

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

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11   **Running title:** Characterization of European eel seminal plasma proteome in relation to  
12   sperm quality

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33     **Abstract**

34     By first time, 2DE protein profile of European eel seminal plasma has been determined.  
35     14 different proteins corresponding to 9 major families were identified in seminal  
36     plasma, through hormonal treatment. Some of them play a part in sperm maturation,  
37     including carbonic anhydrase which is responsible for modulating the pH of seminal  
38     plasma, and warm temperature acclimation protein, which may play an important role in  
39     the final maturation of this species, due to the warm temperature of their spawning  
40     ground (in the Sargasso Sea).

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

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56     Keywords: Sperm motility, proteomics, CASA system, LC-MS/MS, 2D-  
57     Electrophoresis.

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61 **Highlights**

- 62 - For the first time the 2DE protein composition of the seminal plasma of the  
63 European eel has been identified.
- 64 - Lipid transport proteins (apolipoproteins) could play a role in the early phases of  
65 sperm production
- 66 - Immune system proteins (Complement C3) could have an immunologic role  
67 against microbial infection in the final stages of sperm maturation.
- 68 - It seems that proteins located at 19 KDa could be involved in the sperm motility  
69 of the European eel.

70

71     **1. Introduction**

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

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

87         However, only a few studies have focused on the identification of seminal plasma  
88         proteins and their physiological functions in fish. Loir et al. (1990) determined the  
89         concentrations of several organic components such as total proteins, amino acids, lipids,  
90         glucose, fructose and enzymes in rainbow trout and it was observed that the presence of  
91         these components varies, depending on the animals and sampling time. Also in rainbow  
92         trout, a total of 12 proteins were detected by SDS-PAGE and the influence of the  
93         presence of some proteins in the seminal plasma on the sperm quality has been  
94         demonstrated (Lahnsteiner et al., 2007). In another freshwater species, Nile tilapia  
95         (*Oreochromis niloticus*), it has been demonstrated that the presence of a high molecular

96 weight of glycoprotein in seminal plasma contributes to sperm immobilization  
97 (Mochida et al., 1999).

98 Studies about the composition of the seminal plasma of marine fish are even  
99 scarcer. The composition of the seminal plasma of turbot (*Scophthalmus maximus*)  
100 differs from that of salmonids (Billard et al., 1983a) in the total protein content (Suquet  
101 et al., 1993). However, in the case of both species, it seems that a high concentration of  
102 proteins may be linked to a possible role in spermatozoa protection. In turbot, sperm  
103 motility is reduced at high sperm dilutions (Suquet et al., 1992a) and is maintained by  
104 adding BSA (Bovine Serum Albumin, Fauvel et al., 1993a). This discovery is also  
105 supported by evidence showing that seminal proteins protect the spermatozoa against  
106 microbial attack (i.e. transferrin and anti-proteases), oxidative damage (i.e. transferrin,  
107 superoxide dismutase), and premature activation (i.e. parvalbumin) (Wojtczak et al.,  
108 2005a; Dietrich et al., 2010).

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

118 The introduction of proteomics in the study of male fish reproduction provides a  
119 unique opportunity to unravel the physiological mechanisms relating to sperm function,  
120 such as motility and fertilizing ability (Ciereszko et al., 2012). Thus, the use of

121 proteomic studies provides enormous advances in the identification of sperm proteins  
122 (Baker et al., 2007) and the proteins of human seminal plasma (Pilch and Mann et al.,  
123 2006).

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

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

140 Therefore, this decline in the eel population and the popularity of this species in  
141 the food market, has led researchers to look at reproduction in captivity. With this in  
142 mind, our group has worked on the development of extender media including 2% of  
143 BSA, which results in better motilities and viabilities for short-term storage (Peñaranda  
144 et al., 2010a, 2010b). Another example of the role of proteins in sperm quality, was the  
145 improvement in the percentage of motile cells post-thawing thanks to the addition of

146 fetal bovine serum (FBS) in the cryoprotectant medium (Peñaranda et al., 2009). This  
147 means that it is likely that the addition of extra-proteins in the media is related to  
148 enhanced sperm quality.

149 Peñaranda et al. (2010) evaluated the seminal plasma protein content of European  
150 eel, registering mainly four electrophoretic bands around 80, 40, 26 and 12 kDa. Three  
151 of them showed significant differences in concentration during maturation (80, 40 and  
152 12 KDa), and all of them showed the highest value at 8th week (previous to full  
153 spermiation period and best quality sperm period). Indeed, higher concentration of  
154 proteins around 40 KDa was observed at higher motilities. In order to confirm this  
155 possible role of seminal plasma proteins on sperm quality, it is necessary to discover the  
156 identity of these proteins and their precise physiological functions. With this objective,  
157 this study aims to increase our understanding of the reproductive physiology of this  
158 particular species, more specifically with regards to the protein composition of the  
159 seminal plasma. In addition, a study was carried out to determine the presence of the  
160 major proteins and their function in the different categories of sperm motility.

161

## 162 **2. Material and methods**

### 163 *2.1. Fish maintenance and hormonal treatment*

164 A total of 13 adult male European eels (mean body weight  $100\pm2$  g) from the fish  
165 farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were  
166 moved to our facilities, at the Universitat Politècnica de València (Spain). The fish were  
167 reared in a 150 L aquarium equipped with thermostat and cooler, and covered with  
168 black panels to maintain constant darkness. The eels were gradually acclimatized to sea  
169 water (salinity  $37.3\pm0.3$  g/L) over the course of 1 week, and were maintained in sea  
170 water until the end of the experiment.

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

176

177 *2.2. Human and Animal Rights*

178 This study was carried out in strict accordance with the recommendations in the  
179 Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree  
180 53/2013 on the protection of animals used for scientific purposes (BOE 2013). The  
181 protocol was approved by the Committee on the Ethics of Animal Experiments of the  
182 Universitat Politècnica de València (UPV) (Permit Number: 2014/VSC/PEA/00147).  
183 The fish were sacrificed by over-anesthesia with benzocaine (>60 ppm), and all efforts  
184 were made to minimize suffering. The fish were not fed throughout the experiment and  
185 were handled in accordance with the European Union regulations concerning the  
186 protection of experimental animals (Dir 86/609/EEC).

187

188 *2.3. Sperm collection and sampling*

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

196

197 *2.4. Sperm motility evaluation*

198 Sperm motility activation was performed as described by Gallego et al. (2013) by  
199 mixing 1 µl of diluted sperm (dilution 1:25 in P1 extender) with 4 µl of artificial sea  
200 water [SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2]. The  
201 mixture was made in a SpermTrack-10® chamber, with a depth if 10 µm (Proiser R&D,  
202 Paterna, Spain) and observed in a Nikon Eclipse 80i microscope, with a 10x lens (Nikon  
203 negative phase contrast 10x). The frame rate used was 60 fps. Motility was recorded 15  
204 seconds after mixing the sperm with sea water, using a high-sensitivity video camera  
205 HAS-220, and ISAS software (Proiser R&D, Paterna, Spain) was used to determine the  
206 sperm motility parameters. Each sample was evaluated in triplicate. Both the sperm and  
207 the sea water were maintained at 4 °C in a water bath during the sperm motility  
208 evaluation. The sperm samples were classified into three motility categories depending  
209 on the percentage of motile cells observed after sea water activation, I: 0-25%, II: 25-  
210 50% and III: >50%.

