results. Both in Gallego et al. (2013b) and in the present study, calcium-free solutions have been carefully prepared, and the materials have been cleaned with ultrapure water containing EDTA to avoid any Ca^{2+} contamination.

Even if the theory that an increase in $[Ca^{2+}]_i$ is not necessary for sperm motility seems new, it has in fact already been suggested in previous studies. In pufferfish sperm permeabilized to Ca^{2+} by ionophore, when the $[Ca^{2+}]_i$ sperm was set to 100 µM in the quiescent stage, and then activated with seawater with the same amount of Ca^{2+}, motility was activated in a normal way (Krasznai et al., 2003a). However, if the sperm was mixed with an isosmotic media containing a higher $[Ca^{2+}]$ concentration than the sperm, motility was not activated. Then, the authors stated that "*the increase in intracellular Ca^{2+} concentration itself had no significant effect on the motility and velocity of puffer fish sperm. These data suggest that changes in environmental osmolality have priority to intracellular Ca^{2+} in the process of initiation of puffer fish sperm motility*".

Thus, the increase in $[Ca^{2+}]_i$ might be not a universal prerequisite for the initiation of sperm motility in fish. This corroborates Takei et al. (2012) who observed that the decrease in $[Ca^{2+}]_i$ in salmonid sperm caused by hypotonic shock (after a first hypertonic shock caused by glycerol) triggered motility initiation.

Even if freshwater fish sperm needs an increase in $[Ca^{2+}]_i$ for spermatozoa motility, as was clearly demonstrated by Krasznai et al. (2000) in carp sperm, or by Cosson et al. (1989) in rainbow trout, it is possible that marine fish (or European eel) do not. Freshwater and marine fish sperm have different characteristics in the activation of sperm movement. For instance, in common carp and salmonids a decrease in $[K^+]_i$ is observed after sperm activation in a hyposmotic medium (Tanimoto et al., 1994; Krasznai et al., 2003b), while in marine species (pufferfish and European eel) an increase in this ion is observed after sperm activation in an hyperosmotic medium (Takai and Morisawa, 1995; Gallego et al., 2014). There are even differences in sperm activation between freshwater species; while motility initiation is cAMP dependent in salmonids, it is cAMP independent in cyprinids (Morisawa, 2008).

The eels are also very different from other fish species, not only in its biology, but also in the characteristics of the sperm flagellum. The eel flagellum is different from most teleost species, having a structure of 9+0, while in the other teleosts there are 9+2. The eel flagellum lacks the outer dynein arms, radial spokes and spoke heads, the two central tubules and the central tubule projections characteristic of the standard 9+2 flagellum (Gibbons et al., 1985). Gibbons et al. (1985) also stated that eel sperm appear to lack the mechanisms by which Ca^{2+} regulates waveform. Demembranated eel sperm were reactivated in the presence of 0.12 mM or 1 µM Ca^{2+} and their motility was compared to when they were activated in a media with <10 nM Ca^{2+}. No changes in flagellar beat pattern or frequency were observed, whereas the common response in 9+2 cilia and flagella is a change from symmetric to asymmetric bending or even arrest. Thus, in the eel, it seems that Ca^{2+} does not have a direct effect on axonemal structures, as has been proposed in other species (Zilli et al., 2012). This supports the theory that Ca^{2+} plays a different role in sperm

motility in eels compared to other fish species.

In mammals, the hyperactive motility acquired in the female tract, and the acrosomal reaction, have been widely studied (both Ca^{2+}-dependent, see Darszon et al., 2011) but there are few studies about the initial motility activation, which happens by dilution, once the sperm pass from the cauda epididymis (where it is immotile) to the vas deferens prior to ejaculation. Wade et al. (2003) showed, in rat sperm collected from the cauda epididymis, that sperm motility activation was cAMP and calcium-dependent. However, when sperm was activated in a Ca-free media, motility was still activated, although it was reduced by about 60% in comparison to the control. The authors stated that it is possible that calcium was not the only factor influencing motility activation.

4.2. Calcium and sperm motility parameters

Even if total motility was not affected by the absence of extracellular Ca^{2+} in the activation media, other parameters were moderately reduced in this condition, like VCL, VSL, VAP and BFC. These results corroborate previous studies on fish species which related extracellular Ca^{2+} to sperm parameters such as velocity (Alavi et al., 2011) or to the curvature of the spermatozoa movement or VCL (Cosson et al., 1989; Boitano and Omoto, 1992; Cosson et al., 2008; Zilli et al., 2008). In our case, it seems that the increased beat frequency in the medium containing Ca^{2+} (SW) caused an increase in all the velocities, VCL, VSL and VAP, compared to activation in a Ca-free medium.

4.3. Effect of bepridil on sperm motility

Bepridil is a well-known inhibitor of the Na^+/Ca^{2+} exchanger (NCX). The presence of a Na^+/Ca^{2+} exchanger on the spermatozoa membrane of herring, a marine fish, was reported by Vines et al. (2002). In the present study, treatment with bepridil resulted in a post-activation increase in $[Ca^{2+}]_i$ concentrations (when Ca^{2+} was present in the activation medium) and an inhibition of the post-activation increase in $[Na^+]_i$. That means that bepridil partially inhibits the influx of Na^+ from the extracellular environment, and partially prevents the efflux of Ca^{2+} from the spermatozoa.

This study demonstrates, for the second time in a marine fish species, that a NCX is involved in sperm motility. In herring it was reported that the Na^+/Ca^{2+} exchanger acted in reverse, i.e. mediating the efflux of Na^+ and the influx of Ca^{2+} during sperm activation (Vines et al., 2002). NCX is also present in the membrane of human spermatozoa (Krasznai et al., 2006), and is involved in motility initiation, but in this case, it acts in the same way as in European eel sperm, mediating an efflux of Ca^{2+} and a simultaneous influx of Na^+.

4.4. Effect of Ca- ionophore A-23187

In this study, the addition of the Ca^{2+} ionophore A-23187 in an isosmotic solution (Ca-free extender) to the European eel sperm did not cause the start of motility in any case. This is in contrast to the findings of Oda and Morisawa (1993) in pufferfish sperm (*Takifugu niphobles*), but corroborates the results of Krasznai et al. (2003b) from the same species, who observed that in isosmotic extenders containing Ca^{2+} ionophore A231186 and different Ca^{2+} concentrations, sperm did not initiate motility in any case. Also, Oda and Morisawa (1993) found that A-23187 suppressed sperm motility under hypertonic conditions, while in the

present study A-23187 did not suppress sperm motility after hypertonic activation in SW or the Ca-free activator.

Regarding the effect of A23187 on post-activation $[Ca^{2+}]_i$ levels, a higher increase in $[Ca^{2+}]_i$ levels than in the controls was observed after activation with SW (containing Ca^{2+}), but not after activation in a Ca-free hyperosmotic media. For this reason, the observed increase in $[Ca^{2+}]_i$ in A-23187-treated samples (which was 28% higher than in the controls) is likely to be due to the influx from the extracellular environment. The higher increase in $[Ca^{2+}]_i$ levels post-activation observed with bepridil suggests a) a higher influx of Ca^{2+} during activation or b) a lower Ca^{2+} efflux from the cell after activation. This suggests that there is a regulatory mechanism for the homeostasis of intracellular Ca^{2+}, avoiding an excess of $[Ca^{2+}]_i$, which would be disrupted by the treatment with ionophore, which allows the free entry of Ca^{2+} ions through the sperm membrane. Ionophore could disrupt the efflux of Ca^{2+} due to the NCX, or other Ca^{2+} efflux pathways from the sperm cell. Such disruption, however, would not negatively affect sperm motility or kinetic parameters, which were similar to the controls.

4.5. Effect of W-7

W-7 is a calmodulin (CALM) antagonist. CALM is the most ubiquitous Ca^{2+}-binding protein mediating Ca^{2+} signaling, and is a component of both ciliar and flagellar axonemes from organisms as diverse as algae, protozoa and mammals (reviewed by Ignotz and Suarez, 2005). W-7 inhibits calcium calmodulin-dependent kinases (CAMK) and myosin light chain kinase (MYLK) (Ignotz and Suarez, 2005).

Even if a sustained $[Ca^{2+}]_i$ increase is not necessary for motility initiation, W-7 moderately reduced sperm motility when external Ca^{2+} was present, and a strong reduction was seen when external Ca^{2+} was not available. Thus, some internal Ca^{2+} must be involved in this process. Gallego et al (2014) showed by Fluo-4 imaging that European eel sperm in the quiescent stage have $[Ca^{2+}]_i$ concentrated in the mithochondria, but also present in the cytoplasm. Thus, even though in the present study we washed the sperm in a Ca-free medium, some internal Ca^{2+} could still remain and this may bind with calmodulin when the sperm motility is activated. Thus, by adding W-7 in external Ca-free conditions we are inhibiting the enzymatic activity at two levels: 1) by inactivating the whole complex Ca-calmodulin with W-7, which changes its structure and 2) by limiting the amount of Ca-calmodulin, due to the limited amount of Ca^{2+} which can bind to calmodulin, restricted to the internal Ca^{2+} stores.

Our results corroborate the inhibitory effect of W-7 on pufferfish (a marine species) sperm motility (Krasznai et al., 2003a). W-7 also caused a reduction in sperm motility and velocity in several freshwater fish species (tilapia, sterlet, bluegill) (Morita et al., 2006; Zucarelli et al., 2007; Alavi et al., 2011).

5. Conclusions

Our results strongly support the theory that an increase in $[Ca^{2+}]_i$ is not necessary for sperm motility activation in the European eel, although early measurements in the first few seconds post-activation would be necessary to fully confirm this. Ca^{2+} signaling could be a modulator of the sperm velocities and beat frequency rather than being the first signal for sperm motility initiation in this species. The presence of a sodium/calcium exchanger involved in sperm

motility of the European eel has been demonstrated by the increase in $[Ca^{2+}]_i$ and the decrease in $[Na^{+}]_i$ produced by bepridil, which strongly inhibited sperm motility. Also, a calcium-calmodulin complex seems to be involved in sperm motility in this species.

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SUPPLEMENTARY MATERIAL

Set up of the washing protocol for European eel sperm

First, 6 samples with motility >50% were diluted (40 µl sperm plus 960 µl; 1:25 v:v) in P1 extender (125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$ *$6H_20$, 30 mM KCl, 1 mM $CaCl_2$; Peñaranda et al.,2009), which mimics the seminal plasma composition of the European eel (Asturiano et al., 2004). One aliquot from each sample was left without treatment, at 4 ºC, and sperm motility was activated with artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2) at 4 ºC, and recorded with a CASA system (ISAS software, Proiser RandD, Paterna, Spain) as described in section 2.5. Other aliquot was centrifuged at 180 g for 4 minutes at 4 ºC, and other aliquot was centrifuged at 500 g at 4 ºC. After centrifugation, supernatant was carefully removed, the sperm pellet was resuspended in P1 extender (1:25 v:v) with gentle agitation, and sperm motility was checked with the CASA system as described. This process was repeated two more times. Results are shown in Table S1. There were not significant differences in sperm motility or kinetics, and then the washing conditions selected for further experiments were: three centrifugations at 500 g at 4 ºC.

