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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 (Dietrich et al., 2014; Zilli et al., 2005; Lahnsteiner, 2003).

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, 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

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(*Scophthalmus maximus*) differs from that of salmonids (Billard, 1983) 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, MA et al., 2010; Dietrich, M et al., 2010).

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, 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, 1977; 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, 2010b). 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, 2010b, 2010c) 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, 2010b, 2010c) 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. (2013) 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 R&D, Paterna, Spain) and observed in a Nikon Eclipse 80i microscope, with a 10× lens (Nikon negative phase contrast 10×). 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 R&D, 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 °C 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 °C. The protein content of the seminal plasma was determined using the Pierce BCA protein assay (Pierce Chemical Company, Rockford, IL) 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 4 h. The protein bands were photoedited and quantitatively analyzed 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 (7 M urea, 2 M 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 (Fig. 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, Fig. 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 (Fig. 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 (Fig. 1B and C respectively).

Taking the class motility as a basis, significant differences were found in the proteins linked to lipid transport (apolipoprotein, Fig. 2A and B) and the immune system (complement C3, Fig. 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

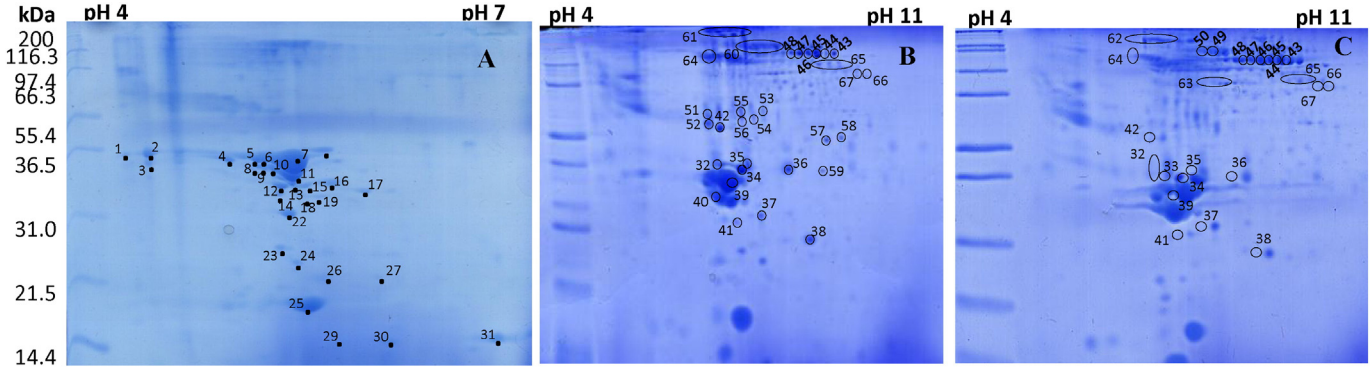

Fig. 1. Two dimensional gel electrophoresis of seminal plasma from European eel. A) Pool (n = 18 sperm samples) representative of sperm motilities; category I, II and III (0–25%, 25–50% and >50% of motile cells, respectively), B) Pool of category I of motility (n = 6 sperm samples), C) Pool of category 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).

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.

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). 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 (Fig. 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 (Fig. 4) and most of them were around 3.5 to 110 kDa standard proteins (Fig. 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. The proteins present around 19 kDa (Fig. 5A) showed a significant increase in the 12th and 13th weeks of treatment. However, the proteins present around 90 kDa (Fig. 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 (Fig. 6A), and therefore they were classified into category I of motility (Fig. 6B). Only one week later (in the 7th week of treatment), 50% of the males had reached category II (Fig. 6B), with more than 25% motile cells (Fig. 6A). The first samples with more than 50% of motile cells (Fig. 6A), were observed at week 8 (30% of males). In the following weeks, in most of the cases, the three motility classes were reported (Fig. 6B).

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 (Fig. 7A) with more than 20% of progressive cells.