211 The parameters considered in this study were total motility (MOT, %), defined as  
212 the percentage of motile spermatozoa; progressive motility (P-MOT, %), defined as the  
213 percentage of spermatozoa which swim forward in 80% of a straight line; curvilinear  
214 velocity (VCL, in µm/s), defined as the time/average velocity of a sperm head along its  
215 actual curvilinear trajectory; average path velocity (VAP, µm/s), defined as the  
216 time/average of sperm head along its spatial average trajectory; straightness (STR, %),  
217 defined as the linearity of the spatial average path; and straight line velocity (VSL,  
218 µm/s), defined as the time/average velocity of a sperm head along the straight line  
219 between its first detected position and its last position; ALH, amplitude of the lateral  
220 movement of the sperm head and beat cross frequency (BCF; beats/s), defined as the

221 average rate at which the curvilinear sperm trajectory crosses its average path trajectory.  
222 Spermatozoa were considered motile if their progressive motility had a straight line  
223 velocity >10 µm/s.

224

225 *2.5. Isolation and concentration of the seminal plasma*

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

232 Once the seminal plasma was obtained and all the samples were classified into the  
233 different motility categories, a representative pool with all three motility categories (I, II  
234 and III, n=6 sperm samples/motility category, in total 18 sperm samples) was used for  
235 2D-Electrophoresis in order to identify the protein profile. In addition, in order to  
236 compare the appearance of different spots in each motility category, 2 different pools  
237 from each motility (in total 6 pools) were used in the 2D- Electrophoresis analysis .

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

241

242 *2.6. Appearance of protein band: 1D-SDS-PAGE*

243 Individual samples of seminal plasma were thawed at room temperature and run  
244 in 1-D sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (gradient  
245 SDS-PAGE; 4-15%) in vertical gels (AMERSHAM ECL™ GEL; BioRad, Madrid,

246 Spain). All the samples were processed under the same conditions: with a protein  
247 concentration of (1 µg/µl), at a constant voltage of 120 v and for 2 h. The gel was  
248 stained with Coomasie brilliant blue R-240 for 4h. The protein bands were photoedited  
249 and quantitatively analysed with GeneTools software (Syngene, IZASA, Spain) for  
250 band detection and molecular weight analysis.

251

252 *2.7. Identification of protein profile: 2D- electrophoresis*

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

265 IPG strips were placed onto second dimension SDS-PAGE (overall gel size 18.3 ×  
266 20.0 × 0.1 cm) which was performed using 1.5 cm 4% stacking gel (0.5 M Tris–HCl pH  
267 8.8, 30% acrylamide, 10% SDS, 10% APS, 0.1% TEMED) and 15% separation gel (1.5  
268 M Tris–HCl pH 8.8, 30% acrylamide, 10% SDS, 10% APS, 0.05% TEMED) using a  
269 Protean IIxi device (BioRad, Hercules, CA, USA). The gels were run at 20 °C at a

270 constant current of 75 V for 30 min, and then at 110-120 V until the dye reached the  
271 bottom of the gel.

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

282

### 283 2.8. Statistical analysis

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

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

294

295     **3. Results**

296     *3.1. Characterization of proteins in the seminal plasma*

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

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

307

308         Taking the class motility as a basis, significant differences were found in the  
309         proteins linked to lipid transport (apolipoprotein, Fig. 2A and B) and the immune  
310         system (complement C3, Fig. 2C and D), with higher amounts (8.425e+006 pixels/unit  
311         area) of apolipoproteins at lower motilities (category I) compared to higher motilities  
312         (2.141e+006 pixels/unit area). Conversely, the complement C3-like family protein was  
313         more abundant (1.129e+007 pixels/unit area) in the samples with the highest percentage  
314         of motile cells (category III) than in those with lower motilities (2.105e+006 pixels/unit  
315         area). No significant differences in the rest of the proteins were found between the  
316         different motility categories.

317

318     *3.2. Concentration of protein in the seminal plasma*

319         The mean protein content of the seminal plasma of all the samples was 384.18

320  $\pm 18.1$  mg/100 ml and no differences were found between the different sperm motility  
321 categories (data not shown). After 5 weeks of hormonal treatment a significant increase  
322 in the total protein content was observed (10<sup>th</sup> week). But two weeks later (12<sup>th</sup> week),  
323 the total content of protein decreased, showing the lowest values (Fig. 3). However,  
324 only one week later (13<sup>th</sup> week) the total protein content increased significantly,  
325 showing the highest values of the experiment with 500 mg/100 ml of protein in the  
326 seminal plasma.

327

### 328 *3.3. Appearance of protein band: 1D-SDS-PAGE*

329 In total, 9 bands were identified by SDS-PAGE (Fig. 4) and most of them were  
330 around 3.5 to 110 kDa standard proteins (Fig. 4). To facilitate the analysis of the results,  
331 areas around the main bands were photoedited (3.5, 10, 15, 20, 30, 40, 50, 60 and 80  
332 kDa) and evaluated using GeneTools software. The proteins present around 19 kDa  
333 (Fig. 5A) showed a significant increase in the 12<sup>th</sup> and 13<sup>th</sup> weeks of treatment.  
334 However, the proteins present around 90 kDa (Fig. 5B) showed a significant decrease in  
335 the last week of treatment (13<sup>th</sup> week).

336

### 337 *3.4. Sperm motility parameters throughout the hormonal treatment*

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

345       Regarding the sperm kinetic parameters, a significant increase was observed from  
346       10th week of treatment, but the highest values were observed during the last two weeks  
347       (Fig. 7A) with more than 20% of progressive cells.

348       A significant increase of VCL and BCF kinetic parameters (Fig. 7B and C,  
349       respectively) was observed from 7th week, registering the highest values in the last  
350       weeks of treatment. No differences were found in the rest of kinetic parameters  
351       analyzed (VAP, ALH and STR).

352

#### 353       **4. Discussion**

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

358       Using high-resolution 2D electrophoresis, we have been able to identify members  
359       of nine protein families with a total of 14 different proteins. Most of the spots analyzed  
360       in the seminal plasma were apolipoproteins. Recent studies have pointed to the presence  
361       of apolipoproteins in the seminal plasma of rainbow trout and carp (Nynca et al., 2010;  
362       Dietrich et al., 2014). These apolipoproteins may be linked to sperm energy resources  
363       and the maintenance of specific carp sperm membrane lipid composition (Dietrich et al.,  
364       2014). Also, apolipoproteins play an important role in the defense (adaptative defense  
365       mechanism) of carp epidermis and mucus against bacteria, as innate response (Concha  
366       et al., 2003).

367       The adaptative immune system is the response of the vertebrate immune system to  
368       a specific antigen that typically generates immunological memory and the  
369       immunoglobulins play varying roles similar to humoral response (Ohta et al., 2006).

370 Our study has shown that immunoglobulins are present in the seminal plasma of the  
371 European eel and may play an adaptative defense mechanism. Nevertheless, proteins  
372 from the innate defense were also found in our study, including the complement C3.  
373 The innate immune system is an ancient evolutionary form and crucial for the first line  
374 of defense (Hoffmann et al., 1999). The complement system mediates a chain of  
375 reactions of proteolysis and assembly of protein complexes, playing a major role in the  
376 body's defense as a part of both the innate and adaptive immune systems (Walport,  
377 2001a,b). One of the most abundant groups of proteins in carp seminal plasma is the  
378 complement group (Dietrich et al., 2014). It is likely that these major proteins, one of  
379 which is complement C3, found in carp seminal plasma, are involved in the protection  
380 of the spermatozoa.

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

385 Another protein identified in this study was the warm temperature acclimation protein,  
386 Wap65. Recent studies using microarray analysis have indicated that this protein which  
387 is related to temperature acclimation may also be involved in immune responses (Sha et  
388 al., 2008). Wap65 was initially identified in the muscle tissue of several species  
389 including goldfish, carp, medaka and pufferfish (Kikuchi et al., 1995; Kinoshita et al.,  
390 2001; Hirayama et al., 2003, 2004). But it wasn't until 2014 that Dietrich et al.  
391 demonstrated the presence of Wap65 in the seminal plasma of common carp. In the case  
392 of the European eel, the temperature of the supposed spawning area (the Sargasso Sea)  
393 is around 20 °C (Boëtius and Boëtius, 1967). Thus, Wap65 may play an important role  
394 in the final stages of maturation of this species, with the levels of Wap65 increasing in

395 the tissues associated with warm temperature, as was observed in goldfish and carp  
396 (Watabe et al., 1993).

