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Additional Information

Effect of the probiotic *Lactobacillus rhamnosus* on the expression of genes involved in European eel spermatogenesis

M. Carmen Vílchez[a*], Stefania Santangeli[b*], Francesca Maradonna[b], Giorgia Gioacchini[b], Cristina Verdenelli[c], Victor Gallego[a], David S. Peñaranda[a], Helge Tveiten[d], Luz Pérez[a], Oliana Carnevali[b**], Juan F. Asturiano[a**].

[a] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia (Spain)

[b] Department of Life and Environmental Sciences, Polytechnic University of Marche, Via Brecce Bianche, 60131 Ancona (Italy)

[c] School of Bioscience and Biotechnology, Via Gentile III da Varano, University of Camerino (Italy)

[d] Norwegian Institute of Fisheries and Aquaculture, Muninbakken 9-13, Breivika, P.O. Box 6122 NO-9291 Tromsø (Norway)

[*]These two authors equally contributed to the production of the paper.

[**]Corresponding author
Dr. Juan F. Asturiano
Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal
Universitat Politècnica de València
Camino de Vera s/n. 46022, Valencia, Spain
E-mail: jfastu@dca.upv.es

[**]Corresponding author
Dr. Oliana Carnevali
Department of Life and Environmental Sciences
Polytechnic University of Marche
Via Brecce Bianche, 60131 Ancona (Italy)
E-mail: o.carnevali@univpm.it

35 **Abstract**
36 Positive effects of probiotics on fish reproduction have been demonstrated in several species. In the
37 present study, 40 male European eels were weekly treated with recombinant hCG for 9 weeks as
38 well as with three different concentrations (10^3, 10^5, 10^6 CFU/ml) of probiotic *Lactobacillus*
39 *rhamnosus* IMC 501® (Sinbyotec, Italy), daily added to the water from the 6th week of the hCG
40 treatment. Males from treated groups and from control were sacrificed after one, two and three
41 weeks of probiotic treatment (7-9th weeks of hCG treatment), sperm and testis samples were
42 collected. Sperm volume was estimated and motility analysed by CASA software. Alternations in
43 transcription of specific genes involved in reproductive process such as *Activin*, Androgen Receptor
44 *α* and *β* (*arα* and *β*), Progesterone Receptor I (*pr1*), Bone Morphogenetic Protein 15 (*bmp15*) and
45 Follicle Stimulating Hormone receptor (*fshr*) were analyzed in the testis.
46 Following two weeks of probiotic treatment, sperm production and sperm motility parameters
47 (percentage of motile cells and percentage of straight swimming spermatozoa) were increased in the
48 European eel treated with 10^5 CFU/ml compared to controls or to the other probiotic doses. These
49 changes were associated with increases in *Activin*, *arα, arβ, pr1* and *fshr* mRNA levels of gene
50 expression. Conversely, after three weeks, *activin* and *pr1* expression were lowered. No significant
51 changes were observed on *bmp15* expression throughout the duration of the treatment with 10^5
52 CFU/ml dose.
53 The lowest (10^3 CFU/ml) and highest (10^6 CFU/ml) probiotic doses, along all the experiment,
54 inhibited the transcription of all genes except for *arα* and *β*, after one week of probiotic treatment
55 when compared to control.
56 The changes observed by transcriptomic analysis and the sperm parameters suggest that *L.*
57 *rhamnosus* at 10^5 CFU/ml for two weeks could improve spermatogenesis process in *Anguilla*
58 *anguilla.*
59

60 **Keywords**
61 *Anguilla anguilla*, probiotic, *Lactobacillus rhamnosus, mRNA levels, CASA system,*
62 *spermatogenesis.*
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68

69 **1. Introduction**

70 The quick decline of European eel (*Anguilla anguilla*) populations has led some research groups to
71 work on the optimization of protocols for maturation in captivity of males and females specimens.
72 Moreover, the European eels are not able to spontaneously mature in captivity and maturation, so
73 the maturation of males and females requires long-term hormonal treatments [1-4] and in this way,
74 a better understanding of the functions of the genes involved in European eel reproduction still
75 needs to be acquired.
76 In addition, control of the timing and maturation between the two sexes would be useful to develop
77 artificial reproduction protocol for this species. Therefore, a good starting point to reach the
78 reproduction of this species could be the development of protocols to obtain high-quality sperm.
79 Thus, as a preliminary study working to reach this goal, this paper tries to expand the current
80 knowledge adding a new research line in the improvement of gametes of quality in the European
81 eel.
82 In large-scale production facilities, where aquatic animals are exposed to stressful conditions,
83 problems related to diseases and deterioration of environmental conditions often occur and result in
84 serious economic losses [5]. Probiotics refereed to beneficial bacteria can control pathogens through
85 a variety of mechanisms. They are increasingly viewed as an alternative to antibiotic treatment.
86 Most probiotics that have been used as biological control agents in aquaculture belong to the lactic
87 acid bacteria class (e.g. *Lactobacillus sp.* and *Carnobacterium sp.*), to the genera *Vibrio* (*V.*
88 *alginolyticus*), *Bacillus*, or *Pseudomonas*, although others have also been mentioned (e.g.
89 *Aeromonas* and *Flavobacterium*) [5]. Each of them has been subjected to species for a specific
90 purpose, ranging from the increasing of survival rates to the spawning induction and early life stage
91 survival of turbot larvae [6], as well as the improvement of survival and production of juveniles and
92 adults of Atlantic salmon [7, 8, 9, 10] or the survival of the post-larval crustaceans [11].
93 Gioacchini et al. [12] demonstrated a positive effect of the probiotic *Lactobacillus rhamnosus* in the
94 induction of oocyte maturation and fecundity in zebrafish (*Danio rerio*) which was through the
95 effects of dietary probiotics on zebrafish reproduction's endocrine control as well as on the
96 auto/paracrine factors involved in the regulation of oocyte maturation.
97 Gonadal development has been studied in eel males, and basically consists of the spermatogenesis
98 process, which is divided into the following stages: spermatogonial stem-cell renewal,
99 spermatogonial proliferation, spermiogenesis and sperm maturation [13-18]. Former studies has
100 shown that human chorionic gonadotropin (hCG) could induce spermatogenesis and sperm
101 maturation in the European eel [4, 19-23]. This treatment promotes a plasma increase of androgen
102 (11-KT) by the Leydig cells [13,15,16,24,25]. As a consequence of this stimulation, Sertoli cells

103 produce growth factors, such as insulin-like growth factor-I (*IGF*-I) and *Activin B*, that are
104 responsible for spermatogonia mitosis [17,18].
105 In the European eel, the supposed temperature of the natural reproduction site is around 20 °C [26].
106 The weekly administration of both males and females of this species have traditionally matured at
107 this water temperature [4,20,27,28]. Nowadays, the weekly administration of hCG under a constant
108 temperature regime of 20 °C [6,28] has been the most widely used hormonal treatment in European
109 eel males.Gallego et al. [23] has shown that recombinant hCG (hCGrec) allows the achievement of
110 better results in almost all sperm parameters throughout the weeks of treatment.
111 In aquaculture, becoming an effective and alternative treatment to induce the spermiation in
112 European eel at 20 °C. In addition, in terms of reproductive aquaculture, the aim is to quickly obtain
113 spermiating males, so to minimize costs and risks related to fish handling. The European eel males
114 become mature under a thermal regime of 20 °C and produce sperm with more than 50% of motile
115 cells after a 6-week hormonal treatment [24]. Males kept in thermal regime of 10 and 15 °C did not
116 show a similar result until the 11th and 9th weeks of hormonal treatment, respectively [24]. In the
117 present study, we have directed our research toward better understanding the effects of hormonal
118 treatment on the European eel reproduction using recombinant hCG at 20 °C and studying the
119 effects of probiotic *L. rhamnosus* to investigate it could result in obtaining a high-quality sperm
120 production. Three different dose of probiotic were used (10^3, 10^5 and 10^6 CFU/ml of water). Sperm
121 quality parameters including density, volume, total motile spermatozoa and progressive motile
122 cells) were investigated. To better understand, we have studied the transcriptional pattern of specific
123 genes involved in the reproductive process. Following, a brief description of our genes of interest
124 such as *Activin*, that was found in the testis of Japanese eel at the initiation of spermatogenesis after
125 hCG stimulation, with its expression site restricted to Sertoli cells, confirming its important roles
126 during the initiation of spermatogenesis induced by 11-KT [29,30].
127 Two androgen receptor (*ar*) subtypes (*α* and *β*), which have been described in fish and found to be
128 predominantly expressed in the gonad. More specifically, *ar* is expressed in Sertoli and interstitial
129 cells, suggesting that androgens have biological activity via the testicular somatic cells [31].
130 Also, sperm maturation is regulated by the endocrine system. In some teleost, including Japanese
131 eel, it is suggested that 17α,20β-dihydroxy-4-pregnen-3-one (DHP) is related to the regulation of
132 sperm maturation [32-34].
133 Progestin receptor, specifically *pr1*, that is associated with increased sperm motility and fertility
134 [33].
135 The gonadotropins LH and FSH, historically recognized as the most important factors in the
136 regulation of testicular physiology. Concerning FSH, it is known to regulate Sertoli cells activities,

