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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
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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.
55

56 Keywords: Sperm motility, proteomics, CASA system, LC–MS/MS, 2D-
57 Electrophoresis.
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59

60
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±2 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±0.3 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/µL) 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 6th 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.

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 10x lens (Nikon negative phase contrast 10x). The frame rate used was 60 fps. Motility was recorded 15 seconds after mixing the sperm with sea water, using a high-sensitivity video camera HAS-220, and ISAS software (Proiser 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 ºC in a water bath during the sperm motility evaluation. The sperm samples were classified into three motility categories depending on the percentage of motile cells observed after sea water activation, I: 0-25%, II: 25-50% and III: >50%.

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

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 ºC. 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 anpholytes, 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

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.

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 acetiltransferases, 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 ±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 (10th week). But two weeks later (12th week),
323 the total content of protein decreased, showing the lowest values (Fig. 3). However,
324 only one week later (13th 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 12th and 13th weeks of treatment.
334 However, the proteins present around 90 kDa (Fig. 5B) showed a significant decrease in
335 the last week of treatment (13th week).
336
337 *3.4. Sperm motility parameters throughout the hormonal treatment*
338 Observation of spermiating 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 7th 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 CO_2 and 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±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±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 (9th) of the increment of the motility (10th 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 fluvialis*), 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
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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	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		449.3	2	AKLEPLVK					
		502.3	2	VQGEDLQSK					
		515.3	2	IQADVDQLK					
		535.3	2	LQPVVEDLR					
		545.8	2	LKPYAEELK					
		579.3	2	IQADVDQLKK					
		604.3	2	AAVGMYLQQVK					
		612.3	2	AAVGMoxYLQQVK					
		623.8	2	DKVQGEDLQSK					
		649.9	2	TKLQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
		490.9	2	DHLSEALTDVKDK					
3	36.5	515.3	2	IQADVDQLK	171	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		535.3	2	LQPVVEDLR					
		670.8	2	TLAEPYVQEYK					
		467.6	2	DKIQADVDQLKK					
5	36.5	535.3	2	LQPVVEDLR	122	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		670.8	2	TLAEPYVQEYK					

		649.9	2	TKLQPVVEDLR					
6	36.5	612.3	2	AAVGMoxYLQQVK	209	*Anguilla japonica*	BAB40960	Apolipoproteins	1A
		614.3	2	DHLSEALTDVK					
		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
		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

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 6th and 13th 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).