As the calcium studies needs the elimination of extracellular Ca^{2+} from the seminal plasma, we first tested if washing in a Ca-free extender (155 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$*$6H_20$, 30 mM KCl, 5 mM EDTA) gives the same results in motility as washing in P1 extender. Both extenders had the same osmolality (325 ±10 mOsm), and the same pH (8.5), which was adjusted the same day of the test. Sperm motility (Figure S1) and the rest of kinetic parameters were similar after washing in both extenders (data not shown).

Table S1. Effect of successive sperm washing under two centrifuge velocities on sperm motility parameters in comparison with the same unwashed samples. Data are expressed as mean ± SEM (n = 6). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity. SE: standard error. P: ANOVA p-value.

Washing number	fresh unwashed	180 g x 1	180 g x 2	180 g x 3	500 g x 1	500 g x 2	500 g x 3	SE	p
MOT (%)	69.3	60.3	52.2	44.8	51.8	59.0	48.9	6.27	0.75
MP (%)	28.5	29.9	32.5	26.0	23.2	23.2	26.2	4.11	0.31
FA (%)	49.6	44.7	38.4	32.9	35.7	37.5	33.4	6.44	0.52
VCL (µm/s)	124.7	132.5	129.2	126.9	122.4	117.5	120.4	10.54	0.96
VSL (µm/s)	59.3	66.9	71.4	67.4	62.4	56.3	60.5	7.95	0.85
VAP (µm/s)	78.6	85.5	86.6	82.9	80.5	75.9	76.4	9.37	0.97
LIN (%)	47.3	49.5	54.0	51.9	50.5	47.5	49.8	2.55	0.55

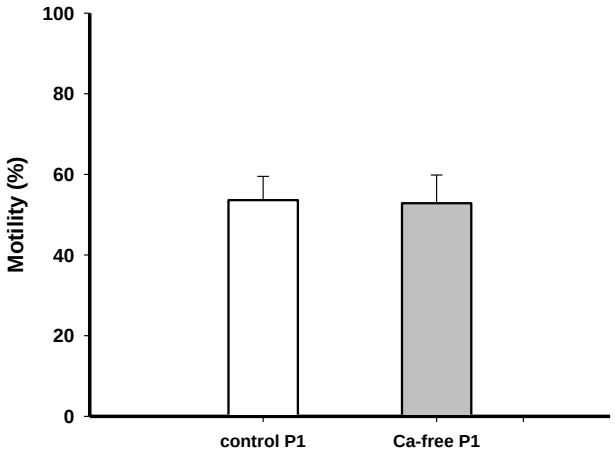

Figure S1. Total sperm motility after washing in Ca-free extender vs control extender (extender P1 Peñaranda et al., 2010, containing 1 mM Ca^{2+}). Data are expressed as mean ± SEM (n=7).

CHAPTER 4

Identification of the major proteins present in the seminal plasma of European eel, and how hormonal treatment affects their evolution. Correlation with sperm quality.

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Abstract

By first time, 2DE protein profile of European eel seminal plasma has been determined. 14 different proteins corresponding to 9 major families were identified in seminal plasma, through hormonal treatment. Some of them play a part in sperm maturation, including carbonic anhydrase which is responsible for modulating the pH of seminal plasma, and warm temperature acclimation protein, which may play an important role in the final maturation of this species, due to the warm temperature of their spawning ground (in the Sargasso Sea).

Sperm samples were classified into three motility categories depending on the percentage of motile cells, I: 0-25%, II: 25-50% and III: >50%. Different protein profiles were observed depending on the sperm motility categories, specifically, with the apolipoproteins and complement C3. Higher numbers of proteins from the apolipoprotein family were registered at lower motilities; whereas the complement C3-like family was higher in the samples with the highest percentage of motile cells. These results suggest that the proteins linked to the transportation of lipids (apolipoprotein) and to the immune system (complement C3) may carry out their functions at different stages of spermatogenesis. Using SDS-PAGE analysis, 13 bands were identified, most of which migrated between 20 to 60 kDa. In the last weeks of treatment significant increases were observed in the percentage of motile spermatozoa, curvilinear velocity and beat cross frequency. This improvement in sperm quality coincided with a higher amount of proteins located at 19 KDa, therefore, this protein could be involved in sperm motility of the European eel.

1. Introduction

Seminal plasma is a multi-functional, heterogeneous and complex protein-rich fluid in which spermatozoa cells are diluted (Rodríguez-Martínez et al., 2011). Numerous findings are consistent in the idea that seminal plasma contains different proteins which are involved in the maintenance of sperm viability and modulate their function (Lahnsteiner et al., 2003; Zilli et al., 2005; Dietrich et al., 2014).

Although interspecies differences have been observed in seminal plasma protein composition (Li et al., 2011), we know that the common role of seminal plasma is to create an optimal environment for the storage of spermatozoa. As a consequence, understanding the mechanism involved in sperm-protein interactions is the main aim of many studies into improving the storage media and therefore, the development of better reproductive technologies. An example of protective effect of the proteins in spermatics cells is the egg yolk. As in rainbow trout (*Oncorhynchus mykiss*), it has been demonstrated that the protection of DNA integrity provided by the egg yolk is greatly improved when only their LDL (low density lipoprotein) fraction is added to the cryopreservation extender (Pérez-Cerezales et al., 2010).

However, only a few studies have focused on the identification of seminal plasma proteins and their physiological functions in fish. Loir et al. (1990) determined the concentrations of several organic components such as total proteins, amino acids, lipids, glucose, fructose and enzymes in rainbow trout and it was observed that the

presence of these components varies, depending on the animals and sampling time. Also in rainbow trout, a total of 12 proteins were detected by SDS-PAGE and the influence of the presence of some proteins in the seminal plasma on the sperm quality has been demonstrated (Lahnsteiner et al., 2007). In another freshwater species, Nile tilapia (*Oreochromis niloticus*), it has been demonstrated that the presence of a high molecular weight of glycoprotein in seminal plasma contributes to sperm immobilization (Mochida et al., 1999).

Studies about the composition of the seminal plasma of marine fish are even scarcer. The composition of the seminal plasma of turbot (*Scophthalmus maximus*) differs from that of salmonids (Billard et al., 1983a) in the total protein content (Suquet et al., 1993). However, in the case of both species, it seems that a high concentration of proteins may be linked to a possible role in spermatozoa protection. In turbot, sperm motility is reduced at high sperm dilutions (Suquet et al., 1992a) and is maintained by adding BSA (Bovine Serum Albumin; Fauvel et al., 1993a). This discovery is also supported by evidence showing that seminal proteins protect the spermatozoa against microbial attack (i.e. transferrin and anti-proteases), oxidative damage (i.e. transferrin, superoxide dismutase), and premature activation (i.e. parvalbumin) (Wojtczak et al., 2005a; Dietrich et al., 2010a,b).

In addition, several studies have been performed regarding the evolution of seminal plasma protein composition during spermatogenesis. In Eurasian perch (*Perca fluviatilis*) the physiological and functional sperm parameters together the seminal plasma proteome was evaluated over the course of their reproductive season (Shaliutina et al., 2012). A similar study, but using a 2D polyacrylamide gel electrophoresis technique, revealed a significant change in 10 protein spots after the third stripping, suggesting that during reproductive season predominantly affected proteins involved in membrane trafficking, organization, cell motility, and oxido-reductase activity (Shaliutina et al., 2012).

The introduction of proteomics in the study of male fish reproduction provides a unique opportunity to unravel the physiological mechanisms relating to sperm function, such as motility and fertilizing ability (Ciereszko et al., 2012). Thus, the use of proteomic studies provides enormous advances in the identification of sperm proteins (Baker et al., 2007) and the proteins of human seminal plasma (Pilch and Mann et al., 2006).

In carp (*Cyprinus carpio*), the major proteins present in fish seminal plasma were identified (Dietrich et al., 2014) using a combination of protein fractionation by one-dimensional gel electrophoresis and high performance liquid chromatography electrospray ionization tandem mass spectrometry. This methodology was also used in a marine species, Senegalese sole (*Solea senegalensis*), to identify and compare the proteins from the seminal plasma of wild-caught and F1 males (Forné et al., 2009). The results of the study contributed to the identification of proteins associated with spermatogenesis previously not observed in teleosts, and suggested potential mechanisms that may be contributing to the poor reproductive performance of Senegalese sole F1 males.

In the present study, the European eel (*Anguilla anguilla*) was used as the

experimental organism. The European eel has a particular life cycle: the prepubertal eel migrates across the Atlantic Ocean for 6-7 months to reach the spawning area, in the Sargasso Sea (Tesch, 1978; Van Ginneken and Maes, 2005). As such they could be considered a marine species. In the last few decades, several factors have contributed to the decline of the European eel: overfishing, migration barriers and habitat reduction.

Therefore, this decline in the eel population and the popularity of this species in the food market, has led researchers to look at reproduction in captivity. With this in mind, our group has worked on the development of extender media including 2% of BSA, which results in better motilities and viabilities for short-term storage (Peñaranda et al., 2010a,b). Another example of the role of proteins in sperm quality, was the improvement in the percentage of motile cells post-thawing thanks to the addition of fetal bovine serum (FBS) in the cryoprotectant medium (Peñaranda et al., 2009). This means that it is likely that the addition of extra-proteins in the media is related to enhanced sperm quality.

Peñaranda et al. (2010a) evaluated the seminal plasma protein content of European eel, registering mainly four electrophoretic bands around 80, 40, 26 and 12 kDa. Three of them showed significant differences in concentration during maturation (80, 40 and 12 KDa), and all of them showed the highest value at 8th week (previous to full spermiation period and best quality sperm period). Indeed, higher concentration of proteins around 40 KDa was observed at higher motilities. In order to confirm this possible role of seminal plasma proteins on sperm quality, it is necessary to discover the identity of these proteins and their precise physiological functions. With this objective, this study aims to increase our understanding of the reproductive physiology of this particular species, more specifically with regards to the protein composition of the seminal plasma. In addition, a study was carried out to determine the presence of the major proteins and their function in the different categories of sperm motility.

2. Material and methods

2.1. Fish maintenance and hormonal treatment

A total of 13 adult male European eels (mean body weight 100±2 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, at the Universitat Politècnica de València (Spain). The fish were reared in a 150 L aquarium equipped with thermostat and cooler, and covered with black panels to maintain constant darkness. The eels were gradually acclimatized to sea water (salinity 37.3±0.3 g/L) over the course of 1 week, and were maintained in sea water until the end of the experiment.