A significant increase of VCL and BCF kinetic parameters (Fig. 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 (Keyvanshokooh et al., 2009; Forné et al., 2009; Li et al., 2010a, 2010b; Zilli et al., 2005) but this is the first time that the protein composition of the seminal plasma of eel has been analyzed.

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 and Flajnik, 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, 2001b). 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

Table 1
Proteins identified using ESI-CID-MS/MS·M_{ox}: 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	1 A
		614.3	2	DHLSEALTDVK					
		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
		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

(continued on next page)

Table 1 (*continued*)

Spot no.	MW (Da)	*m/z*	z	Peptide sequence	MASCOT score	Organism	Accession no.	Protein family	Figure
		791.4	2	YAAELHLVHWNTK					
37	31	656.9	2	TQXEPVVEEXR	*De novo*	*Anguilla japonica*	AAQ10893	Lipocalin	1B,1C
		503.7	2	SYSFXFSR	*De novo*				
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

temperature of the supposed spawning area (the Sargasso Sea) is around 20 °C (Boetius and Boetius, 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., 2010a, 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 (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., 2010a, 2010b, 2010c). 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

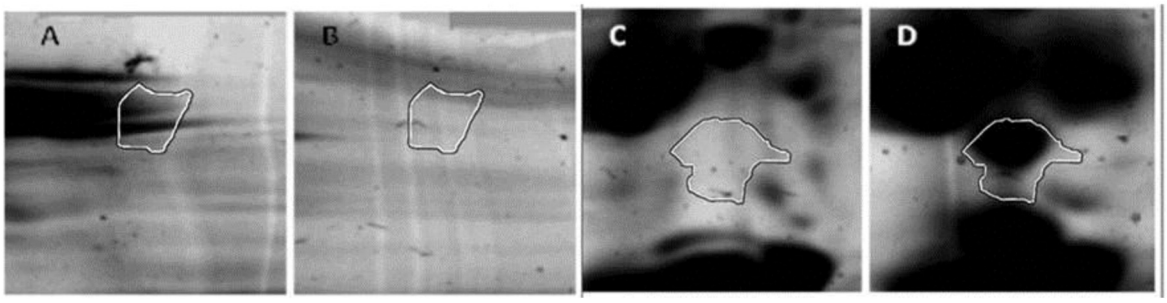

Fig. 2. Images from 2D geles with the presence in seminal plasma of; 28 kDa-2 apolipoprotein in samples showing sperm motility category I (A) and III (B), or presence of complement C3-like in samples showing sperm motility category I (C) and III (D).

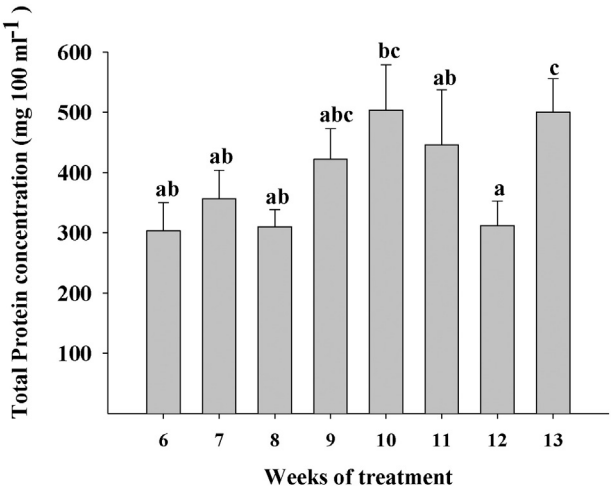

Fig. 3. Mean total protein content in seminal plasma of European eel during the different weeks of the treatment (n = 10 sperm samples by week). Data are expressed as mean ± SEM and different letters indicate significant differences ($P < 0.05$).