137 such as the structural, nutritional, and regulatory (paracrine) support of germ cell development. For
138 this reason, it is important to study the possible effect of the administration of probiotic in the
139 expression of FSH receptor.
140 Past studies have evidenced that many probiotic strains are successfully utilized for growth and
141 health management in aquaculture [35]. In the specific case of the use of *L. rhamnosus* on
142 reproduction, works as those by Lombardo et al. [11] and Gioacchini et al. [36] demonstrated the
143 benefits of these microorganisms in aquatic species.
144 Generally, probiotics have been administered to fish through the feeding. But in this study, during
145 the maturation stage in captivity, males of European eel did not receive any food. Therefore, we
146 were forced to supply the fish with the beneficial bacteria via an alternative method adding the
147 bacterials in the water.
148

149 **2. Material and methods**

150 *2.1. Fish maintenance*

151 A total of 40 adult eel males (mean body weight 115±8 g) were moved to our facilities, in the
152 Aquaculture Laboratory at the Universitat Politècnica de València (Spain) from the fish farm
153 Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain). Eels were randomly
154 distributed (8 males per treatment) in five aquaria (3 priobiotic doses and 2 controls) of 90 liters
155 equipped with separated recirculation systems (total recycling in 24 h), thermostats at 20 ºC and
156 covered to maintain constant shadow. The animals were gradually acclimatized to seawater (salinity
157 37±0.3 g/L) over the course of 1 week, and were maintained in seawater until the end of the
158 experiment.
159 During the experiment the fish were starved and were handled in accordance with the European
160 Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).
161

162 *2.2. Hormonal treatment*

163 The hormonal treatment was initiated with hCGrec (recombinant hCG; Ovitrelle, Madrid) when the
164 animals were kept in sea water. Once a week the animals were anaesthetized with benzocaine (60
165 ppm) and weighed before receiving the administration of hormones by intraperitoneal injection. The
166 hormone was diluted 1:1 (IU/µL) in saline solution (NaCl 0.9%) and a weekly dose of 1.5 IU/g fish
167 was administered until the end of the experiment.
168

169 *2.3. Probiotic administration*

170 Male specimens were treated with three different concentrations of probiotic *L. rhamnosus IMC*

171 *501*® (Sinbyotec, Italy). These were: Higher dose (10^6 CFU/ml of water), Medium dose (10^5
172 CFU/ml of water) and Lower dose (10^3 CFU/ml of water). Probiotic was daily added to the water of
173 the aquaria starting off from the 6th week of hormonal treatment until the end of the experiment.
174
175 *2.4. Sperm collection and sampling*
176 Sperm samples were collected 24 h after the administration of the hormone, on the basis of the
177 sperm quality results found by previous studies [4]. Sperm collection was performed by abdominal
178 pressure after fish anesthetization and the cleaning of the genital area with freshwater in order to
179 avoid sample contamination with feces, urine and seawater. A small aquarium air pump was
180 modified to obtain a vacuum breathing force and to collect the sperm in a tube. A new tube was
181 used for every male and distilled water was used to clean the collecting pipette between the
182 different males.
183 Sperm volume was measured using graduated tubes and samples were maintained at 4 °C until
184 motility analyses were carried out within the first hour after extraction.
185 Eight individuals were sacrificed by each probiotic group and in the case of control group, sixteen
186 fish were sacrificed. Two males/probiotic treatment and 4 males in the control group were
187 sacrificed in the first sampling. In the second and third samplings three males/probiotic treatment
188 and 6 males in the control were used.
189 Testis samples were collected after one, two and three weeks of probiotic exposure (that
190 corresponded respectively to the seventh, eighth and ninth week of the hCGrec treatment) from fish
191 from all groups and conserved in RNA*later*®. In addition, testis samples were also fixed with
192 buffered 10% formalin until histological processing.
193
194 *2.5. Gonad histology*
195 After fixation in 10% buffered formalin (pH 7.4), testis samples were dehydrated in ethanol and
196 embedded in paraffin. Sections of 5-10 µm thickness were taken with a Shandom Hypercut manual
197 microtome and stained by the techniques of Haematoxylin-VOF [37]. Slides were observed with a
198 Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to
199 the microscope.
200 Stages of spermatogenesis were determined according to the most advanced germ cell types and
201 their relative abundance [24].
202
203 *2.6. Evaluation of sperm motility*
204 Samples were maintained at 4 °C until sperm activation. For the activation, 1 µl of sperm was

205 mixed with 4 µl of artificial seawater (Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH
206 adjusted to 8.2; [24]). All the motility analyses were performed following a method standardized by
207 Gallego et al. [38], by duplicate at 5 s post-activation by the motility module of ISAS (Proiser R+D,
208 S.L.; Paterna, Spain) using an ISAS® 782M camera recorder (60 fps; Hz). The chamber used in all
209 experiments was a SpermTrack-10® (Proiser, Paterna, Spain) with 10× negative contrast phase lens
210 in a Nikon Eclipse (E-400) microscope.
211 The parameters considered in this study were: total motility (MOT, %); progressive motility (P-
212 MOT, %), defined as the percentage of spermatozoa which swim forward in 80% of a straight line;
213 curvilinear velocity (VCL, in µm/s), defined as the time/average velocity of a sperm head along its
214 actual curvilinear trajectory; average path velocity (VAP, µm/s), defined as the time/average of
215 sperm head along its spatial average trajectory; and straight line velocity (VSL, µm/s), defined as
216 the time/average velocity of a sperm head along the straight line between its first detected position
217 and its last position. Spermatozoa were considered motile if their progressive motility had straight
218 line velocity over 10 µm/s.
219
220 *2.7. RNA extraction and cDNA synthesis*
221 Total RNA was isolated from testis with the TriReagent solution (Sigma) according to the
222 manufacterer's instructions. Its final concentration and integrity were determined by nanodrop
223 reading and then verified by ethidium bromide staining of 28S and 18S ribosomal RNA fragments
224 on a 1% agarose gel.
225 First-strand cDNA was synthetized by means of reverse transcriptase reaction using the
226 SuperScript-II kit (Invitrogen, Life Technologies) from 1 µg of total RNA.
227
228 *2.8. Real-Time RT-PCR*
229 The relative quantification of the mRNA levelss was performed with the SYBR green method in an
230 iQ5 Multicolor Real-Time PCR Detection system (BioRad). Duplicate PCRs were carried out for
231 each sample.
232 The reactions consisted in 1 µl of diluted (1/10) cDNA, 5 µl of 2X SYBR Green PCR Master Mix
233 (BioRad) containing SYBR Green as a fluorescent intercalating agent, 0.1 µM of both forward and
234 reverse primers (Table 1) and 3.8 µl of milliQ water.
235 The thermal profile for all reactions consisted in a two-step denaturation phase (respectively of 15
236 min and 20 s) followed by 40-45 cycles of 20 s annealing at 60 °C for bone morphogenetic protein
237 15 (*bmp 15*), androgen receptor subtype *β* (*arβ*) and FSH receptor (*fshr*), 57 °C for *Activin* and
238 androgen receptor subtype *α* (*arα*), and 56 °C for *actin* and progestine receptor (*pr1*), 20 s