After sea water acclimatization, the hormonal treatment was initiated with recombinant human chorionic gonadotropin (hCGrec; Ovitrelle, Madrid). Once a week the fish were treated with a dose of 1.5 IU/g fish by intraperitoneal injection. The hormone was diluted 1:1 (to reach 1 IU/μL) in saline solution (NaCl 0.9%) and the individual dose was calculated after weighting each fish.

2.2. Human and Animal Rights

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (UPV) (Permit Number: 2014/VSC/PEA/00147). The fish were sacrificed by over-anesthesia with benzocaine (>60 ppm), and all efforts were made to minimize suffering. 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).

2.3. Sperm collection and sampling

The sperm samples were collected 24 h after hCG administration in order to obtain the highest sperm quality (Pérez et al., 2000), from 6th week of hormonal treatment until the end of the experiment (with a total of 7 samplings during the experiment). Before sperm collection, the fish were anesthetized, and the genital area was cleaned with freshwater, and carefully dried to avoid contamination with feces, urine, or sea water. The sperm was diluted in 1:25 in P1 extender (described by Peñaranda et al., 2010a) and maintained at 4 °C until the motility evaluation.

2.4. Sperm motility evaluation

Sperm motility activation was performed as described by Gallego et al. (2013a) by mixing 1 µl of diluted sperm (dilution 1:25 in P1 extender) with 4 µl of artificial sea water [SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2]. The mixture was made in a SpermTrack-10® chamber, with a depth if 10 µm (Proiser RandD, Paterna, Spain) and observed in a Nikon Eclipse 80i microscope, with a 10x lens (Nikon negative phase contrast 10x). The frame rate used was 60 fps. Motility was recorded 15 seconds after mixing the sperm with sea water, using a high-sensitivity video camera HAS-220, and ISAS software (Proiser RandD, Paterna, Spain) was used to determine the sperm motility parameters. Each sample was evaluated in triplicate. Both the sperm and the sea water were maintained at 4 ºC in a water bath during the sperm motility evaluation. The sperm samples were classified into three motility categories depending on the percentage of motile cells observed after sea water activation, I: 0-25%, II: 25-50% and III: >50%.

The parameters considered in this study were total motility (MOT, %), defined as the percentage of motile spermatozoa; progressive motility (P-MOT, %), defined as the percentage of spermatozoa which swim forward 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; straightness (STR, %), defined as the linearity of the spatial average path; 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; ALH, amplitude of the lateral movement of the sperm head 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 motile if their progressive motility had a straight line velocity >10 µm/s.

2.5. Isolation and concentration of the seminal plasma

In each sampling, the seminal plasma was obtained by centrifuging the sperm samples at 7500 g for 5 min in a microcentrifuge at 4 °C. The seminal plasma was carefully recovered from each sample and stored at -20 ºC. The protein content of the seminal plasma was determined using the Pierce BCA protein assay (Pierce Chemical Company, Rockford, IL; Smith et al., 1985) and was measured in all the males (13 males per sampling) with a total of 78 samples analyzed during the experiment.

Once the seminal plasma was obtained and all the samples were classified into the different motility categories, a representative pool with all three motility categories (I, II and III, n=6 sperm samples/motility category, in total 18 sperm samples) was used for 2D-Electrophoresis in order to identify the protein profile. In addition, in order to compare the appearance of different spots in each motility category, 2 different pools from each motility (in total 6 pools) were used in the 2D- Electrophoresis analysis.

The samples used for 2D-DIGE were concentrated using Millipore's Ultracel® -3K regenerated cellulose membrane (Darmstadt, Germany) until a final concentration of 50 µg protein/µl was achieved.

2.6. Appearance of protein band: 1D-SDS-PAGE

Individual samples of seminal plasma were thawed at room temperature and run in 1-D sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (gradient SDS-PAGE; 4-15%) in vertical gels (AMERSHAM ECL™ GEL; BioRad, Madrid, Spain). All the samples were processed under the same conditions: with a protein concentration of (1 µg/µl), at a constant voltage of 120 v and for 2 h. The gel was stained with Coomasie brilliant blue R-240 for 4h. The protein bands were photoedited and quantitatively analysed with GeneTools software (Syngene, IZASA, Spain) for band detection and molecular weight analysis.

2.7. Identification of protein profile: 2D- electrophoresis

Immobilized pH gradient strips (IPG strips, range: pH:3-11 and 4-7) were hydrated by incubation overnight in 7 M urea, 2 M thiourea, 2% CHAPS, 2% (w/v) DTT, 0.5% IPG buffer and 0.002% bromophenol blue. The different pools of seminal plasma (see section 2.5) were thawed at room temperature and dissolved in a labeling buffer (7M urea, 2M thiourea, 4% w/v CHAPS, 0.5% w/v anpholytes, and 0.002% of bromophenol). The protein components were separated by first-dimension isoelectric focusing (IEF) conducted at 20 °C in an IPGphor (Amersham Bioscience, Uppsala, Sweden) system, with the current limited to 50 µA/strip and the following voltage program: 300 v/15 min, 500 v/1 h, 3500 V/4 h. The IPG strips were then equilibrated by being soaked twice in a SDS equilibration buffer solution containing 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol (v/v), 2% SDS (w/v) and 0.002% bromophenol blue (w/v) with gentle shaking.

IPG strips were placed onto second dimension SDS-PAGE (overall gel size 18.3 × 20.0 × 0.1 cm) which was performed using 1.5 cm 4% stacking gel (0.5 M Tris–HCl pH 8.8, 30% acrylamide, 10% SDS, 10% APS, 0.1% TEMED) and 15% separation gel (1.5 M Tris–HCl pH 8.8, 30% acrylamide, 10% SDS, 10% APS, 0.05% TEMED) using a Protean IIxi device (BioRad, Hercules, CA, USA). The gels were run at 20 °C at a constant current of 75 V for 30 min, and then at 110-120 V until the dye reached the bottom of the gel.

To identify the spots, they were digested with Trypsin and the tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) using a BEH130 C18 column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. Doubly and triply charged ions were selected for collision-induced dissociation (CID) MS/MS. Fragmentation spectra were interpreted manually (*de novo* sequencing), using the on-line form of the MASCOT program, and processed in Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. Images of gels were obtained with the Image Scanner II (GE Healthcare) using Labscan 5 (GE Healthcare) software. The differential analysis between motility categories was performed by Progenesis Samespots program.

2.8. Statistical analysis

Statistical analyses were performed using the statistical package Statgraphics Centurion software (Statistical Graphics Corp., Rockville, MO, USA). Kurtosis and Asymmetry Standard coefficients were used to check the normality of data distribution.

The variables that did not have a normal distribution were log-transformed and their normality was checked again. One-way ANOVA analyses were then performed to check statistical differences among groups. Differences were considered significant if $P<0.05$. Results are presented as the mean ± standard error of the mean. Variance homogeneity was checked using the Bartlett test. The one-way ANOVA analyses were followed by a Duncan post-hoc test. If normality failed after the log transformation, a non-parametric test was carried out (Kruskal-Wallis), followed by a Dunn's test.

3. Results

3.1. Characterization of proteins in the seminal plasma

The analysis of European eel seminal plasma using high-resolution 2D-electrophoresis technology led to the detection of 67 matching spots (Figure 1A,B,C), with a total of 14 different proteins corresponding to 9 major families (Table 1). In the pool which contained samples from different categories (I, II and III, Figure 1A) most of the proteins were classified as apolipoproteins and also, carbonic anhydrase or complement C3, which were present in the pool of motility class I and III (Figure 1B and C, respectively).

The remainder of the proteins identified was: immunoglobulins, transferrins, lipocalins, lectins, hemopexin, ceruloplasmin, and acetiltransferases, located in the category I and III pools (Figure 1B and C respectively).

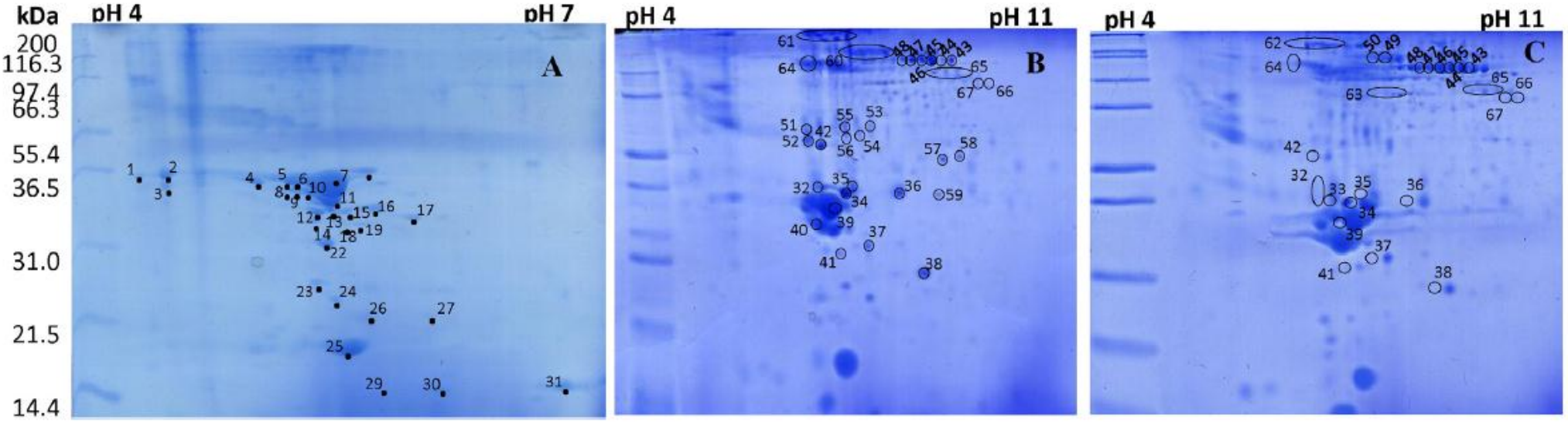

Figure 1: Two dimensional gel electrophoresis of seminal plasma from European eel. A) Pool (n=18 sperm samples) representative of sperm motilities; category I, II and III (0-25%, 25-50% and >50% of motile cells, respectively), B) Pool of category I of motility (n=6 sperm samples), C) Pool of category III of motility (n=6 sperm samples). Numbered protein spots correspond to proteins identified from 2D-MS/MS which are more abundant in seminal plasma. Molecular mass marker (3.5 -200 kDa).