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

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

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

412 An important finding of this study was the presence of carbonic anhydrase (CA).  
413 Little is known on how sperm regulates rises in intracellular bicarbonate. However,  
414 since carbonic anhydrase (CA) is known to participate in the regulation of intracellular  
415 pH (Sly and Hu, 1995), several studies have demonstrated the relationship between CA  
416 and spermatozoa activation. In mammals, CA is key to early activation, catalyzing the  
417 equilibrium between  $\text{CO}_2$  and  $\text{HCO}_3^-$  (Wandernoth et al., 2010). Inaba et al. (2003)  
418 demonstrated that a CA specific inhibitor revealed that this enzyme is involved in the  
419 regulation of sperm motility in flatfish: halibut (*Verasper variegatus*), flounder

420 (*Verasper moserii*) and turbot (*Scophthalmus maximus*).

421 The protein profile found in our study contains a total of 9 bands, 4 of which (80,  
422 40, 20 and 12 kDa) correlate with the bands found in a previous study on European eel  
423 (Peñaranda et al., 2010). In the previous study, a decrease was seen in the band from 80  
424 kDa in the last few weeks of hormonal treatment (weeks 11 and 12). This correlates  
425 with the evolution of the band from 90 kDa in our study. The high amount of 90 kDa  
426 band observed before the peak in motility may be produced by germinal cell types  
427 (spermatocytes and spermatids) present in this gonadal stage, according to the  
428 description of the stages of development by Peñaranda et al. (2010). Proteins with a  
429 molecular weight of around 90 kDa have been observed in the seminal plasma of  
430 common carp (Kowalski et al., 2003b, Drietrich et al., 2014) and have been identified as  
431 serine proteases, probably involved in the protection of the spermatozoa. Thus, the  
432 increment in the 90 kDa band before the peak in motility may be related to the  
433 protection of the spermatozoa cells under formation.

434 Regarding the kinetic parameters, in the last two weeks (weeks 12 and 13) the  
435 percentage of total and progressive motile cells reached similar values (more than 40  
436 and 30% respectively) to those from a previous study (Gallego et al., 2012) with the  
437 same conditions of hormonal treatment and temperature. Therefore, the repetition of  
438 similar results in different experiments suggests that the maturation method (see section  
439 2.1.) is efficient and repetitive.

440 Generally, high protein concentration is a positive characteristic of fish sperm  
441 (Butts et al., 2013). In the present study, the total protein concentration of seminal  
442 plasma (mean content:  $3.84 \pm 18.1$  mg/ml) was higher than the values observed in  
443 Atlantic cod (*Salmo salar*; mean content: ~1 mg/ml, Butts et al., 2011), but lower than  
444 those found in turbot (*S. maximus*; mean content:  $8.8 \pm 1.6$  mg/ml Suquet et al., 1993).

445 Therefore, the total content of protein in the seminal plasma of marine species varies  
446 considerably. Another important finding from our study was that the highest protein  
447 concentrations were found at weeks 10 and 13, coinciding with the best sperm motility  
448 values. Recently it has been proposed that protein composition of the seminal plasma  
449 plays an important role in fertilization (Kaspar et al., 2007, Li et al., 2009). This data  
450 suggests a positive correlation between the concentration of proteins in the seminal  
451 plasma and sperm motility.

452 In addition, the protein band with a molecular weight of 90 kDa showed the major  
453 level in the previous week (9<sup>th</sup>) of the increment of the motility (10<sup>th</sup> week). Also, a  
454 significant increment was seen in the 19 kDa band in the last two weeks of treatment  
455 (weeks 12 and 13), coinciding with high VCL and BFC kinetic parameter values and  
456 also with the highest progressive motility. Therefore, it seems that both bands; 19 and  
457 90 kDa, could be formed by proteins which play some role in sperm motility.  
458 Identifying these proteins is the key to understanding their precise functions.

459 In the present study, the high presence of lipid transport proteins (apolipoproteins)  
460 in sperm samples classified into the motility I category (0-25% of total motility)  
461 suggests that this family of proteins could play a role in the early phases of sperm  
462 production. In a previous study on male European eels (Baeza et al., 2015), it was  
463 shown that certain levels of some polyunsaturated fatty acids (PUFAs) are required  
464 during the early phases of sperm production, and must be transported to the testis from  
465 the muscle and liver. For example EPA (20:5n-3, Eicosapentaenoic acid) appears to be  
466 necessary as a component of the spermatozoa membrane). Thus, this further  
467 corroborates our results, because the higher presence of lipid transport proteins  
468 (apolipoproteins) in the sperm coincides with the presence of samples classified into the  
469 motility category I, probably when the transport of PUFAs is still necessary for the

470 creation of the spermatozoa membrane. At the same time, the decrease in these  
471 apolipoproteins in the sperm samples classified into motility category III of (final sperm  
472 maturation) suggests that the requirement of fatty acids may be lower.

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

481 In this study the presence of carbonic anhydrase (CA) was observed in the  
482 seminal plasma of European eel. However, no variations in the levels of this protein in  
483 the different categories of sperm motility were found. Perhaps, no differences were  
484 observed because sperm motility is a multivariable mechanism in which many factors  
485 are involved, and the necessary internal pH changes can also occur by other  
486 mechanisms, i.e. involving ion channels.

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

490 The present study has improved our understanding of the physiological  
491 mechanisms involved in sperm motility in the European eel. For the first time in eel a  
492 proteomic study has been carried out in order to provide in depth detail of the protein  
493 composition of seminal plasma during spermatogenesis and its correlation with sperm  
494 quality in this species. Understanding the functions of each protein at the different

495 stages of spermatogenesis would allow us to improve the preservation of sperm quality  
496 in marine species, by complementing the dilution media with the most important  
497 proteins.

498

499 **Conclusions**

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

506

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513

514 **References**

- 515 Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L.,  
516 Asturiano., 2015. Relationship between sperm quality parameters and the fatty  
517 acid composition of the muscle, liver and testis of European eel. Comp. Biochem.  
518 Physiol. A 181: 79–86.
- 519 Baker, M.A., Reeves, G., Hetherington, L., Müller, J., Baur, I., Aitken, R.J. 2007.  
520 Identification of gene products presents in Triton X-100soluble and insoluble  
521 fractions of human spermatozoa lysates using LC-MS/MS analysis. Proteomics  
522 Clin. Appl, 1:524-532.