239 elongation at 72 °C and, eventually, 5 min extension at 72 °C. Fluorescence was monitored at the
240 end of each cycle. Dissociation curve analysis showed a single peak in all cases.
241 The quantification of the relative mRNA levels was calculated using *actin* as the housekeeping gene
242 to standardize the results by eliminating variation in mRNA and cDNA quality.
243 No amplification product was observed in negative controls and primer-dimer formation was never
244 observed in control templates. Data were analyzed using Bio-Rad's iQ5 optical system software,
245 version 2.0.
246

247 *2.9. Hormones levels*

248 Plasma concentrations of estradiol-17β (E2), testosterone (T), and 11-ketotestosterone (11-KT)
249 were measured by means of radioimmunoassays, as described previously [39,40]. Assay
250 characteristics and cross-reactivities of the E2 and T antisera have been previously examined by
251 Frantzen et al. [40] and further validated for eel plasma by Mazzeo et al. [41]. Assay characteristics
252 and cross-reactivities of the 17,20βP assay have previously been described by Mazzeo et al. [41]
253 and validated for eel plasma by Peñaranda et al. [24]. The cross-reactivities of a new 11-KT
254 antiserum used in this work have been previously described by Johnsen et al. [42
255]. To validate 11-KT recovery from plasma in the eel assay, a plasma pool were spiked with 45 ng
256 11-KT/mL of plasma and then subjected to ether extraction as described below. The product
257 resulting from this treatment was then assayed by the 11-KT RIA at three different dilutions.
258 Dilutions were found to be parallel to that of the assay standard curve. Steroid recovery after ether
259 extraction was 71.9±2.8%. 11-KT values were corrected for recovery losses. The inter- and intra-
260 assay coefficients of variation (CV) for the 11-KT assay were 15.4% (n=7) and 5.3% (n=10),
261 respectively.
262

263 *2.10. Statistical analysis*

264 All parameters with the exception of mRNA levels analyses were subjected to analysis of variance
265 (General Lineal Model, GLM) including as fixed effect the hormonal treatment. Multiple
266 comparison of means was carried out using a Student-Newman-Keuls test with a signification level
267 of 95%. Differences were considered significant if $p<0.05$. Results are presented as mean ±
268 standard error of the means (SEM). All statistical procedures were run using Statgraphics Plus 5.1.
269 Regarding mRNA levels, the statistical differences among groups were checked with the one-way
270 ANOVA analyses of variance followed by both Tuckey and Bonferroni's multiple comparison tests.
271 Statistical significance was set at $p<0.05$. Results are presented as mean ± standard error. All
272 statistical procedures were run using GraphPad Prism 6.

273 **3. Results**

274 *3.1. Testicular development*

275 Testicular development in each male was classified into six different stages of development (S1–
276 S6). Stages S1–S3 (spermatogenesis process) are characterized by the most advanced germinal cell
277 present: spermatogonia (S1), spermatocytes (S2) or spermatids (S3). Stages S4 (spermiogenesis
278 step) to S6 (spermiation period) differ on one another by the abundance of spermatozoa compared
279 with the other germ cells (Fig. 1).

280 Regarding the results of histological analysis (Fig. 2), after seven weeks of hormonal treatment and
281 one week of probiotic treatment (W1), Control and Medium (10^5 CFU/ml) groups, showed stage 4
282 (characterized by the appearance of first spermatozoa), with 25 and 50 % of males in this stage,
283 respectively. However, Low group (10^3 CFU/mL) showed the highest percentage of males in stage
284 6 (S6, characterized by a proportion between spermatozoa and whole lobules between 75% and
285 95%) with 50% of males in this stage.

286 After eight weeks of hormonal treatment and two weeks of probiotic administration (W2) any group
287 showed males in stage 4 of development. Control group showed 83% of males in stage 5
288 (characterized by the appearance of spermatozoa occupied 50-70% of the total whole lobules)
289 compared to Higher probiotic dose group (10^6 CFU/mL) with 100% of males in stage 6. In this way,
290 Medium and Lower group (10^5 and 10^3 CFU/mL respectively) showed 67% of males in stage 6 of
291 gonadal development.

292 Eventually, following 9 weeks of hormonal treatment and three weeks of probiotics administration
293 (W3), all males exposed to probiotic treatments were in stage 6, or the most advanced gonadal
294 development. However, during this week, Control group showed that 17% of males were still in
295 stage 5.

296

297 *3.2. Sperm volume and motility parameters*

298 Significant higher volumes of sperm, higher sperm density, and higher percentages of motile and
299 progressive motile (straight line swimmers) spermatozoa were found in fish exposed to 10^5
300 CFU/ml, after 2 weeks of probiotic treatment (Fig. 3). No significant differences on sperm
301 parameters were found in other samplings.

302

303 *3.3. mRNA levels of genes involved in spermatogenesis*

304 The lowest dose of the probiotic *L. rhamnosus* (10^3 CFU/mL) showed to be responsible for a
305 decrease of the expression patterns of various genes such as *Activin*, *ara*, *bmp15* and *pr1* (Table 2),
306 with respect to controls, after each of the three weeks of treatment. The differences between treated

307 and control organisms were statistically significant ($p<0.05$), although, as far as *Activin* is
308 concerned, only after the first and the third week of treatment; expression of *arα*, instead, exhibited
309 significant differences both at the second and the third week of treatment; significantly increase of
310 *bmp15* values were found after every week of the study whereas *pr1* showed statistically different
311 expressions patterns only after the third week of treatment. Noteworthy, *bmp15* has a regulatory
312 function in the early stages of spermatogenesis and is therefore identified as a germ cell-specific
313 factor in the testis [43].
314 No significant variations affected *arβ* (Table 2) mRNA levels following the first week, while its
315 mRNA levels were lowered after both the second and the third week in the treated experimental
316 group (significant difference evident solely after the second sampling time).
317 Moreover, *fshr* (Table 2) mRNA levels increased significantly after the first week of treatment, and
318 diminished, in a significant way, after two and three weeks.
319 Conversely, the intermediate probiotic dose (10^5 CFU/mL) caused i) an increase in the mRNA
320 levels of *Activin* (Fig. 4) after the first two weeks of experiment, even though statistical significance
321 was only present after the second one, and a significant reduction of the mRNA levels of the treated
322 fish at the end of the third week; ii) a heightening of the first two weeks' *arα* (Fig.4) expression
323 (significant difference present only after the first week) and a slight decrease during the third week
324 treatment; iii) a statistically significant enhancement of *arβ* (Fig. 1) mRNA quantities at each of the
325 three sampling times; iv) an increase of the expression pattern of *bmp15* (Fig. 4), which was
326 significant at both the second and the third week, when values corresponded to a change of,
327 respectively, two and three fold compared to controls; v) significant increases of *pr1* (Fig. 4) and
328 *fshr* (Fig. 4) transcripts following the first two weeks of treatment as well as minor, non-significant
329 drops of expressions values in either of the above-mentioned genes subsequent to the third week.

330 The administration of the highest dose of probiotic (10^6 CFU/mL) resulted in an inhibition of
331 *Activin*, *arβ* and *bmp15* expressions compared to the controls at all three weeks of treatment (Table
332 3). The variation between treated and control fish mRNA levelss were statistically significant, as far
333 as *Activin* is concerned, at both the first and the third week, while, regarding *arβ* and *bmp15*, at all
334 the of tree weeks of the treatment (Table 3). It is important to note that *Activin* is an important local
335 factor that regulates Sertoli cells number in that it maintains their mitotic potentiality [44].

336 No changes of *pr1* mRNA levels were observed after the first week, while a significant decrease
337 was registered at the end of the second and the third week of treatment (Table 3).

338 *3.4. Hormones levels*

339 During the first and the last week of probiotic treatment, no significant differences were observed in
340 hormones' levels. However, in the second week, the levels of DHP were significantly higher in
341 Medium (10^5 CFU/mL) and High (10^6 CFU/mL) doses of probiotic (Table 4) but no significant
342 differences in the levels of 11 KT and E2 were found between treatments in the same week.

343

344 4. Discussion

345 The present work has found that the use of probiotic has a positive effect on the states of gonadal
346 development in male of European eels.

347 From the first week of probiotic treatment, this study has been able to test the effect of *L.*
348 *rhamnosus* (dissolved in water) on the developmental stages of European eel males.
349 After one week of probiotic treatment (7 weeks of hormone treatment), any male from the group of
350 Low and High doses (Fig. 1; 10^3 and 10^6 CFU/ml, respectively) were in stage 4 of development
351 (characterized by the appearance of first spermatozoa).