Table 1: Proteins identified using ESI-CID-MS/MS.Mox: Methionine oxidation in European eel seminal plasma from; A) Pool (n=18) representative of sperm motility categories I, II and III (0-25%, 25-50% and >50% of motile cells, respectively), B) Pool of category I of motility (n=6), C) Pool of category III of motility (n=6). Numbered protein spots correspond to proteins identified from 2D-Electrophoresis which are more abundant in seminal plasma. Molecular mass marker (3.5-200 kDa).

Spot no.	MW (Da)	m/z	z	Peptide Sequence	MASCOT Score	Organism	Accession no.	Protein family	Figure
2	55.4	416.7	2	HLDEYR	553	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		449.3	2	AKLEPLVK					
		502.3	2	VQGEDLQSK					
		515.3	2	IQADVDQLK					
		535.3	2	LQPVVEDLR					
		545.8	2	LKPYAEELK					
		579.3	2	IQADVDQLKK					
		604.3	2	AAVGMYLQQVK					
		612.3	2	AAVGMoxYLQQVK					
		623.8	2	DKVQGEDLQSK					
		649.9	2	TKLQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
		490.9	2	DHLSEALTDVKDK					
3	36.5	515.3	2	IQADVDQLK	171	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		535.3	2	LQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
5	36.5	535.3	2	LQPVVEDLR	122	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		670.8	2	TLAEPYVQEYK					
		649.9	2	TKLQPVVEDLR					
6	36.5	612.3	2	AAVGMoxYLQQVK	209	*Anguilla japonica*	BAB40960	Apolipoproteins	1A

		614.3	2	DHLSEALTDVK					
Spot no.	**MW (Da)**	**m/z**	**z**	**Peptide Sequence**	**MASCOT Score**	**Organism**	**Accession no.**	**Protein family**	**Figure**
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
		490.9	2	DHLSEALTDVKDK					
7	36.5	604.3	2	AAVGMYLQQVK	238	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		614.3	2	DHLSEALTDVK					
		433.6	2	TKLQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		490.9	2	DHLSEALTDVKDK					
8	36.5	612.3	2	AAVGMoxYLQQVK	95	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		614.3	2	DHLSEALTDVK					
9	36.5	535.3	2	LQPVVEDLR	146	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		612.3	2	AAVGMoxYLQQVK					
		670.8	2	TLAEPYVQEYK					
10	36.5	416.7	2	HLDEYR	235	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		604.3	2	AAVGMYLQQVK					
		612.3	2	AAVGMoxYLQQVK					
		614.3	2	DHLSEALTDVK					
		649.9	2	TKLQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
11	36.5	535.3	2	LQPVVEDLR	289	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		545.8	2	AAVGMoxYLQQVK					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
		490.9	2	DHLSEALTDVKDK					

12	36.5	416.7	2	HLDEYR	335	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
Spot no.	**MW (Da)**	**m/z**	**z**	**Peptide Sequence**	**MASCOT Score**	**Organism**	**Accession no.**	**Protein family**	**Figure**
		449.3	2	AKLEPLVK					
		505.3	2	LVPIVEAIR					
		515.3	2	IQADVDQLK					
		579.3	2	IQADVDQLKK					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
16	36.5	535.3	2	LQPVVEDLR	68	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		612.3	2	AAVGMoxYLQQVK					
20	55.4	491.6	3	QFHFHWGGADDR	107	*Oryzias latipes*	XP_004081218	Carbonic anhydrase	1A
		791.4	3	YAAELHLVHWNTK		*Oryzias latipes*			
22	31	515.3	2	IQADVDQLK	95	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		535.3	2	LQPVVEDLR					
25	21.5	915.9	2	EALEPLAQHIPQSQAAK	90	*Anguilla japonica*	BAB40966	Apolipoproteins	1A
		610.9	2	EALEPLAQHIPQSQAAK					
29	14.4	401.3	2	VGLVAVDK	100	*Tetraodon nigroviridis*	CAG06096	Immune system	1A
		605.8	2	EYVLPSFEVK					
30	14.4	915.9	2	EALEPLAQHIPQSQAAK	82	*Anguilla japonica*	BAB40966	Apolipoproteins	1A
		610.9	2	EALEPLAQHIPQSQAAK					
		677.4	2	AKEALEPLAQHIPQSQAAK					
31	14.4	706.4	2	VATGAAGEXAPXVDK	*De novo*	*Anguilla japonica*	BAB40966	Apolipoproteins	1A
33	31	736.8	2	QFHFHWGGADDR	108	*Oryzias latipes*	XP_004081218	Carbonic anhydrase	1B
		791.4	2	YAAELHLVHWNTK					
34	31	736.8	2	QFHFHWGGADDR	156	*Oryzias latipes*	XP_004081218	Carbonic anhydrase	1C
		791.4	2	YAAELHLVHWNTK					
37	31	656.9	2	TQXEPVVEEXR	*De novo*	*Anguilla japonica*	AAQ10893	Lipocalin	1B,1C

		503.7	2	SYSFXFSR	*De novo*				
Spot no.	**MW (Da)**	**m/z**	**z**	**Peptide Sequence**	**MASCOT Score**	**Organism**	**Accession no.**	**Protein family**	**Figure**
38	31	437.7	2	ATQSAQLR	147	*Anguilla japonica*	Q9I928	Lectin	1B,1C
		491.8	2	YVTVYLPK					
		680.3	2	TFHCPQPMoxIGR					
		453.9	2	TFHCPQPMoxIGR					
		491.8	2	YVTVYLPK					
		680.3	2	TFHCPQPMoxIGR					
		453.9	2	TFHCPQPMoxIGR					
		748.4	2	QVYTITSVTITNR					
42	66.3	501.2	2	AVXDPTDDR	*De novo*	*Lepisosteus oculatus*	XP_006640345	Acetiltransferase	1B,1C
46	116.3	555.7	2	SADFEXXCR	*De novo*	*Takifugu rubripes*	XP_003974413	Transferrin	1B,1C
		983.5	2	(318.1)SFXYXGAEYMSXVR	*De novo*				
48	116.3	661.8	2	CLAEGGGDVAFVK	69	*Takifugu rubripes*	XP_003974413	Transferrin	1B,1C
50	97.4	677.3	2	VGTNFGFNDXNR	*De novo*	*Takifugu rubripes*	XP_003974413	Transferrin	1B,1C
57	66.3	677.3	2	VGTNFGFNDXNR	*De novo*	*Takifugu rubripes*	XP_003974413	Transferrin	1B,1C
60	116.3	510.8	2	DGLGDVAFVK	60	*Oryctolagus cuniculus*	P19134	Transferrin	1B
		682.8	2	CLVEKGDVAFVK					
61	200	565.3	2	GITTLPAVETK	201	*Anguilla anguilla*	ABY73532	Immune system	1B
		764.9	2	GFYPKEVLFSWR					
		782.4	2	TATFACFASEFSPK					
		826.9	2	DFTPDLLTFKWNR					
62	200	707.4	2	TGATYTXXEGYPK	*De novo*	*Lateolabrax japonicus*	CCA29190	Hemopexin	1C
62		650.4	2	XQTVXDAXDAXK	*De novo*				
63	55.4	830.5	2	TPEEEHLGILGPVIR	73	*Lepisosteus oculatus*	XP_006637544	Ceruloplasmin	1C
64	116.3	699.3	2	VYVGTEYFEYK	*De novo*	*Lepisosteus oculatus*	XP_006639097	Hemopexin	1B,1C

		478.8	2	TDSVXFFK	*De novo*				
65	97.4	555.8	2	SADFEXXCR	*De novo*	*Takifugu rubripes*	XP_003974413	Transferrin	1B,1C

Taking the class motility as a basis, significant differences were found in the proteins linked to lipid transport (apolipoprotein, Figure 2A and B) and the immune system (complement C3, Figure 2C and D), with higher amounts (8.425e+006 pixels/unit area) of apolipoproteins at lower motilities (category I) compared to higher motilities (2.141e+006 pixels/unit area). Conversely, the complement C3-like family protein was more abundant (1.129e+007 pixels/unit area) in the samples with the highest percentage of motile cells (category III) than in those with lower motilities (2.105e+006 pixels/unit area). No significant differences in the rest of the proteins were found between the different motility categories.

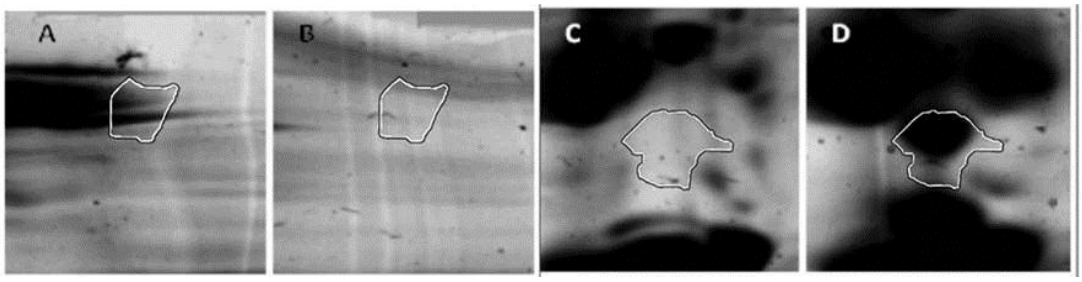

Figure 2: Images from 2D geles with the presence in seminal plasma of; 28kDa-2 apolipoprotein in samples showing sperm motility category I (A) and III (B), or presence of complement C3-like in samples showing sperm motility category I (C) and III (D).

3.2. Concentration of protein in the seminal plasma

The mean protein content of the seminal plasma of all the samples was 384.18 ±18.1 mg/100 ml and no differences were found between the different sperm motility categories (data not shown).

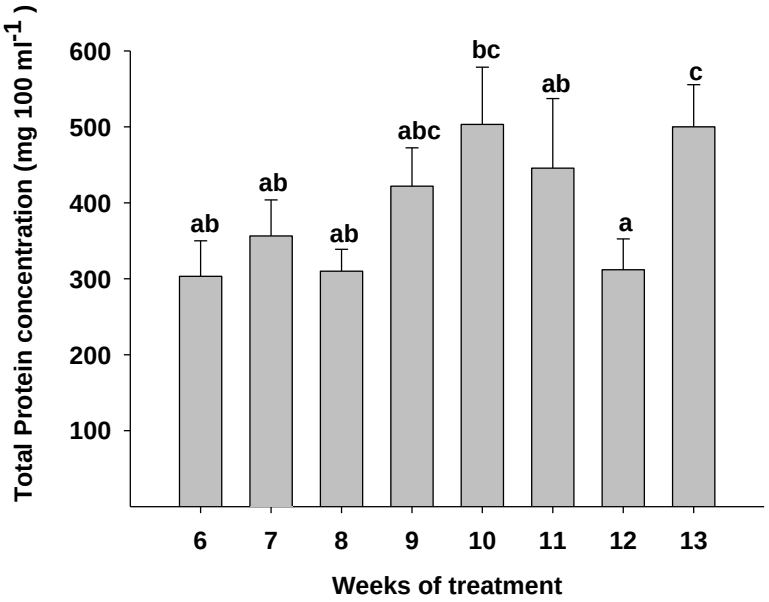

Figure 3: Mean total protein content in seminal plasma of European eel during the different weeks of the treatment (n=10 sperm samples by week). Data are expressed as mean±SEM and different letters indicate significant differences (P<0.05).