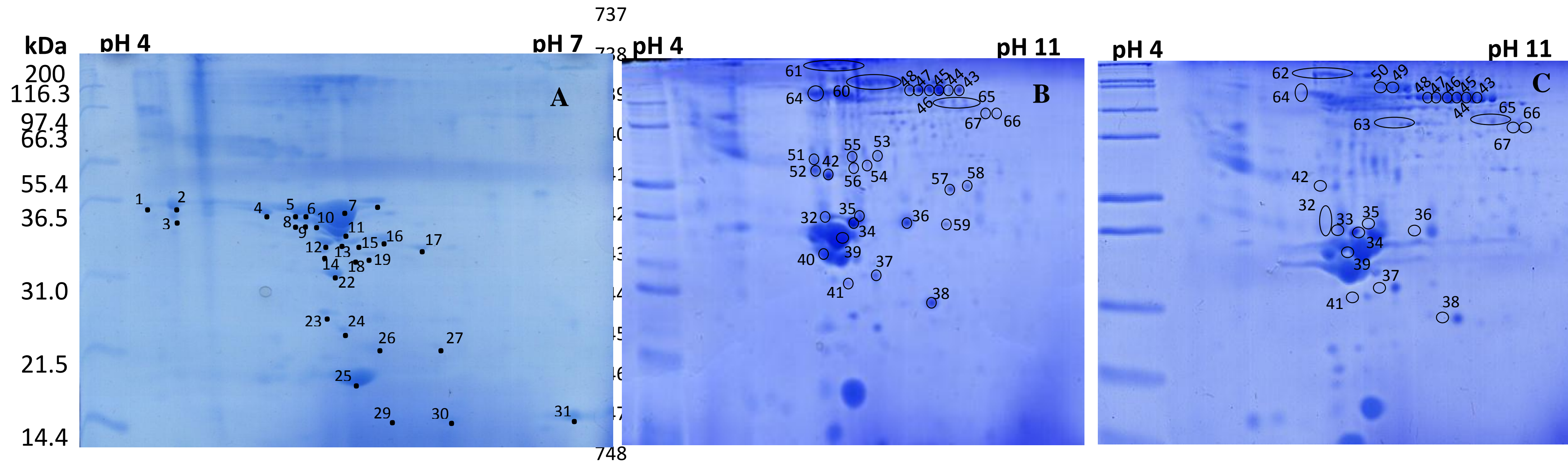
727

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, µm/s) and C) beat frequency (BCF, beats/s). 735 Data are expressed as mean±SEM (n=10 sperm samples) and different letters indicate significant 736 differences ($P<0.05$) between treatments at each week of treatment.

737



748

749

750 **Fig. 1**

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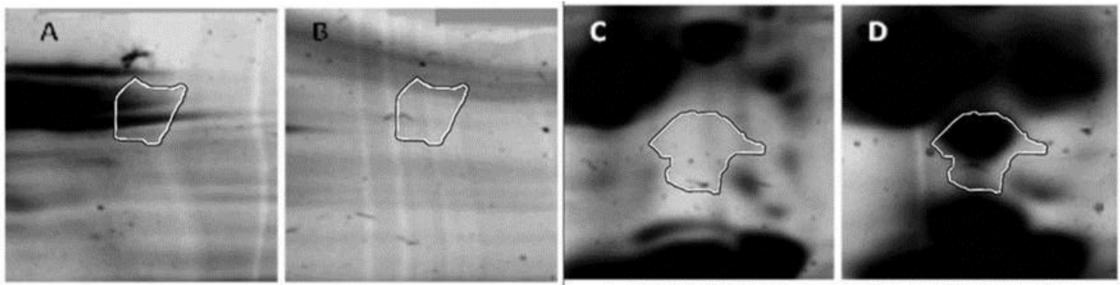
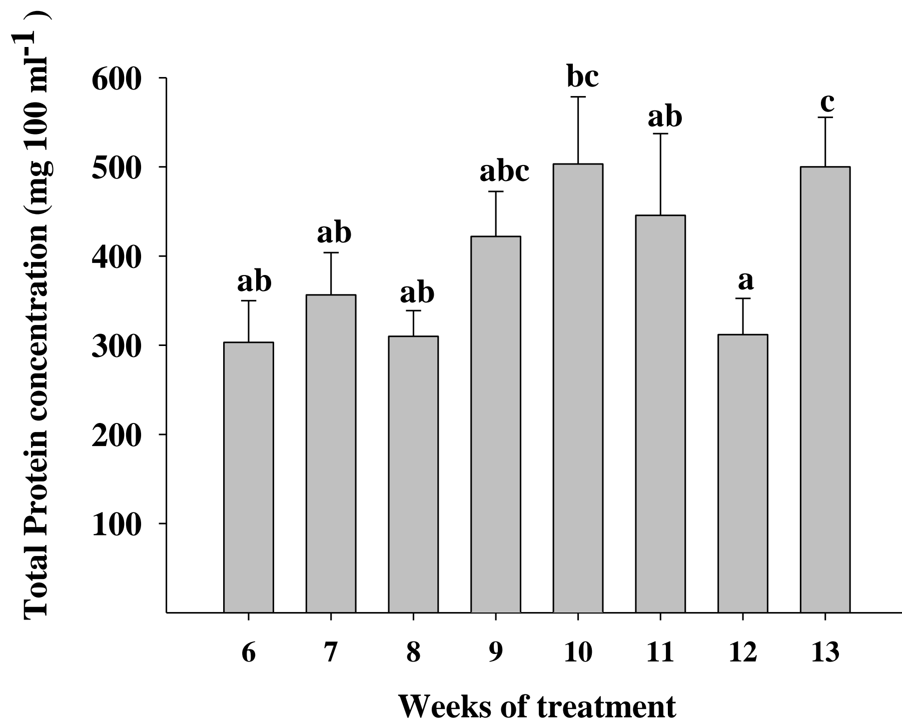