352 In a previous study with males of European eel [24], it was observed that after 7 weeks of hormonal
353 treatment 20% of males were in stage 4 of development. Therefore, both doses of 10^3 and 10^6
354 CFU/ml of *L. rhamnosus* may induce faster gonadal development during the first steps of
355 spermiation.

356 In fact, in the same study [24], males were not observed in stage 4 of development until week 9 of
357 hormonal treatment and the only present stages of the gonadal development were stage 5 and 6 in a
358 1:1 proportion. These results agree with the results from this study after one week of treatment with
359 Low probiotic dose (10^3 CFU/ml) and 7 weeks with hormonal treatment. Therefore, in terms of
360 gonadal development, if we combine the hormonal treatment of 7 weeks and a low dose (10^3
361 CFU/ml) of *L. rhamnosus* treatment during the last one (7th week), it would be possible to achieve
362 the same results of a 9-week hormonal treatment.

363 The results of the present study suggest that two weeks of treatment with *L. rhamnosus* at the two
364 doses of 10^5-10^6 CFU/mL may induce faster gonadal development.

365 The testis of fish have two main functions: they produce germ cells through the processes of
366 spermatogenesis, and they produce sex steroids that are important for the regulation of
367 reproduction. The main interest in DHP in male fishes stems from the discovery of a strong
368 association in several species between blood plasma concentrations of DHP and spermiation
369 [45,46]. In fact, DHP is considered to be the steroid responsible for acquisition of sperm motility in
370 the Japanese eel [17]. In the present study, after two weeks of probiotics treatment (8th week of hCG
371 treatment), DHP levels were significantly higher in the males from Medium and Higher dose of

372 probiotic (10^5 and 10^6 CFU/mL respectively) with respect to the Control group. This result
373 correspond to an advanced stage of gonadal development (stage 6) found in the males from the
374 same experimental groups, supporting a function of DHP during the eel spermatogenesis process.
375
376 From a biological point of view, the sperm quality could be defined as the ability of the sperm to
377 successfully reach and fertilize the oocyte. The interest on the study of sperm quality has been
378 reflected through the years, and there have been several studies regarding the identification of
379 parameters defining this quality. These sperm parameters have so far been documented in scientific
380 papers, like the osmolality, pH and chemical composition of the seminal plasma [1,47]; enzymatic
381 activity [48]; ATP concentration [48]; spermatocrit and sperm density [49,50,51]; sperm motility
382 [52,53]; or sperm morphology and ultrastructure [21,22,54]. All have been linked to the ability of
383 sperm to fertilize the ova.
384 In the present study, the differences of quality sperm parameters (Fig. 3) were observed only in the
385 second week of probiotic treatment (8th week of hCG treatment). At that sampling time, the
386 Medium group (10^5 CFU/mL of *L. rhamnosus*) showed the best values of all sperm parameters
387 analyzed. Regarding the results of sperm volume and density (Fig. 3, A and D), it is important to
388 remark as a possible option the use of a dose of 10^5 CFU/mL for a 2-week period to induce
389 improvement of the above mentioned parameters.
390 In this way, the use of *L. rhamnosus* could be a way to obtain a higher quantity of sperm in less
391 hCG treatment time. A key feature of this study is the fact that the data regarding sperm volume,
392 which agree with those already found by other authors in European eel studies [1,4], were obtained
393 with a completely different approach with respect to the just mentioned works, that being a
394 probiotic treatment.
395 At the same time, the sperm density values we got are far higher than those obtained in the past
396 (values around 1-2×10^9/ mL versus more than 20 x 10^9/mL of sperm in this work).
397 Moreover, previous studies have found significant correlations between the number of motile
398 spermatozoa and fertilization rates in some fish species [54,55] Hence, total motility and
399 progressive motility are recognized as important sperm characteristic for male fertility.
400 Regarding our results of total motility and progressive motility, males of the 10^5 CFU/mL treatment
401 showed the best results of these parameters: 10-12% of progressive motility and 60-80% of total
402 motility. The control group, not receiving any probiotic, showed lower percentages of total motility
403 (4%) and progressive motility (20%).
404 As mentioned previously, in a past work with European eel males [23], we showed the effect of
405 hormonal treatment with hCGrec at 20 ºC. In the same way, the results from [23] were 30% of

406 progressive motility and more than 50% of total motility during the same week of hormonal
407 treatment (8th week). Therefore, comparing the results we are currently discussing with those from
408 Gallego et al. [23], we can definitely appreciate a significant increase in the total motility of 10-30%
409 (60-80% in males under 10^5 CFU/mL of *L. rhamnosus* vs. 50% in [23] study).
410 Similarly to the case of the Japanese eel, the European counterpart has an important advantage in
411 the study of the mechanisms controlling spermatogenesis. This consists in the possibility to
412 artificially induce the process of spermatogenesis by treatment of exogenous gonadotropins. The
413 hormonal treatment allows prolonging the process of spermatogenesis, during which the haploid
414 spermatids develop into spermatozoa. Moreover, the start of hormonal treatment promotes the
415 production of 11-KT by Leydig cells, and the Sertoli cells being its target [17,18]. As a
416 consequence of this stimulation, spermatogenesis begins and Sertoli cells produce growth factors,
417 such as *Activin* [29,30] and *bmp15* [43], which were found to have regulatory functions in its early
418 stages (e.g. spermatogonia mitosis [17,18]). In this study, after two weeks of probiotic treatment,
419 *Activin* and *bmp15* expressions were significantly higher in the testis of animals treated with
420 Medium dose (10^5 CFU/mL) than in the control group.
421 Although the results of this study did not highlight significant differences in the plasma levels of
422 11-KT, the expression of androgen receptors (arα and arβ) and progesterone receptor I (pr1) showed
423 an increase in males treated with Medium dose (10^5 CFU/mL) after two weeks of probiotic
424 treatment (corresponding to 8 weeks of hormonal treatment).
425 In this study, *fshr* (Table 2) mRNA levels increased significantly after the first week of treatment
426 with the lower dose of probiotic, and diminished, in a significant way, after two and three weeks.
427 Therefore, these results support the role of FSH in the first steps of spermatogenesis.
428 The importance of FSHR during the first phases of spermatogenesis was demonstrated also by Ohta
429 et al. [56] who observed that this receptor is activated by the FSH produced by the pituitary gland,
430 stimulates the production of 11-KT level in the Leydig cells which, in turn, stimulates
431 spermatogenesis. As we previously implied, after two weeks of treatment with the intermediate
432 dose of probiotic we can observe an increase in the mRNA levels of the androgen receptors (*arα*
433 and *arβ*) and progesterone receptor I (*pr1*). Both the androgen receptors seems to be expressed in
434 the Sertoli cells and in the interstitial cells, but not in germ cells [33] and, according to Miura et al.
435 [57], they supposedly have a very high affinity for 11-KT.
436 Many authors have focused on the role of *pr1* in the spermatogenesis of teleost [58-61]. This gene is
437 expressed in the germinal, Sertoli and interstitial cells of the testis; it binds the DHP, which is
438 involved in the final part of the spermatogenesis. In addition to it, Miura et al. [62] proposed a
439 possible function for this gene even in the initial part of spermatogenesis, when the spermatogonia

440 are induced to enter in the meiotic prophase. Since we found an increase in the mRNA levels only
441 with the intermediate dose of probiotic but not with the lower and higher dose; we can speculate
442 that this dose of probiotic seems to positively influence the process of spermatogenesis particularly
443 after two weeks of treatment. These results seem to be supported by the analysis of sperm volume
444 and motility even though we did not observe any significant changes in 11-KT plasma levels.
445

446 **Conclusions**

447 The results presented here suggest that treatment with 10^5 CFU/ml dose of probiotic *Lactobacillus*
448 *rhamnosus* for two weeks positively modulates the European eel spermatogenesis process. This
449 hypothesis is supported by the increased sperm volume and motility associated with the changes
450 involved in reproductive process.
451

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457 Valenciana de Acuicultura, S.A. (Puzol, Spain) supplied the eels used in this study.
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459 Department of Zoology, University of Otago, New Zealand.
460

461 **References**

462 [1] Asturiano JF, Pérez L, Garzón DL, Peñaranda DS, Marco-Jiménez F, Martínez-Llorens S, et al.
463 Effect of different methods for the induction of spermiation on semen quality in European eel.
464 Aquac Res 2005;36:1480-7.
465 [2] Huang H, Zhang Y, Huang WR, Li SS, Zhu P, Liu Y, et al. Molecular characterization of
466 marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles
467 during artificially induced gonadal development. Gen Comp Endocrinol 2009; 162:192-202.
468 [3] Ohta H, Kagawa H, Tanaka H, Okuzawa K, Iinuma N, Hirose K. Artificial induction of
469 maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol Biochem 1997;
470 17:163-9.
471 [4] Pérez L, Asturiano JF, Tomás A, Zegrari S, Barrera R, Espinós FJ, et al. Induction of maturation
472 and spermiation in the male European eel: Assessment of sperm quality throughout treatment. J
473 Fish Biol 2000; 57:1488-1504.