After 5 weeks of hormonal treatment a significant increase in the total protein content was observed (10th week). But two weeks later (12th week), the total content of protein decreased, showing the lowest values (Figure 3). However, only one week later (13th week) the total protein content increased significantly, showing the highest values of the experiment with 500 mg/100 ml of protein in the seminal plasma.

3.3. Appearance of protein band: 1D-SDS-PAGE

In total, 9 bands were identified by SDS-PAGE (Figure 4) and most of them were around 3.5 to 110 kDa standard proteins (Figure 4). To facilitate the analysis of the results, areas around the main bands were photoedited (3.5, 10, 15, 20, 30, 40, 50, 60 and 80 kDa) and evaluated using GeneTools software.

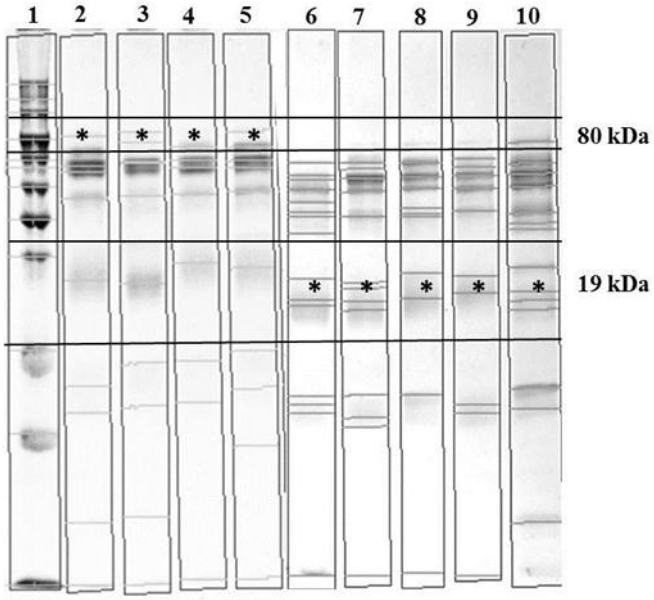

Figure 4: Separation of European eel seminal plasma (1 µg of protein/µl, n=9 sperm samples) by one-dimensional SDS-PAGE (15% acrylamide). The columns 2-5 and 6-10 are different sperm samples in 6th and 13th week of hormonal treatment respectively. Asterisks indicate significant differences between 19 and 90 kDa band. Proteins were stained with Coomassie Brilliant Blue R-240.

The proteins present around 19 kDa (Figure 5A) showed a significant increase in the 12th and 13th weeks of treatment. However, the proteins present around 90 kDa (Figure 5B) showed a significant decrease in the last week of treatment (13th week).

3.4. Sperm motility parameters throughout the hormonal treatment

Observation of spermiating males 6 weeks in to the hormonal treatment showed that they all had less than 10% of total motile cells (Figure 6A), and therefore they were classified into category I of motility (Figure 6B). Only one week later (in the 7th week of treatment), 50% of the males had reached category II (Figure 6B), with more than 25% motile cells (Figure 6A). The first samples with more than 50% of motile cells (Figure 6A), were observed at week 8 (30% of males). In the following weeks, in most of the cases, the three motility classes were reported (Figure 6B).

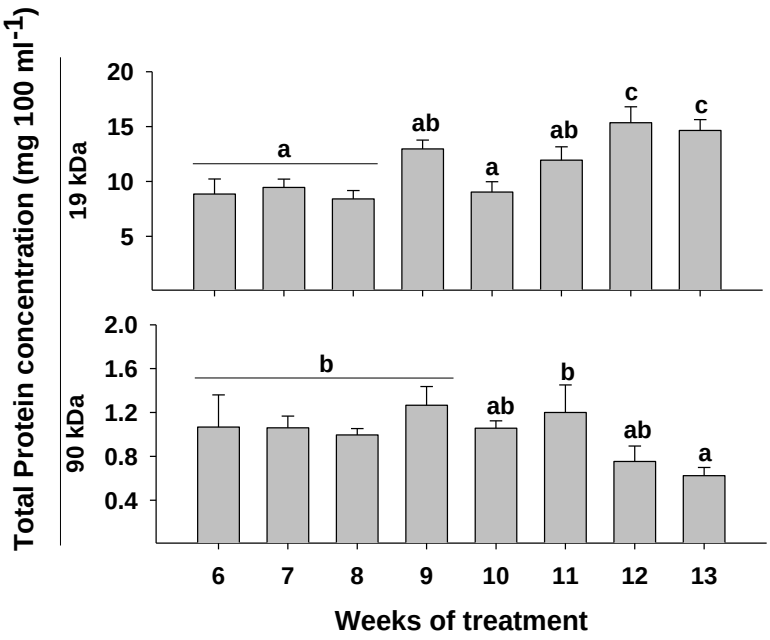

Figure 5: Protein concentration for 19 and 90 kDa band (A and B respectively) present in individual samples of seminal plasma of European eel during the different weeks of the treatment. Data are expressed as mean±SEM (n=10 sperm samples per week). Different letters indicate significant differences (P<0.05).

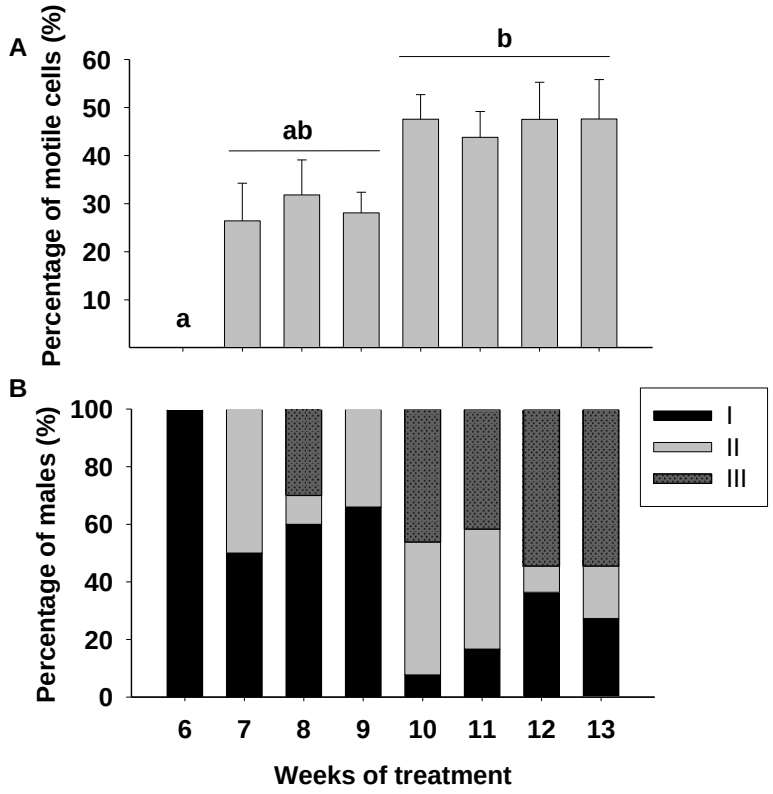

Figure 6: A) Percentage of motile spermatozoa in European eel sperm throughout the different weeks of the hormonal treatment. B) Percentage of the different categories of sperm motility (I, II, III) during the weeks of treatment. Data are expressed as mean±SEM (n=10 sperm samples) and different letters indicate significant differences (P<0.05) between activation samples.

Regarding the sperm kinetic parameters, a significant increase was observed from 10th week of treatment, but the highest values were observed during the last two weeks (Figure 7A) with more than 20% of progressive cells.

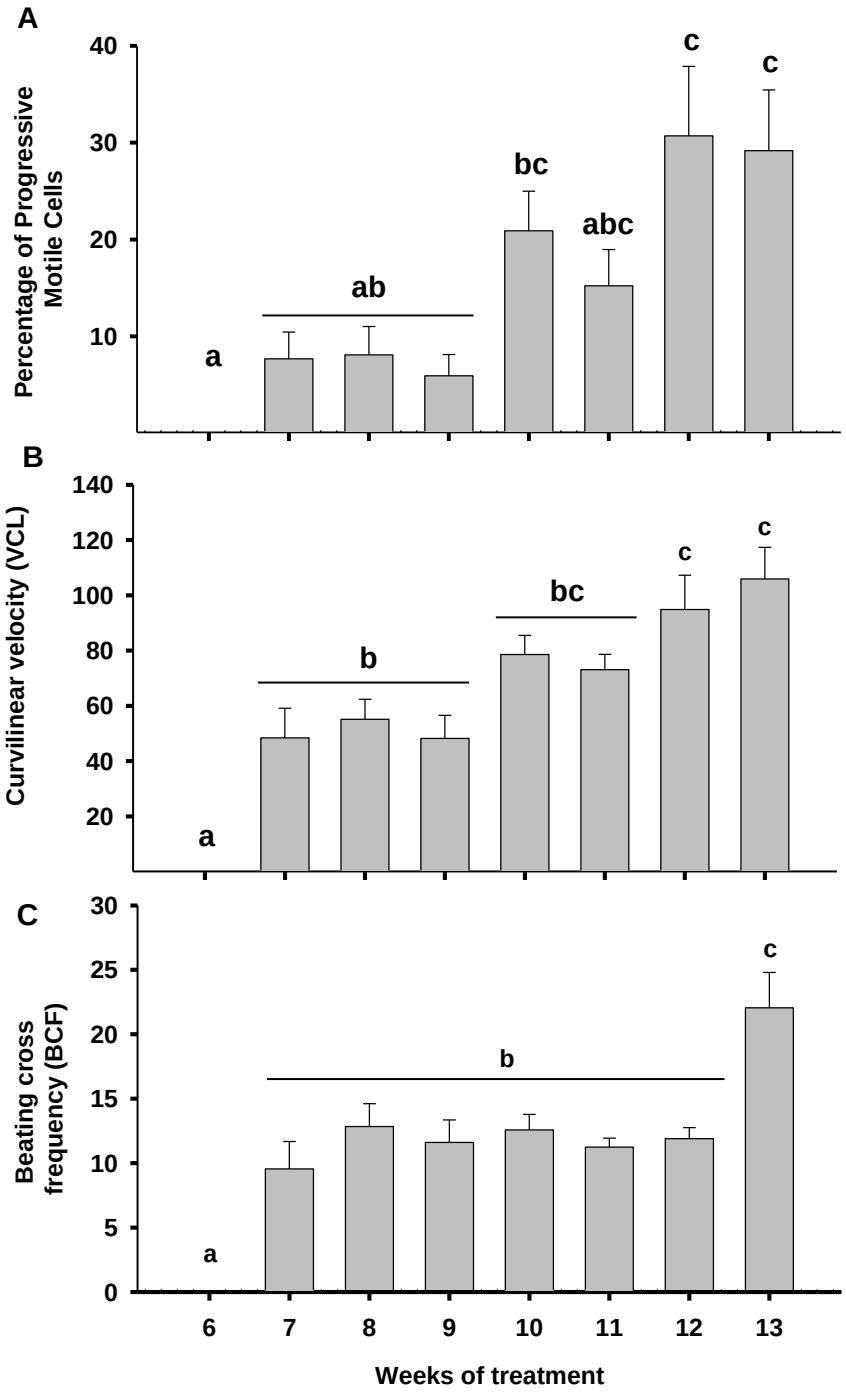

Figure 7: Evolution of sperm quality parameters throughout the hormonal treatment: A) percentage of progressive motile cells, B) curvilinear velocity (VCL, µm/s) and C) beat frequency (BCF, beats/s). Data are expressed as mean±SEM (n=10 sperm samples) and different letters indicate significant differences (P<0.05) between treatments at each week of treatment.