- 523 Billard, R., 1983a: Effects of coelomic and seminal fluids and various saline diluents on  
524 the fertilizing ability of spermatozoa in the rainbow trout, *Salmo gairdneri*. J.  
525 Reprod. Fertil. 68, 77–84.
- 526 Boletín Oficial del Estado (2013) Real Decreto 53/2013 sobre protección de animales  
527 utilizados en experimentación y otros fines científicos. BOE 34 (1); 11370-11371.
- 528 Butts, I.A.E., Alavi, S.M.H., Mokdad, A., Pitcher., T.E. 2013. Physiological functions  
529 of osmolality and calcium ions on the initiation of sperm motility and swimming  
530 performance in redside dace, *Clinostomus elongatus*. Comp. Biochem. Physiol.  
531 A 166; 147–157.
- 532 Ciereszko, A., Dietrich, M. A., and Nynca, J. 2012. The identification of seminal  
533 proteins in fish: from a traditional approach to proteomics. J. Appl. Ichthyol. 28;  
534 865–872.
- 535 Concha, M.I., Molina, S., Oyarzun, C., Villanueva, J. and Amthauer, R. 2003. Local  
536 expression of apolipoprotein A-I gene and a possible role for HDL in primary  
537 defence in the carp skin. Fish Shellfish Immun. 14: 259-73.
- 538 Dietrich, MA, Źmijewski, D, Karol, H, Hejmej, A, Bilińska, B, Jurecka, P, et al. 2010.  
539 Isolation and characterization of transferrin from common carp (*Cyprinus carpio*  
540 L) seminal plasma. Fish Shellfish Immun. 29:66–74.
- 541 Dietrich M, Nynca J, Bilińska B, Kuba J, Kotula-Balak M, Karol H, et al. 2010.  
542 Identification of parvalbumin-like protein as a major protein of common carp  
543 (*Cyprinus carpio* L) spermatozoa which appears during final stage of  
544 spermatogenesis. Comp. Biochem. Physiol. 157:220–7.
- 545 Dietrich, M.A., Arnold, G. J., Nynca, J., Fröhlich, T., Otteb, K., Ciereszko, A. 2014.  
546 Characterization of carp seminal plasma proteome in relation to blood plasma. J.  
547 Proteomics 98; 218-232.
- 548 Fauvel C., Omnes, M. H., Mugnier, C., Normant, Y., Dorange, G., Suquet, M 1993a. La  
549 reproduction du turbot (*Scophthalmus maximus*): aspects biologiques et gestion  
550 des reproducteurs. Piscic Fr., 112, 23-39.
- 551 Forné, I., Agulleiro, M. J., Asensio, E., Abián, J., Cerdà, J. 2009. 2-D DIGE analysis of  
552 Senegalese sole (*Solea senegalensis*) testis proteome in wild-caught and hormone-  
553 treated F1 fish. Proteomics, 9, 2171–2181.
- 554 Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L.,  
555 Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative  
556 hormonal treatments on the reproductive performance of European eel males

- 557 (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354–355, 7–  
558 16.
- 559 Gallego, V., Carneiro, P.C.F., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Soler, C.,  
560 Pérez, L., Asturiano, J.F., 2013a. Standardization of European eel (*Anguilla  
anguilla*) sperm motility evaluation by CASA software. Theriogenology 79,  
561 1034–1040.
- 563 Hashemitabar, M., Bahmanzadeh, M., Mostafaie, A., Orazizadeh, M., Farimani, M.,  
564 Nikbakht, R. 2014. A Proteomic Analysis of Human Follicular Fluid: Comparison  
565 between Younger and Older Women with Normal FSH Levels. Int. J. Mol. Sci.,  
566 15, 17518-17540.
- 567 Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., Ezekowitz, R.A., 1999. Phylogenetic  
568 perspectives in innate immunity. Science 284, 1313–1318.
- 569 Hirayama, M., Nakaniwa, M., Ikeda, D., Hirazawa, N., Otaka, T., Mitsuboshi, T.,  
570 Shirasu, K., Watabe, S., 2003. Primary structures and gene organizations of two  
571 types of Wap65 from the pufferfish (*Takifugu rubripes*). Fish Physiol. Biochem.  
572 29, 211–224.
- 573 Hirayama, M., Kobiyama, A., Kinoshita, S., Watabe, S., 2004. The occurrence of two  
574 types of hemopexin-like protein in medaka and differences in their affinity to  
575 heme. J. Exp. Biol. 207, 1387–1398.
- 576 Inaba, K. 2003. Molecular architecture of the sperm flagella: molecules for motility and  
577 signaling. Zool Sci. 20:1043–1056.
- 578 Kandar, R., Drabkova, P., Myslikova, K., Hampl, R. 2014. Determination of retinol and  
579 α-tocopherol in human seminal plasma using an HPLC with UV detection.  
580 Andrologia, 46, 472–478.
- 581 Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., Alavi,  
582 SMH., Hulak, M., Linhart, O., 2007. Equalizing sperm concentrations in a  
583 common carp (*Cyprinus carpio*) sperm pool does not affect variance in  
584 proportions of larvae sired in competition. Aquaculture 27, 204–209.
- 585 Keyvanshokooh, S., Kalbassi, M.R., Hosseinkhani, S., Vaziri, B. 2009. Comparative  
586 proteomics analysis of male and female Persian sturgeon (*Acipenser persicus*)  
587 gonads. Anim. Reprod. Sci. 111; 361–368.
- 588 Kikuchi, K., Yamashita, M., Watabe, S., Aida, K., 1995. The warm temperature  
589 acclimation-related 65-kDa protein, Wap65, in goldfish and its gene expression. J.  
590 Biol. Chem. 270, 17087–17092.

- 591 Kinoshita, S., Itoi, S., Watabe, S., 2001. cDNA cloning and characterization of the  
592 warm-temperature-acclimation-associated protein Wap65 from carp *Cyprinus*  
593 *carpio*. Fish Physiol. Biochem. 24, 125–134.
- 594 Kowalski, R., Glogowski, J., Kucharczyk, D., Goryczko, K., Dobosz, S., and Ciereszko.  
595 A. 2003b. Proteolytic activity and electrophoretic profiles of proteases from  
596 seminal plasma of teleosts. J. Fish. Biol. 63, 1008–1019.
- 597 Lahnsteiner, F., Radner, M. 2010. Lysozyme activities and immunoglobulin  
598 concentrations in seminal plasma and spermatozoa of different teleost species and  
599 indications on its significance for sperm function. Theriogenology 74: 246–254.
- 600 Lahnsteiner F. 2003. Morphology, fine structure, biochemistry, and function of the  
601 spermatic ducts in marine fish. Tissue Cell;35:363–73.
- 602 Lahnsteiner. F. 2007. Characterization of seminal plasma proteins stabilizing the sperm  
603 viability in rainbow trout (*Oncorhynchus mykiss*). Anim. Reprod. Sci. 97; 151–  
604 164.
- 605 Li, P., Hulak, M., Rodina, M., Sulc, M., Li, Z-H., Linhart. O. 2010d. Comparative  
606 protein profiles: Potential molecular markers from spermatozoa of  
607 Acipenseriformes (*Chondrostei, Pisces*). Comp. Biochem. and Physiol. D 5; 302–  
608 307.
- 609 Li, P., Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M.,  
610 Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O. 2010. Ice-age  
611 endurance: the effects of cryopreservation on proteins of sperm of common carp,  
612 *Cyprinus carpio* L. Theriogenology 74; 413–423.
- 613 Li, P., Hulak, M., Linhart, O., 2009. Sperm proteins in teleostean and chondrostean  
614 (sturgeon) fishes. Fish Physiol. Biochem. 35, 567–581.
- 615 Li, P., Rodina, M., Hulak, M., Gela, D., Psenicka, M., Li, Z.H., Linhart, O. 2011.  
616 Physico-chemical properties and protein profiles of sperm from three freshwater  
617 chondrostean species: a comparative study among Siberian sturgeon (*Acipenser*  
618 *baerii*), starlet (*Acipenser ruthenus*) and paddlefish (*Polyodon spathula*). J Appl  
619 Ichthyol; 27:673–7.
- 620 Loir, M., Labbe, C., Maisse, G., Pinson, A., Boulard, G., Mourot, B., Chambeiron, F.  
621 1990. Proteins of seminal fluid and spermatozoa of rainbow trout (*Oncorhynchus*  
622 *mykiss*): Partial characterization and variations. Fish Physiol. Biochem. 8; 485–95.
- 623 Mochida, K., Kondo, T., Matsubara, T., Adachi, S., Yamauchi, K. 1999. A high  
624 molecular weight glycoprotein in seminal plasma is a sperm immobilizing factor