474 [5] Balcázar JL, de Blas I, Ruiz-Zazuela I, Cunningham D, Vandrell D, Muzquiz JL. The role of
475 probiotics in aquaculture. Vet Microbiol 2006;114:173-86.
476 [6] Gatesoupe FJ. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus*
477 *maximus*, against pathogenic *Vibrio*. Aquat Living Resour 1994;7:277–82.
478 [7] Robertson P, O´Dowd C, Burrells C, Williams P, Austin B. Use of Carnobacterium sp. as a
479 probiotic for Atlantic salmon (*Salmo salar*, Linnaeus) and rainbow trout (*Oncorhynchus mykiss*,
480 Walbaum) Aquaculture 2000;185:235–43.
481 [8] Lombardo F, Gioacchini G, Carnevali O. Probiotic-based Nutritional Effects on Killifish
482 Reproduction. Fish Aquacult J 2011; Vol. 2011: FAJ-33.
483 [9] Gioacchini G, Giorgini E, Vaccari L, Carnevali O. Can probiotics affect reproductive processes?
484 In: Probiotics, prebiotics and gut health in aquaculture nutrition. Wiley-Blackwell. 2014 pp328-348.
485 doi:10.1002/9781118897263.ch12
486 [10] Carnevali O, Avella MA, Gioacchini G. Effect of probiotic administration on zebrafish
487 development and reproduction. Gen Comp Endocrinol 2013: 297-302.
488 [11] Rengpipat S, Phianphak W, Piyatiratitivorakul S, Menasaveta P. Effects of a probiotic
489 bacterium in black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 1998;167:
490 301–13.
491 [12] Gioacchini G, Maradonna F, Lombardo F, Bizzaro D, Olivotto I, Carnevali C. Increase of
492 fecundity by probiotic administration in zebrafish (*Danio rerio*). Reproduction 2010a;140:953-59.
493 [13] Miura T, Kawamura S, Miura C, Yamauchi K. Impaired spermatogenesis in the Japanese eel,
494 *Anguilla japonica*: Possibility for the existence of factors that regulate entry of germ cells into
495 meiosis. Dev Growth Differ 1997;39:685-91.
496 [14] Miura T, Kudo N, Miura C, Yamauchi K, Nagahama Y. Two testicular cDNA clones
497 suppressed by gonadotropin stimulation exhibit ZP2- and ZP3- like structures in Japanese eel. Mol
498 Reprod Dev 1998;51:235–42.
499 [15] Miura T, Miura C, Konda Y, Yamauchi K. Spermatogenesis-preventing substance in Japanese
500 eel. Development 2002a;129:2689–97.
501 [16] Miura T, Ando N, Miura C, Yamauchi K. Comparative studies between in vivo and in vitro
502 spermatogenesis of Japanese eel (*Anguilla japonica*). Zool Sci 2002b;19:321–29.
503 [17] Miura T, Miura C. Japanese eel: a model for analysis of spermatogenesis. Zool Sci 2001;18:
504 1055–63.
505 [18] Miura T, Ohta T, Miura CI, Yamauchi K. Complementary deoxyribonucleicacid cloning of
506 spermatogonial stem cell renewal factor. Endocrinology 2003;144:5504–10.
507 [19] Asturiano JF, Pérez L, Pérez-Navarro FJ, Garzón DL, Martínez S, Tomás A, et al.

508 Optimization trial of methods for induction of spermiation in European eel (*Anguilla anguilla*) and
509 test of different sperm activation media. Aquaculture Europe '04 Biotechnologies for Quality 2004a;
510 34:130-1.
511 [20] Asturiano JF, Marco-Jiménez F, Pérez L, Balasch S, Garzón DL, Peñaranda DS, et al. Effects
512 of hCG as spermiation inducer on European eel semen quality. Theriogenology 2006;66:1012-20.
513 [21] Asturiano JF, Marco-Jiménez F, Peñaranda DS, Garzón DL, Pérez L, Vicente JS, et al. Effect
514 of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology.
515 Reprod Dom Anim 2007;42:162-66.
516 [22] Marco-Jiménez F, Pérez L, Castro MPVD, Garzón DL, Peñaranda DS, Vicente JS, et al.
517 Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa
518 analysis and scanning electron microscopy. Theriogenology 2006;65:1302-10.
519 [23] Gallego V, Mazzeo I, Vílchez MC, Peñaranda DS, Carneiro PCF, Pérez L, et al. Study of the
520 effects of thermal regime and alternative hormonal treatments on the reproductive performance of
521 European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 2012;354-
522 355:7-16.
523 [24] Peñaranda DS, Pérez L, Gallego V, Jover M, Tveiten H, Baloche S, et al. Molecular and
524 physiological study of the artificial maturation process in European eel males: from brain to testis.
525 Gen Comp Endocrinol 2010;166:160–71.
526 [25] Baeza R, Peñaranda DS, Vílchez MC, Tveiten H, Pérez L, Asturiano JF. Exploring correlations
527 between sex steroids and fatty acids and their potential roles in the induced maturation of the male
528 European eel. Aquaculture 2015;435:328–35.
529 [26] Boëtius I, Boëtius J. Studies in the European eel, *Anguilla anguilla* (L.). Experimental
530 induction of the male sexual cycle, its relation to temperature and other factors. Meddelelser fra
531 Danmarks Fiskeri-og Havunderøgelser 1967;4:339–405.
532 [27] Asturiano JF, Pérez L, Tomás A, Zegrari S, Espinós FJ, Jover M. Hormonal induction of
533 gonadal maturation and spawning in European eel females *Anguilla anguilla* L., 1758:
534 Morphological changes and oocyte development. Boletín del Instituto Español de Oceanografía
535 [Newsletter of the Spanish Institute of Oceanography] 2002;18:127-37 [in Spanish].
536 [28] Pedersen BH. Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation
537 of the eggs. Aquaculture 2003;224:323-38.
538 [29] Miura T, Miura C, Yamauchi K, Nagahama Y. Human recombinant activin induces
539 proliferation of spermatogonia *in vitro* in the Japanese eel *Anguilla japonica*. Fisheries Sci 1995a;
540 63:434–37.
541 [30] Miura T, Miura C, Yamauchi K, Nagahama Y. Activin B is a major mediator of hormone-