A significant increase of VCL and BCF kinetic parameters (Figure 7B and C, respectively) was observed from 7th week, registering the highest values in the last weeks of treatment.

No differences were found in the rest of kinetic parameters analyzed (VAP, ALH and STR).

4. Discussion

Several proteomic studies have been performed on the sperm of many fish species (Zilli et al., 2005; Keyvanshokooh et al., 2009; Forn et al., 2009; Li et al., 2010; Li et al., 2010d) but this is the first time that the protein composition of the seminal plasma of eel has been analysed.

Using high-resolution 2D electrophoresis, we have been able to identify members of nine protein families with a total of 14 different proteins. Most of the spots analyzed in the seminal plasma were apolipoproteins. Recent studies have pointed to the presence of apolipoproteins in the seminal plasma of rainbow trout and carp (Nynca et al., 2010; Dietrich et al., 2014). These apolipoproteins may be linked to sperm energy resources and the maintenance of specific carp sperm membrane lipid composition (Dietrich et al., 2014). Also, apolipoproteins play an important role in the defense (adaptative defense mechanism) of carp epidermis and mucus against bacteria, as innate response (Concha et al., 2003).

The adaptative immune system is the response of the vertebrate immune system to a specific antigen that typically generates immunological memory and the immunoglobulins play varying roles similar to humoral response (Ohta et al., 2006). Our study has shown that immunoglobulins are present in the seminal plasma of the European eel and may play an adaptative defense mechanism. Nevertheless, proteins from the innate defense were also found in our study, including the complement C3. The innate immune system is an ancient evolutionary form and crucial for the first line of defense (Hoffmann et al., 1999). The complement system mediates a chain of reactions of proteolysis and assembly of protein complexes, playing a major role in the body's defense as a part of both the innate and adaptive immune systems (Walport, 2001a,b). One of the most abundant groups of proteins in carp seminal plasma is the complement group (Dietrich et al., 2014). It is likely that these major proteins, one of which is complement C3, found in carp seminal plasma, are involved in the protection of the spermatozoa.

In addition, other proteins such as the retinol binding protein (RBP) are related to the protection of spermatozoa in a similar way to non-enzymatic antioxidants (Kandar et al., 2014) in humans. Thus, RBP plays an important role in protecting the spermatozoa against oxidative stress.

Another protein identified in this study was the warm temperature acclimation protein, Wap65. Recent studies using microarray analysis have indicated that this protein which is related to temperature acclimation may also be involved in immune responses (Sha et al., 2008). Wap65 was initially identified in the muscle tissue of several species including goldfish, carp, medaka and pufferfish (Kikuchi et al., 1995; Kinoshita et al., 2001; Hirayama et al., 2003, 2004). But it wasn´t until 2014 that Dietrich et al. demonstrated the presence of Wap65 in the seminal plasma of common carp. In the case of the European eel, the temperature of the supposed spawning area (the Sargasso Sea) is around 20 °C (Boëtius and Boëtius, 1967). Thus, Wap65 may play an important role in the final stages of maturation of this species, with the levels of Wap65 increasing in the tissues associated with warm temperature, as was observed in goldfish and carp (Watabe et al., 1993).

Moreover, in teleosts, Wap65 has high structural similarities with mammalian hemopexin (Sha et al., 2008), which also was identified in our study. Free heme is a potential source of iron that is toxic for cells and catalyzes the formation of free radicals. Plasma hemopexin promotes the metabolic processing of heme and inhibits the toxicity resulting from its oxidative catalytic activity (Hashemitabar et al., 2014).

Thus, the presence of both proteins; Wap65 and hemopexin, in the seminal plasma of the eel may be related to an immune response acting as protection against the oxidative damage that free heme causes during bacterial infections (Sha et al., 2008).

This study also discovered the presence of the iron-binding superfamily of proteins, transferrins (TF), in the seminal plasma of eel. Among them, serotransferrin (STF) and melanotransferrin (MTF) were identified. In fish, TF is recognized as a component of non-specific humoral defense mechanisms which act against bacteria. For example, in common carp TF are the major proteins present in the seminal plasma and their function is likely to involve the protection of spermatozoa from bacteria and heavy metal toxicity (Dietrich et al., 2010b).

An important finding of this study was the presence of carbonic anhydrase (CA). Little is known on how sperm regulates rises in intracellular bicarbonate. However, since carbonic anhydrase (CA) is known to participate in the regulation of intracellular pH (Sly and Hu, 1995), several studies have demonstrated the relationship between CA and spermatozoa activation. In mammals, CA is key to early activation, catalyzing the equilibrium between CO_2 and HCO_3^- (Wandernoth et al., 2010). Inaba et al. (2003) demonstrated that a CA specific inhibitor revealed that this enzyme is involved in the regulation of sperm motility in flatfish: halibut (*Verasper variegatus*), flounder (*Verasper moserii*) and turbot (*Scophthalmus maximus*).

The protein profile found in our study contains a total of 9 bands, 4 of which (80, 40, 20 and 12 kDa) correlate with the bands found in a previous study on European eel (Peñaranda et al., 2010b). In the previous study, a decrease was seen in the band from 80 kDa in the last few weeks of hormonal treatment (weeks 11 and 12). This correlates with the evolution of the band from 90 kDa in our study. The high amount of 90 kDa band observed before the peak in motility may be produced by germinal cell types (spermatocytes and spermatids) present in this gonadal stage, according to the description of the stages of development by Peñaranda et al. (2010b). Proteins with a molecular weight of around 90 kDa have been observed in the seminal plasma of common carp (Kowalski et al., 2003b; Drietrich et al., 2014) and have been identified as serine proteases, probably involved in the protection of the spermatozoa. Thus, the increment in the 90 kDa band before the peak in motility may be related to the protection of the spermatozoa cells under formation.

Regarding the kinetic parameters, in the last two weeks (weeks 12 and 13) the percentage of total and progressive motile cells reached similar values (more than 40 and 30% respectively) to those from a previous study (Gallego et al., 2012a) with the same conditions of hormonal treatment and temperature. Therefore, the repetition of similar results in different experiments suggests that the maturation method (see section 2.1.) is efficient and repetitive.

Generally, high protein concentration is a positive characteristic of fish sperm (Butts et al., 2013). In the present study, the total protein concentration of seminal plasma (mean content: 3.84±18.1 mg/ml) was higher than the values observed in Atlantic cod (*Salmo salar*; mean content: ~1 mg/ml; Butts et al., 2011), but lower than those found in turbot (*S. maximus*; mean content: 8.8±1.6 mg/ml; Suquet et al., 1993). Therefore, the total content of protein in the seminal plasma of marine species varies considerably. Another important finding from our study was that the highest protein concentrations were found at weeks 10 and 13, coinciding with the best sperm motility values.

Recently it has been proposed that protein composition of the seminal plasma plays an important role in fertilization (Kaspar et al., 2007; Li et al., 2009).This data suggests a positive correlation between the concentration of proteins in the seminal plasma and sperm motility.

In addition, the protein band with a molecular weight of 90 kDa showed the major level in the previous week (9^{th}) of the increment of the motility (10^{th} week). Also, a significant increment was seen in the 19 kDa band in the last two weeks of treatment (weeks 12 and 13), coinciding with high VCL and BFC kinetic parameter values and also with the highest progressive motility. Therefore, it seems that both bands; 19 and 90 kDa, could be formed by proteins which play some role in sperm motility. Identifying these proteins is the key to understanding their precise functions.

In the present study, the high presence of lipid transport proteins (apolipoproteins) in sperm samples classified into the motility I category (0-25% of total motility) suggests that this family of proteins could play a role in the early phases of sperm production. In a previous study on male European eels (Baeza et al., 2015), it was shown that certain levels of some polyunsaturated fatty acids (PUFAs) are required during the early phases of sperm production, and must be transported to the testis from the muscle and liver.

For example EPA (20:5n-3, Eicosapentaenoic acid) appears to be necessary as a component of the spermatozoa membrane).Thus, this further corroborates our results, because the higher presence of lipid transport proteins (apolipoproteins) in the sperm coincides with the presence of samples classified into the motility category I, probably when the transport of PUFAs is still necessary for the creation of the spermatozoa membrane. At the same time, the decrease in these apolipoproteins in the sperm samples classified into motility category III of (final sperm maturation) suggests that the requirement of fatty acids may be lower.

In our study, the complement C3 was present in high quantities in the motility III samples (>50% of total motility), suggesting this protein has an immunologic role against microbial infection, especially during the final sperm maturation stages. In a study on several freshwater species, brown trout (*Salmo trutta f. fario*), burbot (*Lota lota*) and perch (*Perca fluvialis*), Lahnsteiner et al. (2010) observed a correlation between complement C3 levels, sperm motility parameters and the presence of immunoglobulins, indicating that C3/immunoglobulins play important physiological role in the sperm.

In this study the presence of carbonic anhydrase (CA) was observed in the seminal plasma of European eel. However, no variations in the levels of this protein in the different categories

of sperm motility were found. Perhaps, no differences were observed because sperm motility is a multivariable mechanism in which many factors are involved, and the necessary internal pH changes can also occur by other mechanisms, i.e. involving ion channels.

Taken together, these results suggest that proteins linked to lipid transport (apolipoproteins) and to the immune system (complement C3) may carry out their functions during different stages of the spermatogenic process.