- 625       in the teleost Nile tilapia, *Oreochromis niloticus*. Develop. Growth Differ. 41,  
626       619–627.
- 627       Nynca J., Dietrich, M.A., Karol, H., Ciereszko, A. 2010. Identification of  
628       apolipoproteins C-I in rainbow trout seminal plasma. Reprod. Fertil. Dev.  
629       22:1183–7.
- 630       Ohta, Y., Flajnik, M. 2006. IgD, like IgM, is a primordial immunoglobulin class  
631       perpetuated in most jawed vertebrates. PNAS 103(28):10723–10728.
- 632       Peñaranda, D. S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J. F. 2010a.  
633       European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 45,  
634       407–415.
- 635       Peñaranda, D.S., Marco-Jiménez, F., Pérez, L., Gallego, V., Mazzeo, I., Vicente, J.S.,  
636       Jover, M., Asturiano, J.F. 2010b. Evaluation of different diluents for short-term  
637       storage of European eel sperm under air-limited conditions. J. Appl. Ichthyol. 26  
638       659-664.
- 639       Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Asturiano, J. F. 2009. Improvement  
640       of European eel sperm cryopreservation method by preventing spermatozoa  
641       movement activation caused by cryoprotectants. Cryobiology 59, 119–126.
- 642       Peñaranda, D.S., Marco-Jiménez, F., Pérez, L., Gallego, V., Mazzeo, I., Jover M.,  
643       Asturiano, J.F. 2010. Protein profile study in European eel (*Anguilla anguilla*)  
644       seminal plasma and its correlation with sperm quality. J. Appl. Ichthyol. 26, 746–  
645       752.
- 646       Pérez, L., Asturiano, J. F., Tomás, A., Zegrari, S., Barrera, R., Espinós, F.J., Navarro,  
647       J.C., Jover, M., 2000. Induction of maturation and spermiation in the male  
648       European eel: assessment of sperm quality throughout treatment. J. Fish Biol. 57,  
649       1488–1504.
- 650       Pérez-Cerezales, S., Martínez-Páramo S., Beirão, J., Herráez M.P. 2010. Evaluation of  
651       DNA damage as a quality marker for rainbow trout sperm cryopreservation and  
652       use of LDL as cryoprotectant. Theriogenology 74: 282–289.
- 653       Pilch, B., Mann, M., 2006. Large-scale and high-confidence proteomic analysis of  
654       human seminal plasma. Genome Biol 7:R40.
- 655       Rodríguez-Martínez, H., Kvist, U., Ernerudh, J., Sanz, L., Calvete, J.J. 2011. Seminal  
656       Plasma Proteins: What Role Do They Play? Am. J. Reprod. Immunol. 66 (Suppl.  
657       1) 11–22.

- 658 Suquet, M., Omnes, M.H., Normant, Y., Fauvel, C. 1992a. Assessment of sperm  
659 concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture 101;  
660 177-185.
- 661 Suquet, M., Dorange, G., Omnes, M.H., Normant, Y., Roux, Le., Fauvel, C. 1993.  
662 Composition of the seminal fluid and ultrastructure of the spermatozoon of turbot  
663 (*Scophthalmus maximus*). J. Fish Biol. 42,509-516.
- 664 Shaliutina, A., Hulak, M., Dzuyba, B., Linhart, O. 2012. Spermatozoa motility and  
665 variation in the seminal plasma proteome of Eurasian perch (*Perca fluviatilis*)  
666 during the reproductive season. Mol Reprod Dev; 79:879–87.
- 667 Sha, Z., Xu, P., Takano, T., Liu, H., Terhune, J., Liu, Z. 2008. The warm temperature  
668 acclimation protein Wap65 as an immune response gene: Its duplicates are  
669 differentially regulated by temperature and bacterial infections. Mol. Immunol.  
670 45:1458–1469.
- 671 Sly, W.S., Hu, P.Y. 1995. Human carbonic anhydrases and carbonic anhydrase  
672 deficiencies. Ann. Rev. Biochem. 64: 375– 401.
- 673 Tesch, W.W., 1977. The Eel. Biology and Management of Anguillid Eels. Chapman &  
674 Hall, London.
- 675 Van Ginneken, V.J.T., Maes, G.E., 2005. The European eel (*Anguilla anguilla*, L.), its  
676 lifecycle, evolution and reproduction: a literature review. Rev. Fish Biol. Fish. 15,  
677 367–398.
- 678 Walport, M.J., 2001a. Complement. First of two parts. N. Engl. J. Med. 344, 1058–  
679 1066.
- 680 Walport, M.J., 2001b. Complement. Second of two parts. N. Engl. J. Med. 344, 1140–  
681 1144.
- 682 Watabe, S., Kikuchi, K., Aida, K., 1993. Cold- and warm-temperature acclimation  
683 induces specific cytosolic protein in goldfish and carp. Nippon Suisan Gakkaishi  
684 59, 151–156.
- 685 Wandernoth, P. M., Raubuch, M., Mannowetz, N., Becker, H.M., Deitmer, J.M., Sly,  
686 W.S., Wennemuth, G., 2010. Role of Carbonic Anhydrase IV in the Bicarbonate-  
687 Mediated Activation of Murine and Human Sperm. Volume 5. Issue 11.e15061.
- 688 Wojtczak, M., Dietrich, G.J., Ciereszko, A. 2005a. Transferrin and antiproteases are  
689 major proteins of common carp seminal plasma. Fish Shellfish Immun. 19:387–  
690 91.

691 Zilli, L., Schiavone, R., Zonno, V., Rossano, R., Storelli, C., Vilella, S. 2005. Effect of  
692 cryopreservation on sea bass sperm proteins. Biol. Reprod. 72; 1262–1267.

693 **Tables captions**

694

695 **Table 1:** Proteins identified using ESI-CID-MS/MS. $M_{ox}$ : Methionine oxidation in European eel seminal plasma from; A) Pool (n=18) representative  
 696 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  
 697 category III of motility (n=6). Numbered protein spots correspond to proteins identified from 2D-Electrophoresis which are more abundant in  
 698 seminal plasma. Molecular mass marker (3.5-200 kDa).

699

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	<i>Anguilla japonica</i>	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					
3	36.5	515.3	2	IQADVDQLK	171	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins	1A
		535.3	2	LQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
5	36.5	490.9	2	DHLSEALTDVKDK	122	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins	1A
		535.3	2	IQADVDQLK					
		670.8	2	LQPVVEDLR					