542 induced spermatogonial proliferation in the Japanese eel. *In*: the Fifth International Symposium on
543 the Reproductive Physiology of the Fish 1995b; 284–286. Edited by F.W. Goetz and P. Thomas,
544 University of Texas, Austin.
545 [31] Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. Two subtypes of androgen and progestogen
546 receptors in fish testes. Comp Biochem Physiol 2001;129:449–55.
547 [32] Miura T, Yamauchi K, Takahashi H, Nagahama Y. Hormonal induction of all stages of
548 spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci. USA
549 1991b;88:5774–78.
550 [33] Miura T, Yamauchi K, Takahashi H, Nagahama Y. Involvement of steroid hormones in
551 gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*). Biomed Res
552 1991d;12:241–48.
553 [34] Miura T, Yamauchi K, Takahashi H, Nagahama Y. The role of hormones in the acquisition of
554 sperm motility in salmonid fish. J Exp Zool 1992;261: 359–63.[35] Dimitroglou A, Merrifield D,
555 Carnevali O, Picchietti S, Avella M, Daniels C. et al. Microbial manipulations to improve fish
556 health and production:a Mediterranean perspective. Fish Shellfish Immun 2011;30:1-16.
557 [36] Gioacchini G, Lombardo F, Merrifield DL, Silvi S, Cresci A, Avella MA, et al. Effects of
558 probiotic on zebrafish reproduction. J Aquac Res Development 2001;
559 http://dx.doi.org/10.4172/2155-9546, S1-002.
560 [37] Sarasquete C, Gutiérrez M. New Tetrachromic VOF Stain (Type III G.S) for Normal and
561 Pathological Tissues. Eur J Histochem 2005;49:211-20.
562 [38] Gallego V, Carneiro PCF, Mazzeo I, Vílchez MC, Peñaranda DS, Soler C, Pérez L, Asturiano
563 JF. Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA
564 software. Theriogenology 2013;79:1034–40.
565 [39] Schulz R. Serum levels of 11b-oxotestosterone in male and 17b-estradiol in female rainbow
566 trout (*Salmo gairdneri*) during the first reproductive cycle. Gen Comp Endocrinol 1984;56:111–20.
567 [40] Frantzen M, Arnesen AM, Damsgård B, Tveiten H, Johnsen HK. Effects of photoperiod on sex
568 steroids and gonad maturation in Arctic charr. Aquaculture 2004;240:561–74.
569 [41] Mazzeo I, Peñaranda D S, Gallego V, Baloche S, Nourizadeh-Lillabadi R, Tveiten H, Pérez
570 L. Temperature modulates the progression of vitellogenesis in European eel. Aquaculture
571 2014;434:38-47.
572 [42] Johnsen H, Tveiten H, Torgersen JS, Andersen O. Divergent and sex-dimorphic expression of
573 the paralogs of the Sox9-Amh-Cyp19a1 regulatory cascade in developing and adult Atlantic cod
574 (*Gadus morhua* L.). Mol Reprod Dev 2013;80:358-70.
575 [43] Nicholls P K, Harrison C A, Gilchrist RB, Farnworth PG, Stanton PG. Growth differentiation

576 factor 9 is a germ cell regulator of Sertoli cell function. Endocrinology 2009;150:2481-90.
577 [44] Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a
578 defined period of rat testis development. Endocrinology 1995;136:5438-44.
579 [45] Tveiten H, Frantzen M, Scott AM, Scott AP. Synthesis of 17,20β,21-trihydroxypregn-4-en-3-
580 one by ovaries of reproductively mature Atlantic cod *Gadus morhua*. J Fish Biol 2010;77:33–5.
581 [46] Scott AP, Sumpter JP, Stacey P and N. The role of the maturation-inducing steroid, 17,20*β*-
582 dihydroxypregn-4-en-3-one, in male fishes: a review. J Fish Biol 2010;76:183–224.
583 [47] Ciereszko A. Chemical composition of seminal plasma and its physiological relationship with
584 sperm motility, fertilizing capacity and cryopreservation in fish. In: Fish Spermatology. Eds: Alavi
585 SMH, Cosson JJ, Coward K, Rafiee G. Oxford, London, 2008; 215–240.
586 [48] Burness G, Moyes CD, Montgomerie R. Motility, ATP levels and metabolic enzyme activity
587 of sperm from bluegill (*Lepomis macrochirus*). Comp Biochem Physiol 2005;140:11–17.
588 [49] Groison AL, Suquet M, Cosson J, Mayer I, Severe A, Bouquet JM, et al. Sperm motility in
589 European hake, *Merluccius merluccius*, and characterization of its spermatozoa concentration and
590 volume, spermatocrit, osmolality and pH. Aquaculture 2010;301:31–6.
591 [50] Harald BT, Tillmann JB, Deborah JM, Joanne P. The relationship between sperm density,
592 spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus*
593 *hippoglossus.* Aquaculture 2001;194:191–200.
594 [51] Sorensen S, Gallego V, Pérez L, Butts I, Tomkiewicz J, Asturiano JF. Evaluation of methods
595 to determine sperm density for the European eel, *Anguilla anguilla*. Reprod Domest Anim
596 2013;48:936-44.
597 [52] Sadiqul M, Akhter T. Tale of fish sperm and factors affecting sperm motility: a review.
598 AdvanLife Sci 2011;1:11-9.
599 [53] Liu QH, Li J, Xiao ZZ, Ding FH, Yu DD, Xu XZ. Use of computer-assisted sperm analysis
600 (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*).
601 Aquaculture 2007;263:20-5.
602 [54] Rurangwa E, Kime DE, Ollevier F, Nash JP. The measurement of sperm motility and factors
603 affecting sperm quality in cultured fish. Aquaculture 2004;234:1-28.
604 [55] Ottesen OH, Babiak I, Dahle G. Sperm competition and fertilization success of Atlantic halibut
605 (*Hippoglossus hippoglossus* Linnaeus). Aquaculture 2009;286:240-5.
606 [56] Ohta T, Miyake H, Miura C, Kamei H, Katsumi A, Takeshi M. Follicle-Stimulating Hormone
607 Induces Spermatogenesis Mediated by Androgen Production in Japanese Eel, *Anguilla japonica*.
608 Biol Reprod 2007;**77**:970-77.
609 [57] Miura T, Yamauchi K, Nagahama Y, Takahashi H. Induction of a spermatogenesis in male

610 Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool Sci
611 1991; 8:63-73.
612 [58] Ueda H, Kambegawa A, Nagahama Y. Involvement of gonadotrophin and steroid hormones in
613 spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. Gen
614 Comp Endocrinol 1985;59:24-30.
615 [59] Baynes SM, Scott AP. Seasonal variations in parameters of milt production and in plasma
616 concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). Gen Comp Endocrinol
617 1985;57:150-60.
618 [60] Yueh WS, Chang CF. 17α,20β,21-trihydroxy-4-pregnen-3-one and 17α,20β-dihydroxy-4-
619 pregnen-3-one stimulated spermiation in protandrous black porgy, *Acanthopagrus schlegeli*. Fish
620 Physiol Biochem 1997;17:187-93.
621 [61] Tubbs C, Thomas P. Functional characteristic of membrane progestin receptor alpha
622 (mPRalpha) subtypes: a review with new data showing mPRalpha expression in seatrout sperm and
623 its association with sperm motility. Steroids 2008;73:935-41.
624 [62] Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C. Progestin is an essential factor for the
625 initiation of the meiosis in spermatogenetic cells of the eel. Proceedings of the National Academy of
626 Sciences 2006;103:7333-8.
627 [63] Peñaranda DS, Mazzeo I, Gallego V, Hildahl J, Nourizadeh-Lillabadi R, Pérez L, Weltzien F-
628 A, Asturiano JF. The Regulation of Aromatase and Androgen Receptor Expression During Gonad
629 Development in Male and Female European Eel. Reprod Dom Anim 2014;49:512–521.
630 [64] Peñaranda DS, Morini M, Vílchez MC, Gallego V, Mazzeo I, Dirks RP, et al. Temperature
631 affects the European eel spermiation modulating the testis steroidogenesis: physiological and
632 migratory implications. 17th International Congress of Comparative Endocrinology. ICCE 2013:
633 Book of abstracts (digital).
634 [65] Minegishi Y, Dirks RP, de Wijze DL, Brittijn SA, Burgerhout E, Spaink HP, van de Thillart
635 GEEJM. Quantitative bioassays for measuring biologically functional gonadotropins based on eel
636 gonadotropic receptors. Gen Comp Endocrinol 2012; 178:145-152.
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643 **Figure captions**

644

645 **Fig. 1:** Percentage of three different stages of development in each week throughout the probiotics
646 treatments (control, 10^3 CFU/mL, 10^5 CFU/mL, 10^6 CFU/mL) Photomicrographs of histological
647 sections for the different stages found during treatment. Testis at stage 4 (**A**), testis at stage 5 (**B**),
648 and testis at stage 6 (**C**). Scale bar 100 µm.

649

650 **Fig. 2**: Sperm quality parameters throughout the probiotics treatments (control, 10^3 CFU/mL, 10^5
651 CFU/mL, 10^6 CFU/mL) in males of *A. anguilla* treated for two weeks (8th week of hCG treatment).
652 A) sperm volume; B) percentage of motile cells; C) percentage of progressive motile cells; D)
653 sperm density. Data are expressed as mean±SEM and different letters indicate significant
654 differences between treatments.