755

756 **Fig. 2**

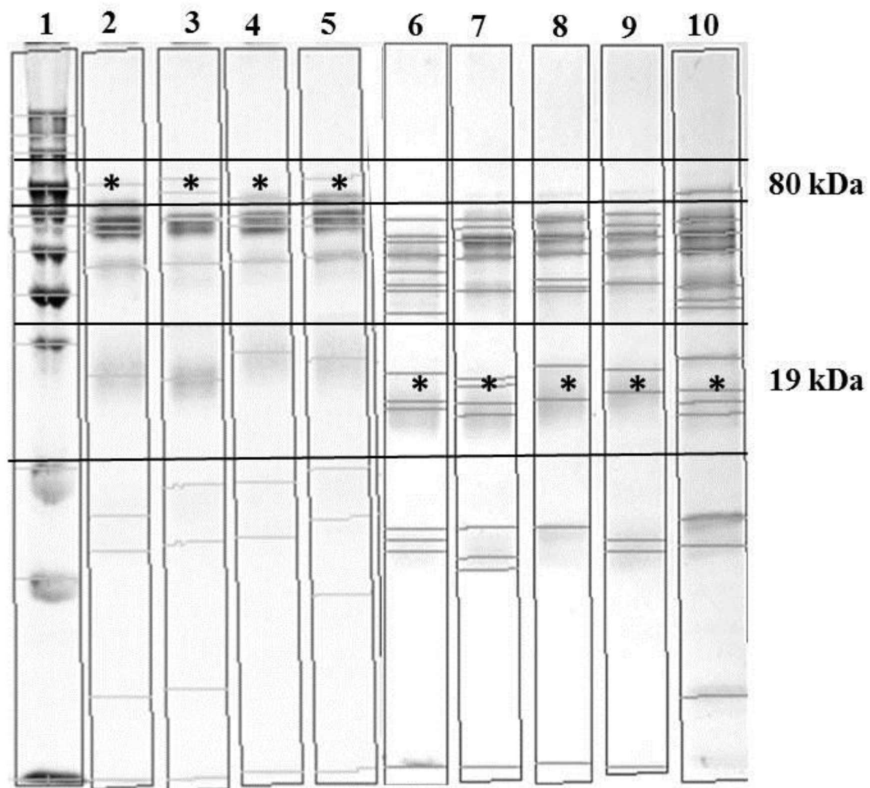
757



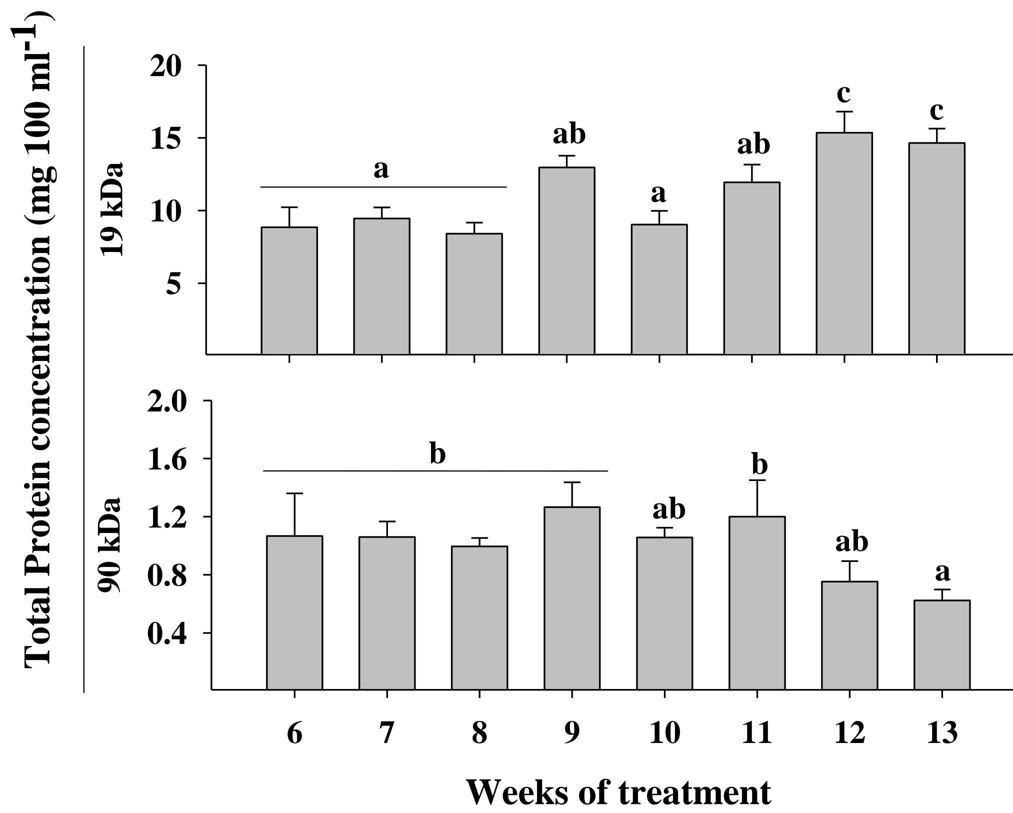