		649.9	2	TKLQPVVEDLR				
6	36.5	612.3	2	AAVMoxYLQQVK	209	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins 1A
		614.3	2	DHLSEALTDVK				
		670.8	2	TLAEPYVQEYK				
		467.6	2	DKIQADVDQLKK				
		490.9	2	DHLSEALTDVKDK				
7	36.5	604.3	2	AAVGMYLQQVK	238	<i>Anguilla japonica</i>	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	AAVMoxYLQQVK	95	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins 1A
		614.3	2	DHLSEALTDVK				
9	36.5	535.3	2	LQPVVEDLR	146	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins 1A
		612.3	2	AAVMoxYLQQVK				
		670.8	2	TLAEPYVQEYK				
10	36.5	416.7	2	HLDEYR	235	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins 1A
		604.3	2	AAVGMYLQQVK				
		612.3	2	AAVMoxYLQQVK				
		614.3	2	DHLSEALTDVK				
		649.9	2	TKLQPVVEDLR				
		670.8	2	TLAEPYVQEYK				
		467.6	2	DKIQADVDQLKK				
11	36.5	535.3	2	LQPVVEDLR	289	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins 1A
		545.8	2	AAVMoxYLQQVK				
		670.8	2	TLAEPYVQEYK				
		467.6	2	DKIQADVDQLKK				
		490.9	2	DHLSEALTDVKDK				
12	36.5	416.7	2	HLDEYR	335	<i>Anguilla japonica</i>	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	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins
		612.3	2	AAVG <b>Mox</b> YLQQVK				1A
20	55.4	491.6	3	QFHFHWGGADDR	107	<i>Oryzias latipes</i>	XP_004081218	Carbonic anhydrase
		791.4	3	YAAELHLVHWNTK		<i>Oryzias latipes</i>		1A
22	31	515.3	2	IQADVDQLK	95	<i>Anguilla japonica</i>	BAB40960	Apolipoproteins
		535.3	2	LQPVVEDLR				1A
25	21.5	915.9	2	EALEPLAQHIPQSQAAK	90	<i>Anguilla japonica</i>	BAB40966	Apolipoproteins
		610.9	2	EALEPLAQHIPQSQAAK				1A
29	14.4	401.3	2	VGLVAVDK	100	<i>Tetraodon nigroviridis</i>	CAG06096	Immune system
		605.8	2	EYVLPSFEVK				1A
30	14.4	915.9	2	EALEPLAQHIPQSQAAK	82	<i>Anguilla japonica</i>	BAB40966	Apolipoproteins
		610.9	2	EALEPLAQHIPQSQAAK				1A
		677.4	2	AKEALEPLAQHIPQSQAAK				
31	14.4	706.4	2	VATGAAGE <b>X</b> APXVDK	<i>De novo</i>	<i>Anguilla japonica</i>	BAB40966	Apolipoproteins
33	31	736.8	2	QFHFHWGGADDR	108	<i>Oryzias latipes</i>	XP_004081218	Carbonic anhydrase
		791.4	2	YAAELHLVHWNTK				1B
34	31	736.8	2	QFHFHWGGADDR	156	<i>Oryzias latipes</i>	XP_004081218	Carbonic anhydrase
		791.4	2	YAAELHLVHWNTK				1C
37	31	656.9	2	TQXEPVVEEXR	<i>De novo</i>	<i>Anguilla japonica</i>	AAQ10893	Lipocalin
		503.7	2	SYSFXFSR	<i>De novo</i>			1B,1C
38	31	437.7	2	ATQSAQLR	147	<i>Anguilla japonica</i>	Q9I928	Lectin
		491.8	2	YVTVYLPK				1B,1C
		680.3	2	TFHCPQPM <b><i>x</i></b> IGR				
		453.9	2	TFHCPQPM <b><i>x</i></b> IGR				
		491.8	2	YVTVYLPK				
		680.3	2	TFHCPQPM <b><i>x</i></b> IGR				
		453.9	2	TFHCPQPM <b><i>x</i></b> IGR				

		748.4	2	QVYTITSVTITNR					
42	66.3	501.2	2	AVXDPTDDR	<i>De novo</i>	<i>Lepisosteus oculatus</i>	XP_006640345	Acetiltransferase	1B,1C
46	116.3	555.7	2	SADFEXXCR	<i>De novo</i>	<i>Takifugu rubripes</i>	XP_003974413	Transferrin	1B,1C
		983.5	2	(318.1)SFXYXGAEYMSXVR	<i>De novo</i>				
48	116.3	661.8	2	CLAEGGGDVAFVK	69	<i>Takifugu rubripes</i>	XP_003974413	Transferrin	1B,1C
50	97.4	677.3	2	VGTNFGFNDXNR	<i>De novo</i>	<i>Takifugu rubripes</i>	XP_003974413	Transferrin	1B,1C
57	66.3	677.3	2	VGTNFGFNDXNR	<i>De novo</i>	<i>Takifugu rubripes</i>	XP_003974413	Transferrin	1B,1C
60	116.3	510.8	2	DGLGDVAFVK	60	<i>Oryctolagus cuniculus</i>	P19134	Transferrin	1B
		682.8	2	CLVEKGDVAFVK					
61	200	565.3	2	GITTLPAVETK	201	<i>Anguilla anguilla</i>	ABY73532	Immune system	1B
		764.9	2	GFYPKEVLFWSWR					
		782.4	2	TATFACFASEFSPK					
		826.9	2	DFTPDLTTFKWNR					
62	200	707.4	2	TGATYTXXEGYPK	<i>De novo</i>	<i>Lateolabrax japonicus</i>	CCA29190	Hemopexin	1C
62		650.4	2	XQTVXDAXDAXK	<i>De novo</i>				
63	55.4	830.5	2	TPEEEHLGILGPVIR	73	<i>Lepisosteus oculatus</i>	XP_006637544	Ceruloplasmin	1C
64	116.3	699.3	2	VYVGTEYFEYK	<i>De novo</i>	<i>Lepisosteus oculatus</i>	XP_006639097	Hemopexin	1B,1C
		478.8	2	TDSVXFFK	<i>De novo</i>				
65	97.4	555.8	2	SADFEXXCR	<i>De novo</i>	<i>Takifugu rubripes</i>	XP_003974413	Transferrin	1B,1C

700 **Figure captions**

701

702 **Fig. 1:** Two dimensional gel electrophoresis of seminal plasma from European eel. A) Pool (n=18  
703 sperm samples) representative of sperm motilities; category I, II and III (0-25%, 25-50% and >50%  
704 of motile cells, respectively), B) Pool of category I of motility (n=6 sperm samples), C) Pool of  
705 category III of motility (n=6 sperm samples). Numbered protein spots correspond to proteins  
706 identified from 2D-MS/MS which are more abundant in seminal plasma. Molecular mass marker  
707 (3.5-200 kDa).

708

709

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

713

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

717

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

722

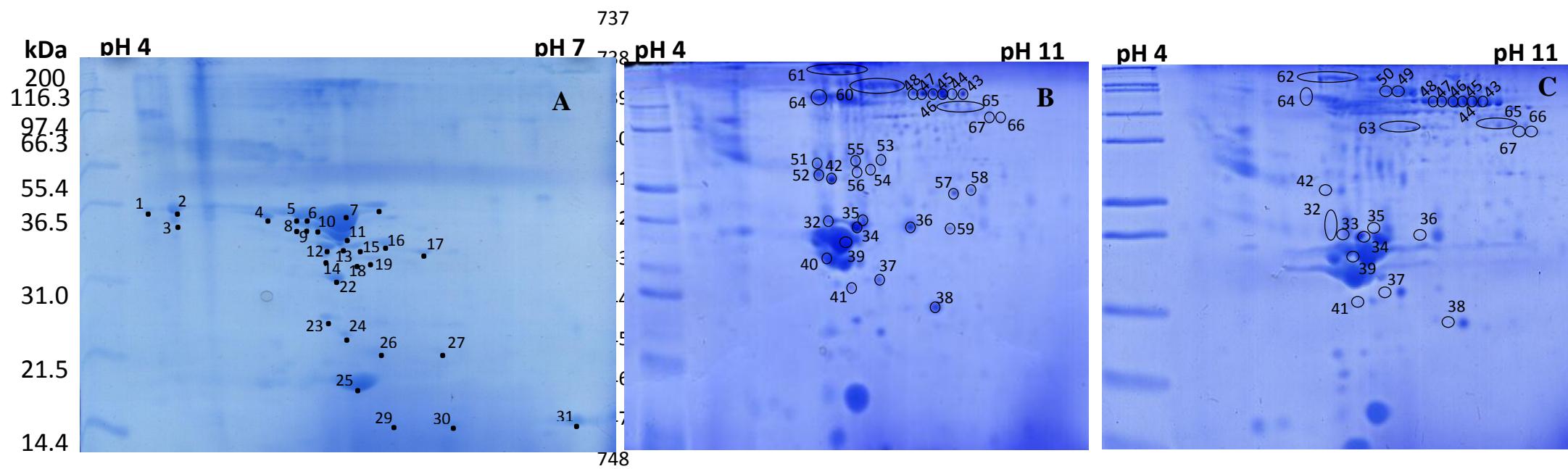
723 **Fig. 5:** Protein concentration for 19 and 90 kDa band (A and B respectively) present in individual  
724 samples of seminal plasma of European eel during the different weeks of the treatment. Data are  
725 expressed as mean±SEM (n=10 sperm samples per week). Different letters indicate significant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