655

656 **Fig. 3:** *Activin*, *arα*, *arβ*, *bmp15*, *fshr* and *pr1* mRNA levels in the testis of *A. anguilla* treated for
657 one (w1), two (w2) and three (w3) weeks with 10^5 CFU/mL of *L. rhamnosus.* Data are expressed as
658 mean±SD and different letters indicate significant differences between treatments. Statistical
659 differences among groups were checked with the one-way ANOVA analyses of variance followed
660 by both Tuckey and Bonferroni's multiple comparison tests. Statistical significance was set at $p <$
661 0.05.

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676 **Table captions**

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678 **Table 1**: Forward and reverse primers used for detection of mRNA levelss. *actin= Anguilla*
679 *Anguilla beta-actin; Activin= Anguilla anguilla activin beta B subunit precursor; arα = Anguilla*
680 *anguilla mRNA for androgen receptor alpha; arβ = Anguilla anguilla mRNA for androgen receptor*
681 *beta; pr1= Anguilla anguilla Progesterone Receptor I; bmp15= Anguilla anguilla Bone*
682 *Morphogenetic Protein 15; fshr: Anguilla anguilla fshr mRNA for follicle-stimulating hormone*.

683

684 Primer´s references: (1) designed by authors, (2) [63], (3) [64], (4) provided by Dr. Lockman, (5) [65].

685

686 **Table 2**: *Activin*, *arα*, *arβ*, *bmp15*, *fshr* and *pr1* mRNA levels in the testis of *A. anguilla* treated for
687 one (w1), two (w2) and three (w3) weeks with 10^3 CFU/mL of *L. rhamnosus.* Data are expressed as
688 mean±SEM and different letters indicate significant differences between treatments.

689

690 **Table 3**: *Activin*, *arα*, *arβ*, *bmp15*, *fshr* and *pr1* mRNA levels in the testis of *A. anguilla* treated for
691 one (w1), two (w2) and three (w3) weeks with 10^6 CFU/mL of *L. rhamnosus.* Data are expressed as
692 mean±SEM and different letters indicate significant differences between treatments.

693

694 **Table 4**: Plasma 11-ketotestosterone (11-KT), 17α,20β-dihydroxy-4-pregnen-3-one (DHP), and
695 estradiol-17β (E2) levels (ng/mL) in males of *A. anguilla* treated for two weeks (8th week of hCG
696 treatment) in control or *L. rhamnosus*-treated fish. Data are expressed as mean±SEM and different
697 letters indicate significant differences between treatments.

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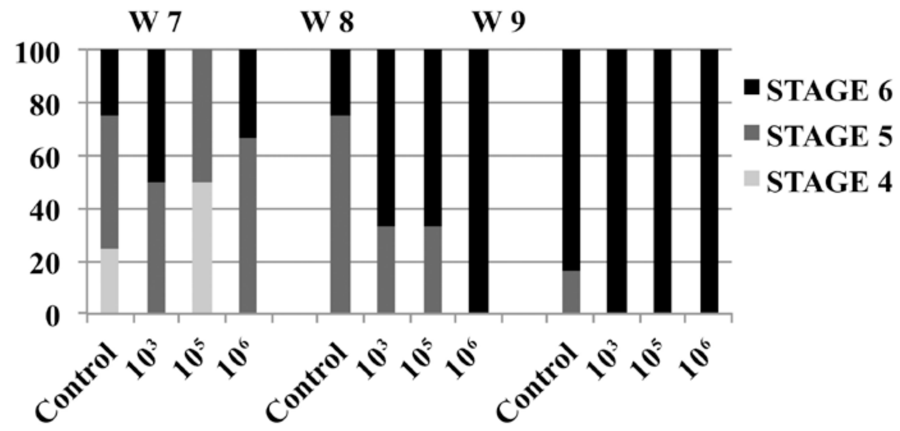

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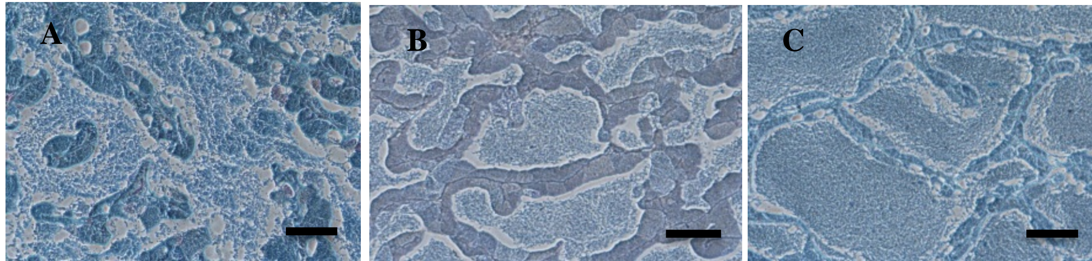

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725 **Fig.1**
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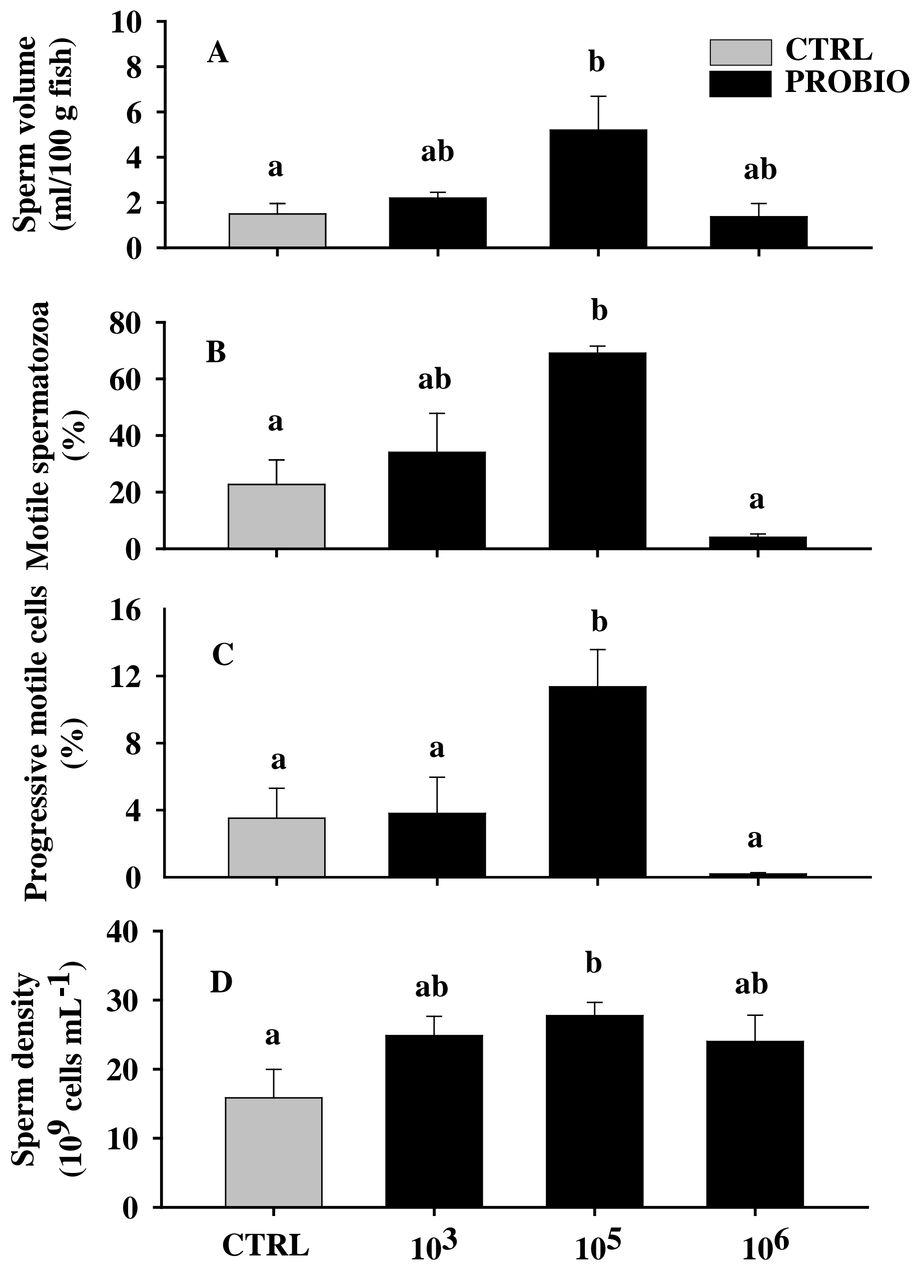