758

759 **Fig. 3**

760



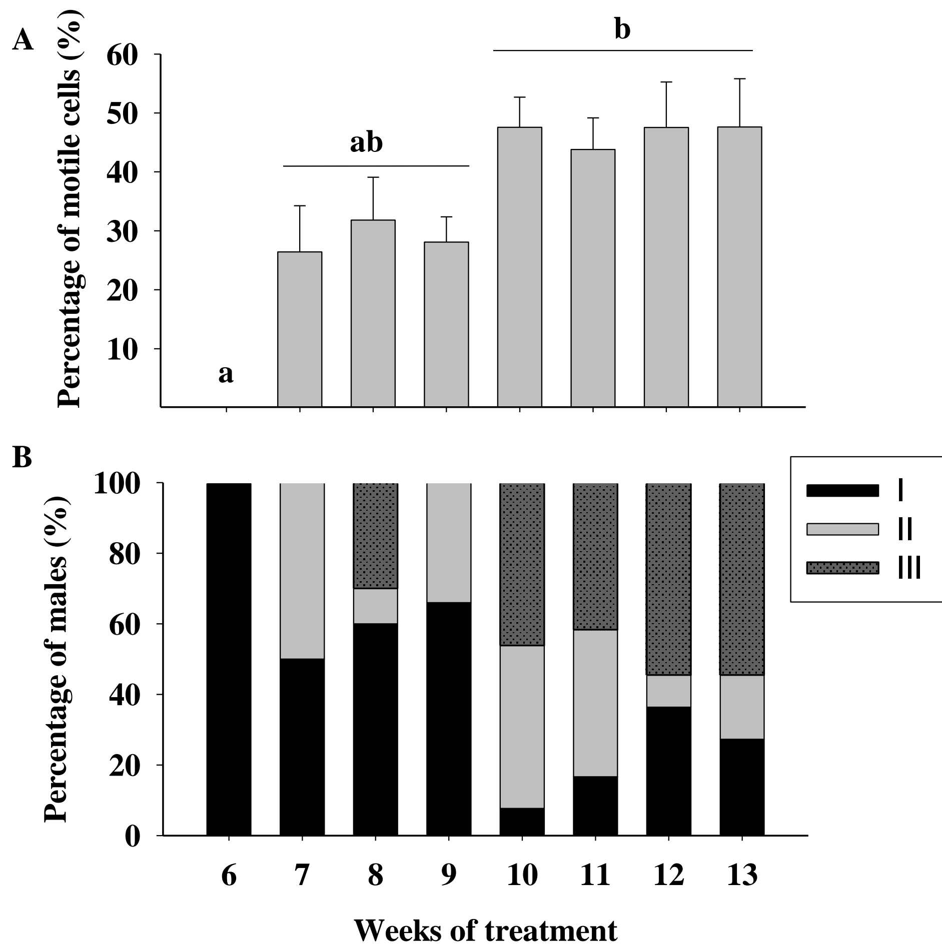
761
762
763 **Fig. 4**



764