The present study has improved our understanding of the physiological mechanisms involved in sperm motility in the European eel. For the first time in eel a proteomic study has been carried out in order to provide in depth detail of the protein composition of seminal plasma during spermatogenesis and its correlation with sperm quality in this species. Understanding the functions of each protein at the different stages of spermatogenesis would allow us to improve the preservation of sperm quality in marine species, by complementing the dilution media with the most important proteins.

5. Conclusions

Although no differences were found in the protein profile of the different sperm motility groups, these results suggest that the proteins related to lipid transport (apolipoprotein) and to the immune system (complement C3) may carry out their functions during different stages of spermatogenesis. In addition, there were higher levels of proteins in the 20-60 kDa range in sperm samples with enhanced motility, suggesting that these proteins may have a role in determining spermatozoa motility.

Acknowledgements

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GENERAL DISCUSSION

1. The role of ionic agents during sperm motility activation: seminal plasma vs. activation medium

In this thesis a combination of different techniques was used to evaluate spermatozoa motility and size, including: intracellular ion measurement by flow cytometry and CASA-ASMA systems. Different ion channels inhibitors and ionophores were used in order to understand the physiological mechanisms of sperm motility activation in this particular marine fish, the European eel.

But, which are the most important agents in the regulation of sperm motility?. Are they those present in the seminal plasma or in the activating media?.

Ionic composition of the seminal plasma

Our research group has previously described the ionic composition of the seminal plasma of European eel in relation to the variation of sperm motility during the spermiation period (Asturiano et al., 2004). In the study, an increase in K^+ concentrations was observed, while Ca^{2+} and Mg^{2+} levels showed a progressive reduction in correlation with sperm motility improvement. Na^+ was maintained at constant levels.

In this thesis we have demonstrated that the presence of the ions K^+ and Na^+ in the seminal plasma (or in the extender medium) is necessary in order to maintain sperm motility (Chapter 1 and 2, respectively), but Ca^{2+} is not needed (Chapter 3). In fact, when Na^+ or K^+ were removed from the seminal plasma, sperm motility activation in SW was strongly reduced. These same results were not observed when Ca^{2+} was removed from the seminal plasma.

Regarding K^+, our results agree with those previously published by Ohta et al. (2001), who demonstrated a decrease in sperm motility after 30 min of incubation in a K-free extender.

Regarding Na^+, although this is the most abundant ion present in the seminal plasma of European eel (Asturiano et al., 2004), little is known about its role in the sperm of marine fish. In a freshwater fish species (brook trout, *Salvelinus fontinalis*), Na^+ has been shown to play a role in spermatozoa activation (Bondarenko et al., 2014). In this thesis, the importance of the presence of Na^+ in the seminal plasma of a marine fish has been demonstrated for the first time (Chapter 2).

In this thesis one important hypothesis has been suggested: the effect of Na^+ and K^+ presence is important for the further sperm motility. One of the reasons to explain this fact is that both ions are involved in the membrane potential (Em). If we consider that in quiescent stage Em due to the Na^+ is 5.75 mV, based on eel seminal plasma (Asturiano et al., 2004; $[Na^+]_e$=120 mM), and results from Chapter 2 (Vílchez et al., 2016; $[Na^+]_i$=96.72 mM). Em due to Na^+ would become even more depolarized (Em=34.6 mV) after contact with seawater, as $[Na^+]$ is higher in seawater ($[Na^+]$=355 mM) than in seminal plasma ($[Na^+]$=96.72 mM). Thus, considering Na^+, the seawater activation will depolarize the sperm cell.

However, if external Na^+ is removed from the seminal plasma, Em due to Na+ will become hyperpolarized (Em=-183.31 mV), as $[Na^+]_e$ would be lower than $[Na^+]_i$ (96.72 mM). So, if we compare the Em in a medium K-free (Em =-186 mV, Chapter 1) with a medium Na-free

(Em=-183.3 mV) we will observe that, in both cases, the effect of the absence of these ions is a hyperpolarization of the spermatozoa membrane.

Therefore, this hyperpolarization before the hyperosmotic shock could be related with the sperm motility initiation

Regarding the pH of the seminal plasma, we have reported that a pH gradient seems to be present in the European eel sperm in the quiescent stage. This finding is supported by the pH_i measurements taken in the quiescent stage (pH_i = 7.2) which were 1.3 units lower than the pH measured in the seminal plasma (pH= 8.5, Asturiano et al., 2004). This gradient seems to be important, because when the sperm was incubated in P1 with a pH of 6.5 (0.6 units lower than pH_i) sperm motility was supressed after activation (Peñaranda et al., 2009).

On the other hand, the results obtained in this study using flow cytometry (see Chapter 1) show the influence pH_i has on sperm motility, as the pH_i alkalinization (by NH_4Cl) in the quiescent stage inhibited sperm motility activation, while pH_i acidification (by Na-acetate) had no effect on sperm motility.

Nowadays, the ion Ca^{2+} is considered the most important ion for sperm motility activation (Boitano and Omoto, 1992; Tanimoto et al., 1994; Krasznai et al., 2000; Kho et al., 2004; Alavi et al., 2011; Takei et al., 2012). Within this context, Ca^{2+} has been shown to be a major element in triggering fish sperm motility in both freshwater and marine species (salmonids (Boitano and Omoto, 1992), (Tanimoto et al., 1994, Kho et al., 2004, Takei et al., 2012); common carp, (Krasznai et al., 2000); sturgeon, (Alavi et al., 2011). Moreover, there are several types of Ca^{2+} channels which control the initiation of spermatozoa motility (sea urchin; Darszon et al., 2011). However, this thesis has demonstrated for the first time that the presence Ca^{2+} in the seminal plasma or the activation media is not essential for sperm motility activation in the European eel (Chapter 3). The most important difference between our thesis and previous studies (pufferfish; Oda and Morisawa, 1993) is that in all of our experiments, ion-free solutions have been carefully prepared and all the materials have been cleaned with ultrapure water containing EDTA to avoid any Ca^{2+} contamination. This Ca^{2+} chelator was not used in the activation medium in previous studies (Oda and Morisawa, 1993), thus trace elements of this ion Ca^{2+} could be masking the results. Therefore, in contrast to K^+ and Na^+ removal, Ca^{2+} removal from the seminal plasma (or from the extender medium) did not decrease the further sperm motility activation in the European eel (Chapter 3).

Ionic composition of the activation media

Regarding the ion composition of the activation media, we have reported that eel spermatozoa were able to initiate movement without K^+, Na^+, or Ca^{2+} in the activation medium. That supports the fact that European eel sperm can be activated in non-ionic hyperosmotic solutions (sucrose, mannitol, data not shown), like the sperm of many other marine fish species such as pufferfish (Morisawa and Suzuki, 1980; Gallego et al., 2013b), European sea bass (Dreanno et al., 1999) or cod (Cosson et al., 2008).

Several sperm velocities (VCL, VSL, VAP) decreased when sperm was activated in the Ca-free activator, thus supporting the theory that Ca^{2+} has a modulatory effect on sperm motility.

These results support Krasznai et al. (2003a) who reported that puffer fish spermatozoa was activated in either Ca^{2+} free or Ca^{2+} containing artificial sea water. However, in another marine fish species, the herring, it was shown that extracellular Ca^{2+} was necessary for the initiation of sperm motility (Vines et al., 2002).

Thus, that the fact that the presence of K^+ and Na^+ in the activation media does not affect sperm motility, and that sperm can be activated in non-ionic media (for example sucrose or mannitol, data not shown), suggests that European eel has an osmotic mode of motility activation rather than an ionic mode of motility activation. In other words, hypertonic shock is the main requirement for motility activation, irrespective of the ionic composition, with the exception of the pH (or $[H^+]$).

2. The role of seminal plasma ions on the sperm volume regulation

The reaction of the spermatozoa on their release into hypotonic or hypertonic solutions varies among freshwater or sea water fish species. Bondarenko et al. (2013) observed an increase in carp spermatozoa volume in hypotonic conditions, during the motility period. Also, Cabrita et al. (1999) demonstrated that rainbow trout (*Oncorhynchus mykiss*) spermatozoa swelled immediately after transfer into a hypotonic non-activating environment.

Although the shrinkage of marine fish spermatozoa after sea water activation has been postulated for more than 30 years this has been the first study to make sue of an ASMA system to measure the sperm head area before and after sea water activation (Chapters 1 and 2). There was approximately a 5.7% decrease in spermatozoa head area after sea water activation compared to quiescent sperm.

It is interesting that the shrinkage of the sperm head was observed in the quiescent stage, by the removal of K^+ or Na^+ from the media. It means the shrinkage is derived not only hyperosmolarity shock but also another reason. When we observed the measurements of head area from control samples and ion free samples (Na^+ and K^+ free), it was observed that the head area of control samples post-activated in ASW (an hyperosmotic medium, 7.6 μm^2) was similar to the head area of the samples Na^+ free and K^+ free in quiescent stage (an isoosmotic medium, 7.7 μm^2 and 7.6 μm^2, respectively). That means that the factor that could influence in the regulation of sperm volume changes is not only osmolality, but also the presence of ions such as Na^+ and K^+.

Moreover, the decrease of the head area of control samples post-activated in ASW could be the responsible for the intracellular sodium increase, from 96.72 mM (quiescent stage) to 152.21 mM (post-activation).

This reduction of spermatozoa head size seems to be necessary for cell activation, as when the spermatozoa head area was reduced in the quiescent stage, by the removal of K^+ or Na^+ from the seminal plasma, sperm motility was reduced noticeably (Chapters 1, 2). Thus, our results suggest a close relationship between sperm volume changes and the presence of Na^+ and K^+ in the seminal plasma.

It has been suggested that aquaporins, molecular water channels, play a role in spermatozoa motility activation in different sea water species (Cosson et al., 1999, 2004). The reduction in the spermatozoa head area observed in this study could be linked to the efflux of water

through the aquaporins (AQPs), whose presence in fish sperm was demonstrated by Zilli et al. (2009).

3. New data about which are the ions pathways during the sperm motility activation in marine fish

In this thesis, the effects of the sperm membrane modifications through the use of specific ionophores or ion channel inhibitors have been tested. In terms of the permeabilization of the spermatozoa membrane to specific ions in the quiescent stage, several ionophores were tested, including valinomycin (K^+ ionophore), A-23187 (Ca^{2+} ionophore) and monensin (Na^+ ionophore).