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736 **Fig.2**
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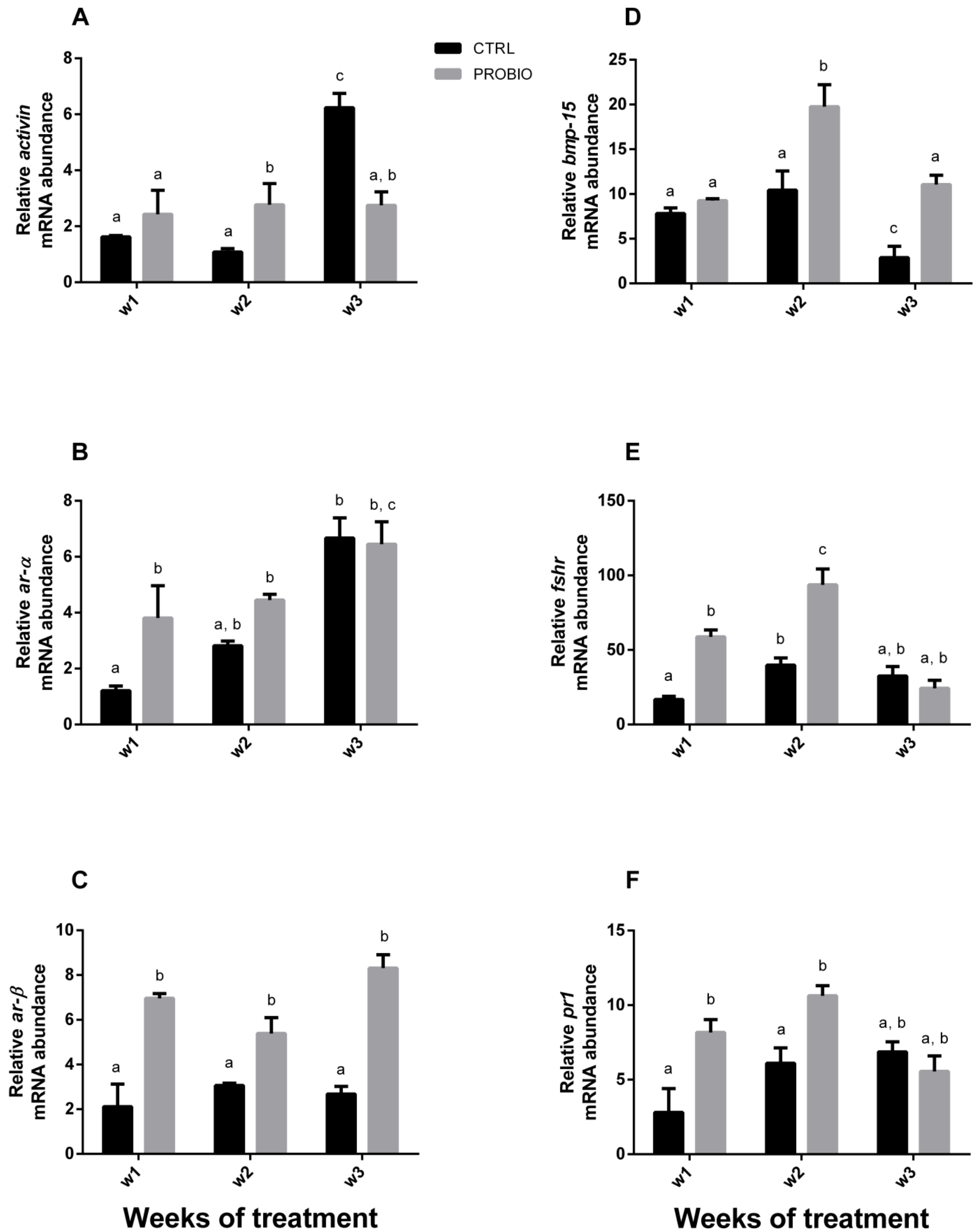

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749 **Fig. 3**

750 **Table 1**.

Gene	Sequence (5'- 3')	Orientation	GenBank Accession number (Reference)	Annealing Temperature (ºC)
actin	CGGAATCCACGAGACC TCCAGACGGAGTATTTGC	Forward Reverse	DQ286836 [(1)]	56
Activin	GGGCTTGGACAACCAGAAGA GTCACCATTGCAGCTTTCGG	Forward Reverse	GU269543 [(1)]	57
arα	CGGAAGGGAAACAGAAGTACC AGCGAAGCACCTTTTGAGAC	Forward Reverse	FR668031 [(2)]	57
arβ	CGCTGAAGGAAAACAGAGGT CATTCCAGCCTCAAAGCACT	Forward Reverse	FR668032 [(2)]	60
pr1	AGTTTGCCAATCTCCAGGTG ATCAAACTGTGGCTGGCTCT	Forward Reverse	AZBK00000000 [(3)]	56
bmp15	AAGCGGTTCTCAGTGTCGTT AAGGTACGCGAGAAAGCACA	Forward Reverse	[(4)]	60
fshr	AGCTAAGCTTGGATCCACCATGACACCTCTGTGGGTCCTCCT AGCTGCGGCCGCTCAGTGGGGTTGTTGATGGGCAC	Forward Reverse	AB700600 [(5)]	60

751

752 **Table 2**.

GENE	Week 1		Week 2		Week 3	
	CTRL	PROBIO	CTRL	PROBIO	CTRL	PROBIO
Activin	4.82±0.87[a]	1.82±0.62[b]	3.16±0.52[a,b]	1.46±0.55[b]	15.59±1.99[c]	1.93±0.90[b]
arα	2.79±1.03[a]	1.34±0.37[b]	5.05±0.84[b]	1.91±0.72[a]	7.92±1.10[c]	1.22±0.19[a]
arβ	1.45±0.49[a]	1.61±0.45[a]	3.75±0.54[b]	1.76±0.58[a]	2.68±0.34[a,b]	2.10±0.40[a]
bmp15	7.82±0.76[a]	5.36±0.78[b]	12.43±0.64[c]	4.57±0.51[b,e]	5.52±1.10[b]	3.51±0.29[e]
fshr	16.78±2.12[a]	39.81±4.87[b]	39.86±4.86[b]	18.90±2.07[a]	32.51±6.42[b,c]	28.67±4.64[c]
pr1	2.81±1.59[a]	2.09±0.72[a]	6.11±0.11[b]	4.11±0.51[a,b]	9.21±0.75[c]	4.66±1.04[a,b]

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777 **Table 3.**

GENE	Week 1		Week 2		Week 3	
	CTRL	PROBIO	CTRL	PROBIO	CTRL	PROBIO
Activin	23.20±0.48[a]	11.56±0.45[b]	16.57±2.27[a,b]	7.77±0.51[b]	86.20±7.01[c]	3.53±2.33[b]
arα	2.87±0.53[a]	4.52±0.46[a]	6.61±0.99[b]	2.14±0.02[a]	14.27±1.10[c]	1.27±0.45[a,b]
arβ	3.75±1.04[a]	9.72±1.08[b]	8.53±0.39[b]	3.88±0.53[a]	7.29±0.47[b]	1.92±1.34[a]
bmp15	186.66±18.16[a]	80.85±15.86[b]	299.54±13.21[c]	70.50±3.32[b]	131.79±22.72[d]	4.06±3.33[e]
fshr	16.78±2.12[a]	20.34±3.54[a]	39.86±4.86[b]	16.68±2.23[a]	32.51±3.05[b]	4.02±2.96[c]
pr1	3.12±0.94[a]	3.18±0.35[a]	6.77±0.18[b]	2.38±0.05[a]	10.21±0.16[c]	1.73±0.98[a]

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802 **Table 4.**

ANALYZED HORMONES	CONTROL	10^3 CFU/mL	10^5 CFU/mL	10^6 CFU/mL
11KT	23.22 ± 7.93	12.40 ± 0.46	15.75 ± 6.94	14.9 ± 4.9
DHP	0.68 ± 0.05[a]	0.89 ± 0.03[ab]	1.14 ± 0.11[b]	1.11 ± 0.22[b]
E2	0.86 ± 0.17	0.43 ± 0.12	0.58 ± 0.09	0.76 ± 0.31

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