727

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

732

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



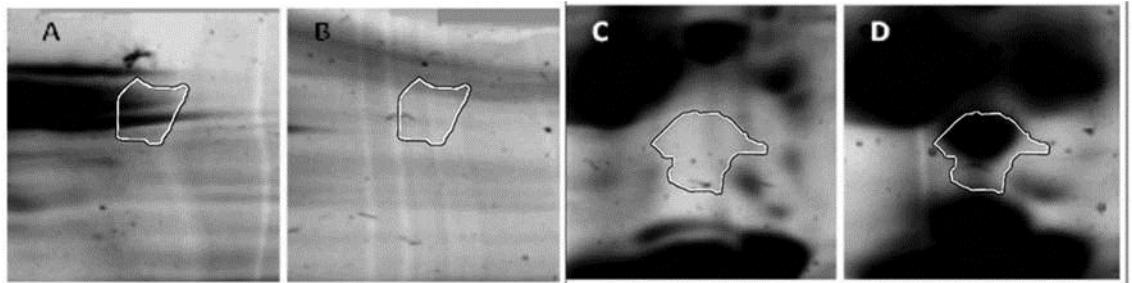
749

750 **Fig. 1**

751

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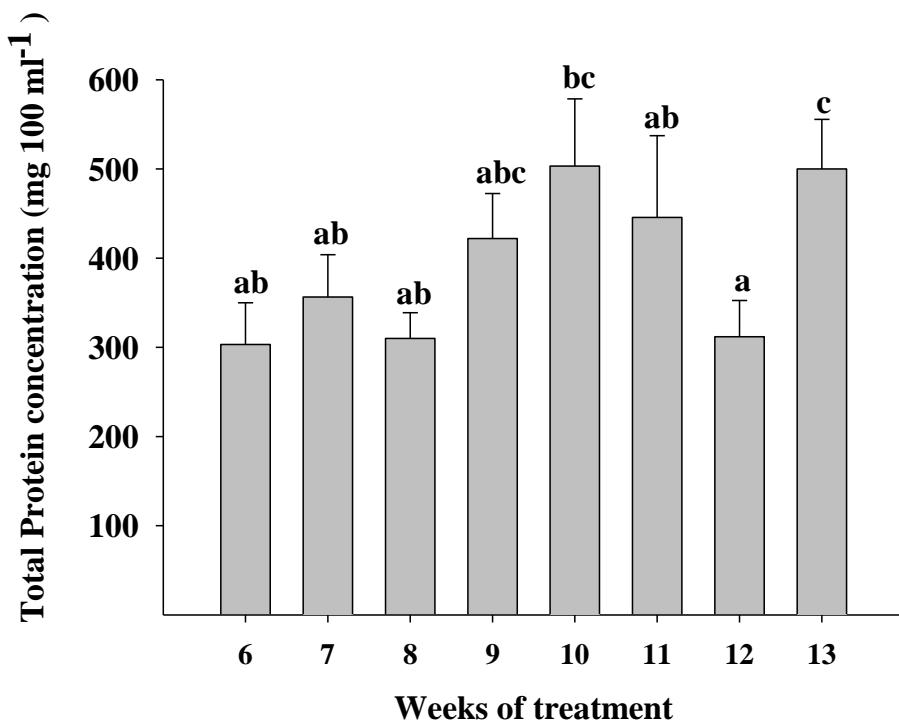


754

755

756 **Fig. 2**

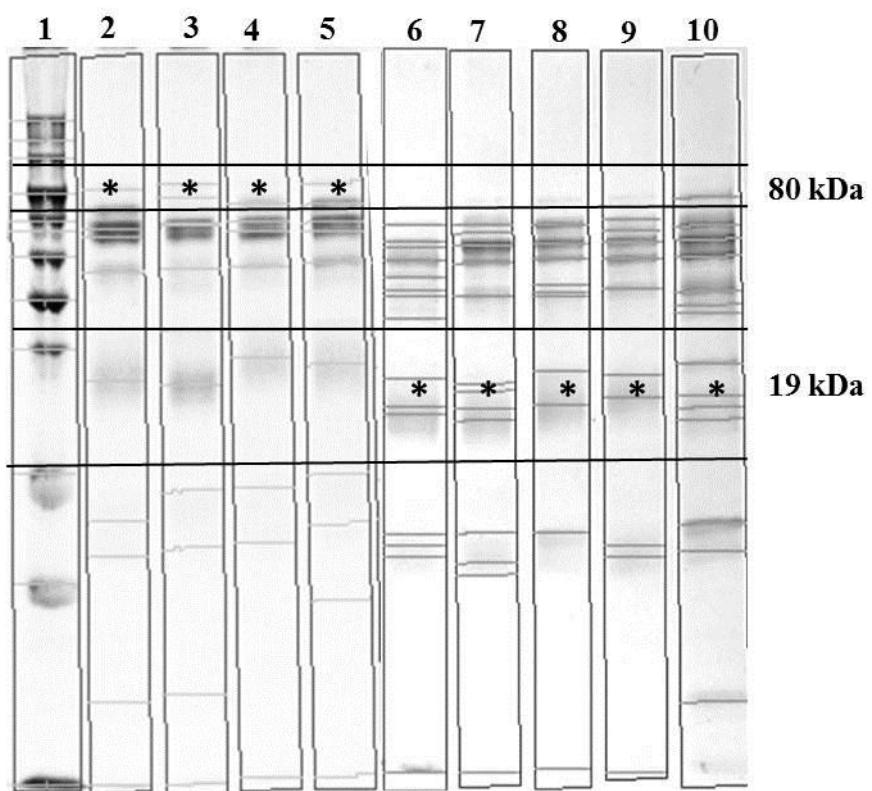
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758