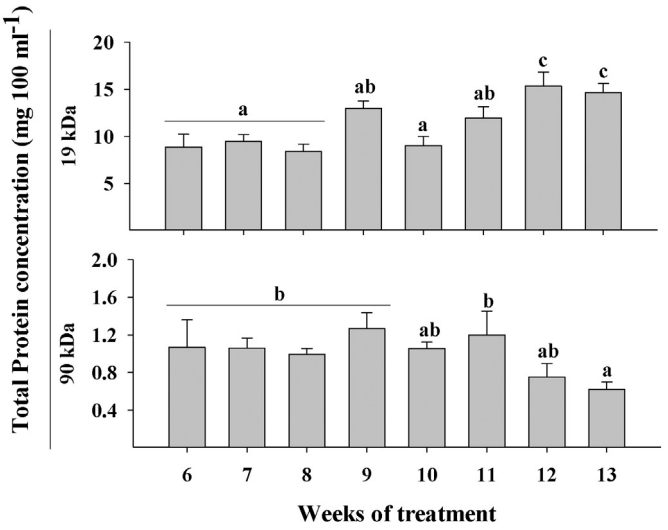

Fig. 5. Protein concentration for 19 and 90 kDa band (A and B respectively) present in individual samples of seminal plasma of European eel during the different weeks of the treatment. Data are expressed as mean ± SEM (n = 10 sperm samples per week). Different letters indicate significant differences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

of development by Peñaranda et al. (2010a, 2010b, 2010c). Proteins with a molecular weight of around 90 kDa have been observed in the seminal plasma of common carp (Kowalski et al., 2003b, Dietrich 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., 2012) 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., 2013), 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.

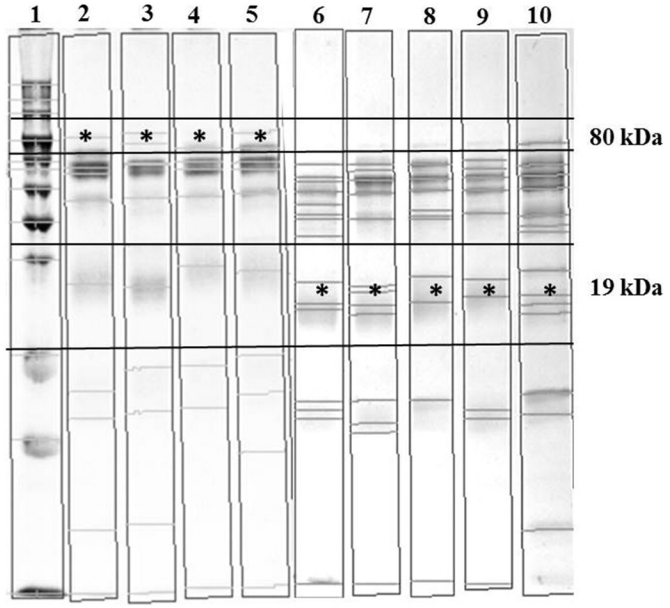

Fig. 4. Separation of European eel seminal plasma (1 μg of protein/μL, $n = 9$ sperm samples) by one-dimensional SDS-PAGE (15% acrylamide). The columns 2–5 and 6–10 are different sperm samples in 6th and 13th week of hormonal treatment respectively. Asterisks indicate significant differences between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