765 **Fig. 5**

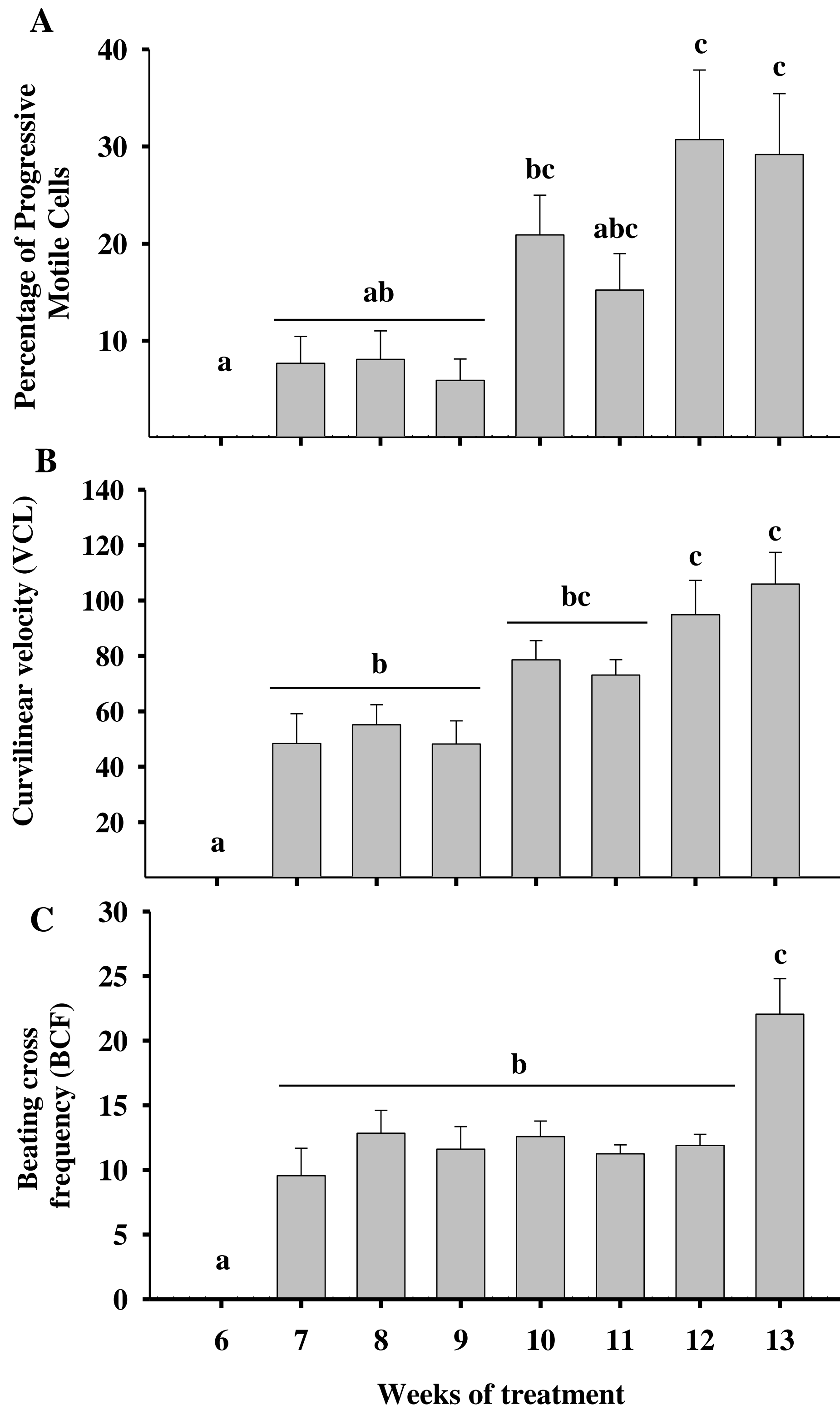
766



767

768 **Fig. 6**

769



770

771 **Fig. 7**