759 **Fig. 3**

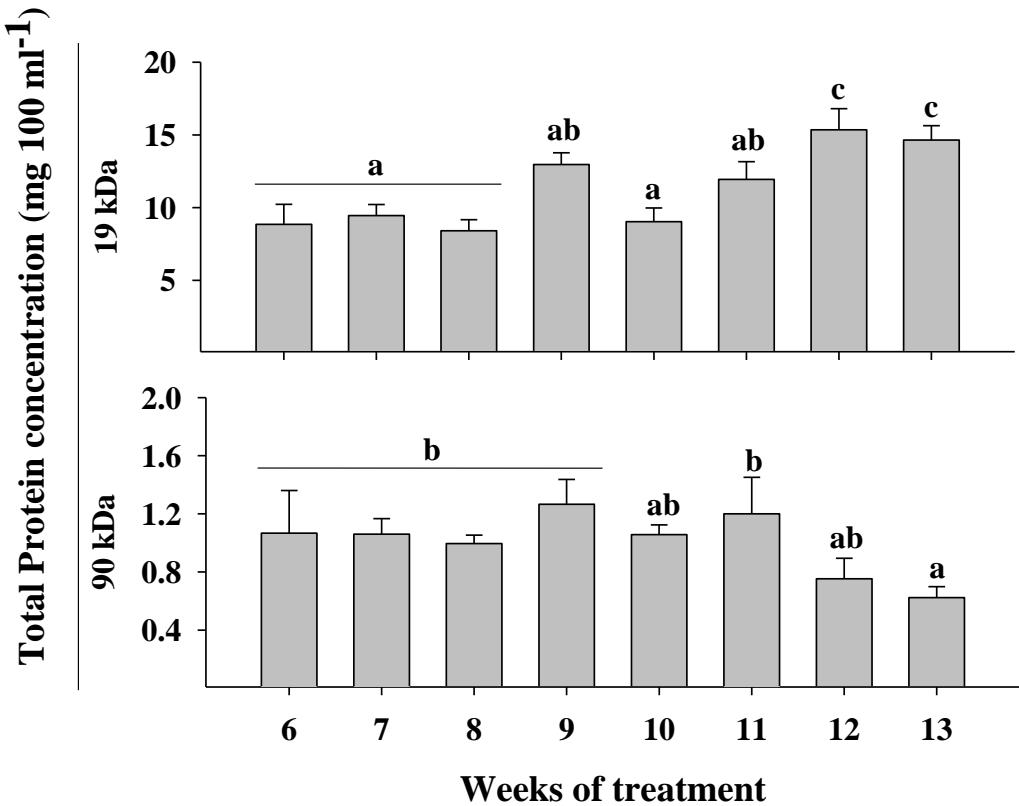
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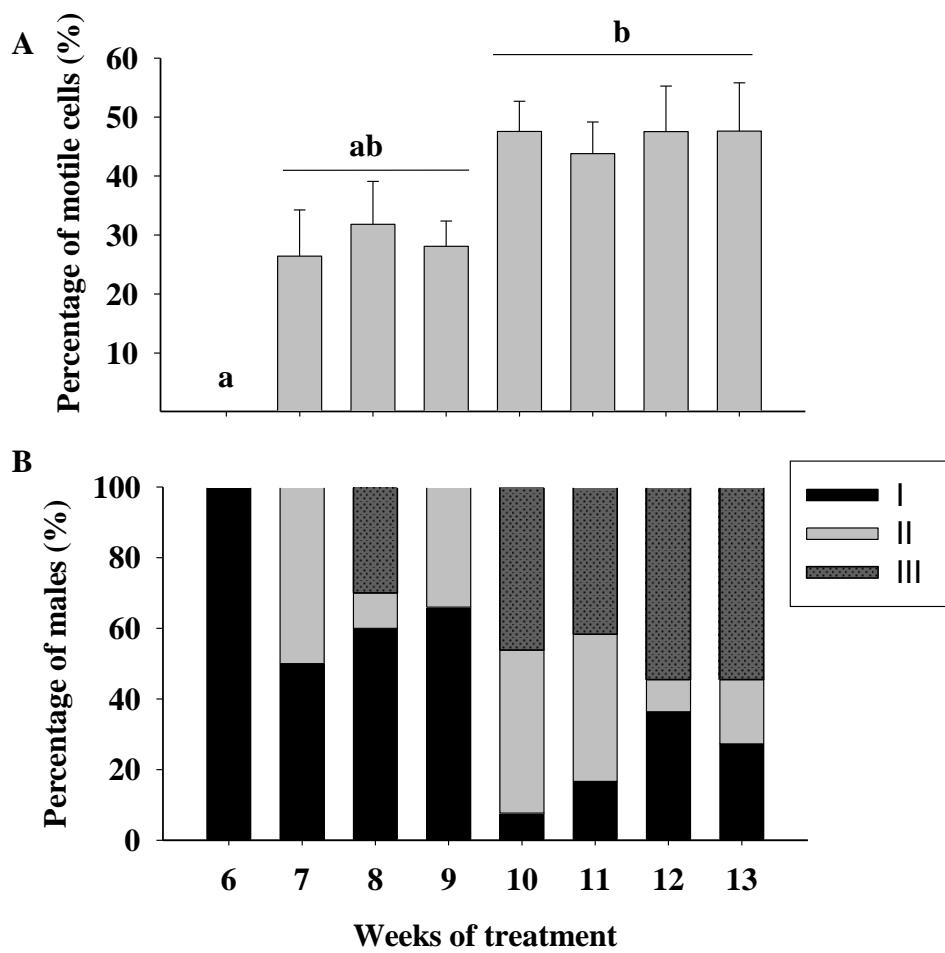
763 **Fig. 4**



764

765 **Fig. 5**

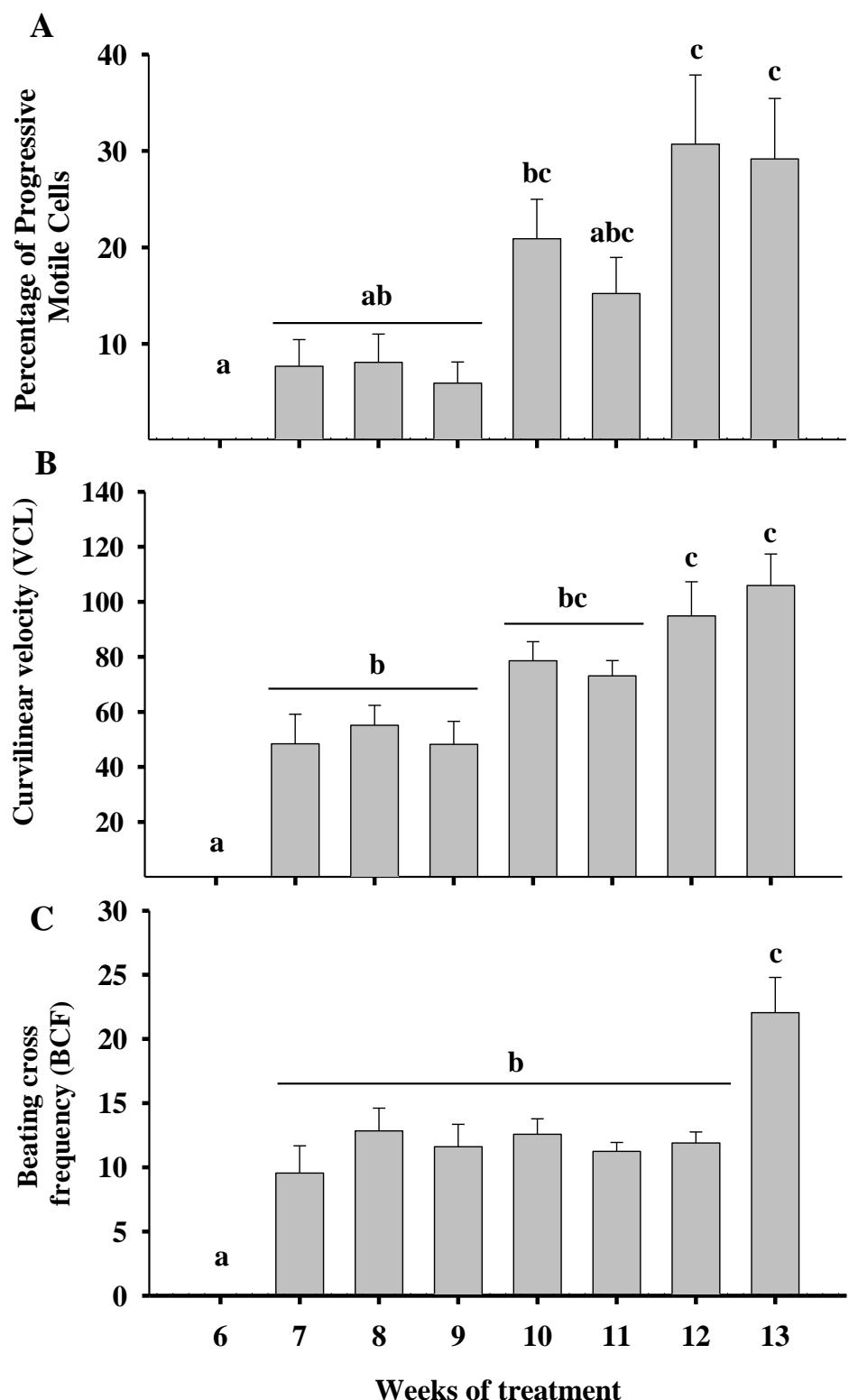
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767

768   **Fig. 6**

769



770

771 Fig. 7