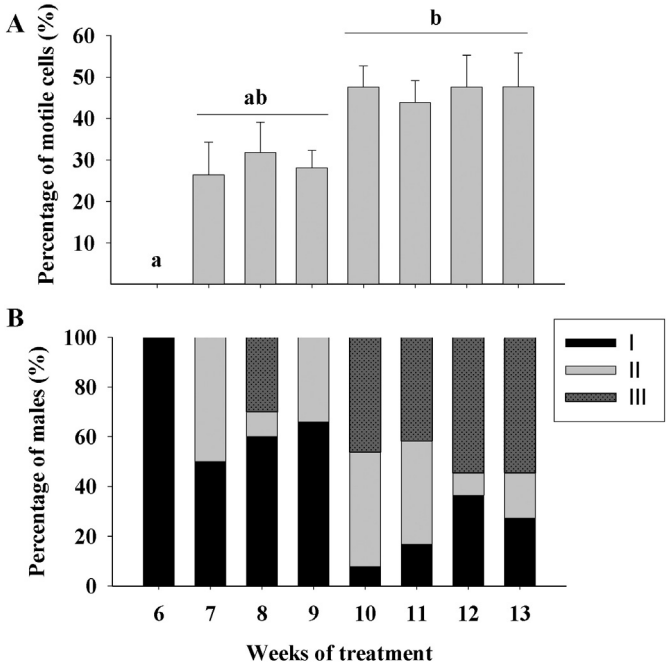

Fig. 6. A) Percentage of motile spermatozoa in European eel sperm throughout the different weeks of the hormonal treatment. B) Percentage of the different categories of sperm motility (I, II, III) during the weeks of treatment. Data are expressed as mean ± SEM (n = 10 sperm samples) and different letters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

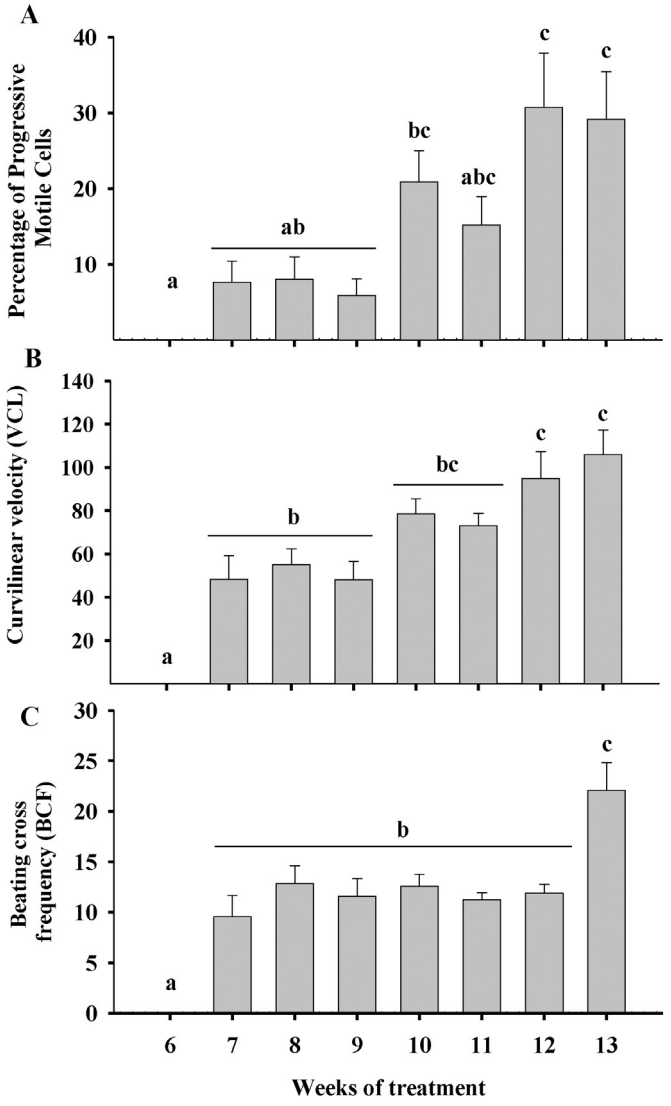

Fig. 7. Evolution of sperm quality parameters throughout the hormonal treatment: A) percentage of progressive motile cells, B) curvilinear velocity (VCL, μm/s) and C) beat frequency (BCF, beats/s). Data are expressed as mean ± SEM (n = 10 sperm samples) and different letters indicate significant differences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

In addition, the protein band with a molecular weight of 90 kDa showed the major level in the previous week (9th) of the increment of the motility (10th 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 and Radner (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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