The inophore K^+ (valinomycin) inhibited sperm motility. When valinomycin was added, and sperm was activated, a slight (non-significant) increase in $[K^+]_i$ was observed at the same time as a significant increase in $[H^+]_i$ (or a decrease in the pH_i). In addition, when external K^+ was removed (prior to activation) a significant decrease in $[H^+]_i$ (thus a significant increase in the pH) was observed. Thus, both ions changed in the same direction; when K^+ increased, the protons increased, and when K^+ decreased, the protons also decreased. This would suggest there is a co-transport of K^+ and H^+ at work during activation, rather than a K^+/H^+ exchange which is what has generally been accepted in studies on animal sperm (Mac Aulay and Zeuthen, 2010; Zeuthen, 2010). Therefore, during sperm motility activation, the transport of K^+ and H^+ through the membrane would occur via co-transporters and be accompanied by an efflux of water, thus affecting the volume of fish spermatozoa and further sperm motility.

Several studies about fish sperm motility come from experiments using ion channel inhibitors. In this thesis several ion channel inhibitors were tested: TEA, $BaCl_2$ and 4-AP (K^+ channel inhibitors, Chapter 1); amiloride (Na^+ inhibitor, Chapter 2); Bepridil and W-7 (Ca^{2+} inhibitors, Chapter 3). Most of them caused a reduction in sperm motility, especially 4-AP (a voltage-gated K^+ channel inhibitor), amiloride (epithelial sodium channel blocker) and bepridil (inhibitor of the Na^+/Ca^{2+} exchanger, NCX). These results support previous studies on marine species (see Table 1), where similar results showing a reduction in sperm motility were observed (Herring, Vines et al., 2002; Japanese eel, Tanaka et al., 2004; and Atlantic croacker, *Micropogonias undulatus*, Detweiler and Thomas, 1998).

Table 1. Ion channel inhibitors/modulators tested in marine fish species.

Study	Species	Inhibitor	Effect
Tanaka et al., 2004	Japanese eel	4AP	Inhibition
Detweiler and Thomas, 1998	Atlantic croaker	4AP	Inhibition
Detweiler and Thomas, 1998	Atlantic croaker	amiloride	Inhibition
Zilli et al., 2008	Gilthead seabream	$BaCl_2$	No inhibition
Vines et al., 2002	Herring	bepridil	Inhibition
Krasznai et al., 2003a	Puffer fish	W-7	Inhibition

Therefore, the results from this thesis suggest that a Na^+ channel (sensitive to amiloride), a voltage-gated K^+ channel (inhibited by 4-AP), and a Na^+/Ca^{2+} exchanger (NCX, sensitive to bepridil), are involved in European eel sperm motility activation.

Quantitative measurements

In this thesis, a method for the quantitative analysis of $[Na^+]_i$ and $[H+]_i$ was set up, and used to study the variations in these ions during motility activation for the first time in European eel (*Anguilla anguilla*). Sperm motility activation induced a 1.5-fold increase in $[Na^+]_i$, with levels moving from 97 mM in the quiescent stage to 152 mM post-activation in sea water.

After sperm motility activation in a hyperosmotic medium (ASW or an ion-free activator medium), the intracellular fluorescence emitted by different dyes (FLUO-4 for $Ca^{2+}{}_i$, CoroNa Green for $Na^+{}_i$ and PBFI for $K^+{}_i$), increased compared to the levels recorded in the quiescent stage. However, the increase in $Na^+{}_i$ is not necessarily related to sperm motility, as a similar increase was also seen when the sperm was activated but motility was low (for instance, when the sperm was washed in a Na-free extender and then activated in seawater). Also, in some cases, no increase in $Na^+{}_i$ was observed, but motility was normal (when sperm was activated in a Na-free activation media). Also, the increase in $K^+{}_i$ does not appear to be related to sperm motility, as it also happened when sperm was activated but motility was low (4-AP inhibition, Chapter 1).

Our results therefore suggest that the increase in intracellular concentrations of Na^+ and K^+ could be a consequence of the reduction of the sperm volume observed after hyperosmotic shock (Chapter 1 and 2), rather than triggers of sperm motility initiation. However, when bepridil, (a Na^+/Ca^{2+} exchanger inhibitor) inhibited sperm motility, the increase in $Na^+{}_i$ was also inhibited. In this Na^+/Ca^{2+} exchange, maybe the Ca^{2+} efflux is more important than the Na^+ influx for the sperm motility, as the Na^+ influx does not seem very important for that process.

One of the most important results to come out of this thesis it is that the increase in $[Ca^{2+}]_i$ does not seem to be a pre-requisite for the induction of sperm motility in European eel. This result is very important, as flagellum movement in all animals, including fish sperm is thought to be Ca^{2+}-dependent. However, our Ca^{2+} measurement was taken 30 s post-activation, and Ca^{2+} increases can be transient (pufferfish, Oda and Morisawa, 1993), or occur in several fast waves (sea urchin, Alvarez et al., 2012). To confirm that Ca^{2+} is not necessary for eel sperm motility, Ca^{2+} measurements would have to be taken in the first milliseconds after sperm activation, as per the method used for other species (sea urchin, Darszon et al., 2006).

Quantitative pH measurements indicated a slight decrease in pH_i (7.2 in quiescent stage and 7.1) post-activation in sea water. It is generally accepted that European eel seminal plasma has a pH of 8.5 (Asturiano et al., 2004). Thus, in the quiescent stage, a pH gradient between the sperm cell and the external media exists, with a lower pH inside the cell. Also, it is well known that sea water has a pH about 8.2, and that European eel sperm cannot be activated at 6.5 but motility is still activated at pH 7.2. Taken together, these pH_i results indicate that for successful sperm motility activation the pH_i should be lower than the seminal plasma pH, and lower or equal to the activation medium pH.

4. Protein composition of the seminal plasma: key to improving sperm quality

For the first time proteomic methodologies have been used to determine the protein profile of European eel seminal plasma during spermiogenesis (see Chapter 4). The protein profile

determined in this thesis is the same as/similar to the protein components previously published in other fish seminal plasma (common carp; Dietrich et al., 2014). In addition both studies agree on the presence of proteins in the seminal plasma, including: transferrins, apolipoproteins, carbonic anhydrase or proteins from immunologic system like complement C3.

Indeed, the present results go one step further, because different protein profiles were observed in relation to the percentage of motile sperm cells. To be specific, apolipoproteins and complement C3 increased/decreased when sperm motility increased. These results suggest that proteins linked to lipid transport (apolipoprotein) and to the immune system (complement C3) may carry out their functions at different stages of spermatogenesis.

Therefore, understanding the functions of each protein at the different stages of spermatogenesis would allow us to improve sperm quality preservation in marine species, by complementing the dilution media with the most important proteins. For example, according to our results, the apolipoprotein family may be included in the artificial seminal plasma at low sperm motilities (during the early phases of spermiogenesis process), and complement C3 may be included in the artificial seminal plasma at higher sperm motilities (during advanced phases of spermatogenesis).

The results obtained in this study suggest there are no variations in the levels of carbonic anhydrase (CA) in the seminal plasma of sperm from different motility categories. Thus, it seems that the CA levels of the seminal plasma in both motility classes I and III are the same (I: 0–25% and III: 50% motile cells, respectively).

These results support a previous study on European eel (Asturiano et al., 2004) where the seminal plasma pH of different of sperm motility classes did not vary. Thus, it seems that in normal conditions, both the CA and pH levels of the seminal plasma remain constant, irrespective of the percentage of motile sperm cells. However, several studies have shown that modifications in both intracellular pH_i, and extracellular pH_e have a direct impact on sperm motility activation in fish.

During this thesis, when the pH of ASW was lower (6.2) than the pH_i (pH_i= 7.1, see Chapter 1) a strong reduction in sperm motility was observed (data not shown). Also, if the pH of seminal plasma was low (6.5), sperm motility was inhibited (Peñaranda et al., 2009). Moreover, when the pH_i was increased with Na-acetate, the motility was supressed, but when the pH_i was decreased by ammonium chloride, the motility was similar to that of the controls. These results indicate that for activation to occur eel sperm needs a low pH_i and a high extracellular pH. This is also supported by the fact that an increase in pH_i was observed at the same time as a notable reduction in motility after the removal of K^+ from the seminal plasma.

Therefore, our results show/prove that sperm motility is a multivariable mechanism involving many factors, and that the pH_i changes occur not only due to CA (carbonic anhydrase) but also as a result of other mechanisms, i.e. changes in ion levels.

In summary, we are one step closer to understanding the sperm motility activation of marine fish, in particular of the peculiar species that is the European eel.

New studies about membrane potential and its relation to K^+, Na^+ and pH, the role of sperm volume changes on sperm motility initiation, as well as the ion fluxes occurring in the first milliseconds after sperm activation would add to our understanding of the motility activation process occurring in marine fish sperm.

As a general conclusion, in this particular species the ionic composition of the seminal plasma is much more important for sperm motility than the ionic composition of the activating media.

CONCLUSIONS

i. For the first time in a marine fish, a significant decrease in sperm head area has been demonstrated post activation in sea water.

ii. The presence of K^+ and Na^+ in the extender medium and in the seminal plasma is important for the preservation of sperm motility in the European eel, at least in part by maintaining the right sperm cell volume which decreases during the normal sperm activation process. However, the presence of the ion Ca^{2+} in the seminal plasma or the activation media is not essential for sperm motility activation in the European eel.

iii. The intracellular pH of eel spermatozoa has been quantified before and after sperm motility activation (7.2 and 7.1, respectively), and for successful sperm motility activation the intracellular pH has to be lower than the seminal plasma pH, and lower or equal to the activation medium pH.

iv. This study has determined for the first time the absolute intracellular [Na^+] concentration of a marine fish before and after sperm activation (96.72 mM and 152.21 mM, respectively). The fact that the levels observed were very similar to the [Na^+] levels found in the seminal plasma of the European eel (109 mM) indicates that, in the quiescent stage, there is a Na^+ equilibrium outside/inside the spermatozoa.

v. Although an increase in intracellular Na^+ occurs after sperm activation, this increase is not related to sperm motility. This increase in intracellular Na^+ after activation is the result both of the decrease in cell volume and the influx of external Na^+

vi. An increase in intracellular [Ca^{2+}] is not necessary for sperm motility activation in the European eel, however sperm velocities and flagellum beat frequencies are reduced in Ca-free conditions.

vii. A 4-AP-sensitive voltage gated potassium channel and amiloride-sensitive sodium channel are involved in the sperm-motility activation of the European eel.

viii. The increase in intracellular [Ca^{2+}] and the decrease in intracellular [Na^+] produced by bepridil, and the subsequent strong inhibition of sperm motility.demonstrates that a sodium/calcium exchanger is involved in the sperm motility of the European eel.

ix. For the first time the 2DE protein composition of the seminal plasma of the European eel has been identified, showing a total of 14 different proteins corresponding to 9 major families.

x. The fact that apolipoprotein levels in the seminal plasma are higher at lower motilities, whereas complement C3 levels are greater at high motilities suggests that the proteins linked to the transportation of lipids (apolipoprotein) and to the immune system (complement C3) carry out their functions at different stages of sperm maturation.

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