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3 **Using specific recombinant gonadotropins to induce spermatogenesis**

4 **and spermiation in the European eel (*Anguilla anguilla*)**

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Abstract

New specific European eel (*Anguilla anguilla*) recombinant gonadotropins (aarGths) produced in the ovarian cells of Chinese hamsters (CHO) were used to induce maturation in captive male eels. In the first experiment, five different hormonal treatments were assayed: one group was given a constant dose of recombinant European eel follicle-stimulating hormone (aarFsh; 4 µg/fish) for 9 weeks, and the second group received a constant dose of recombinant European eel luteinizing hormone (aarLh; 2 µg/fish) also for 9 weeks. The other three groups were injected with different combinations of both aarGths (some doses constant, some variable). All five treatments stimulated androgen synthesis, but the increase was more pronounced in the fish treated with a combination of both aarGths. Unlike aarLh, aarFsh alone was able to induce spermiation, the best results were achieved in the fish that were treated with a constant dose of aarFSH and an increasing dose of aarLH, with spermiation being induced (20% motile cells) despite the fact that these fish were immature at the start of the experiment. In order to improve sperm quality, a second experiment was performed. Immature males received three constant doses of aarFsh (2.8, 1.4 or 0.7 µg/fish) and increasing doses of aarLh (every 3 weeks; 1, 2, 6 µg/fish). All the treatments induced spermiation, however the best sperm quality (with ≥50% motile cells) was observed in the males treated with the highest dose of aarFsh. In conclusion, these specific recombinant gonadotropins have demonstrated their capacity to induce spermatogenesis and spermiation *in vivo* in a teleost fish, the European eel.

1. Introduction

The European eel (*Anguilla anguilla*) population has decreased by 95-99%, compared to levels in 1960-80 [1], and as a result the species has been placed on the Red List of Threatened Species and listed as "Critically Endangered" by the International Union for Conservation of Nature (IUCN). However, the wild eel populations still sustain important fisheries and aquaculture industries (based on harvesting juvenile 'glass eels' from the wild) in Europe. To reduce the dependency of eel industry on wild populations, and even for stocking purposes, the development of new protocols to reproduce this species in captivity is crucial. The full life-cycle of the European eel has yet to be successfully closed in captivity, thus limiting aquaculture to ranching of wild caught glass eel. Some of the main hurdles encountered include the stagnation of puberty [2], finding alternative treatments to induce full maturation [3,4] and obtaining high quality gametes [5].

Gonadal activity (steroidogenesis and gametogenesis) is controlled by pituitary gonadotropin hormones (Gths), responsible for the control of reproduction in both teleost fish and other vertebrates. The follicle-stimulating hormone (Fsh) regulates the Sertoli cell activities, including structural, nutritional and regulatory (paracrine) support of germ cell development, and the luteinizing hormone (Lh) regulates the Leydig cell sex steroid production [6]. Both Gths, Lh and Fsh, are considered the most important pituitary hormones regulating testicular physiology.

At present, it is possible to induce eel maturation using exogenous hormones in both males [3,7], and females [8], but these hormonal treatments result in low rates of fertilization and hatching, mainly attributed to low gamete quality [9]. The traditional method used to induce eel maturation has involved the use of human chorionic gonadotropin (hCG) in males [10] and carp or salmon pituitary extract in females [11]. However, the administration of non-native Lh or equivalents has not resulted in a reliable level of percentage of matured fish nor gamete quality [12,13]. Therefore, in order to solve the reproductive problems encountered when using the current hormonal treatments in both male and female eels, a native eel Fsh purified from immature Japanese eel (*Anguilla japonica*) was tested to induce spermatogenesis *in vitro* [14]. The native Fsh was able to stimulate androgen synthesis but, due to the difficulty in obtaining an adequate amount of native Gths, tools for producing recombinant gonadotropins (rGths) in eel were developed [15].

Later on, a recombinant Japanese eel Fsh (ajrFsh) was synthesized using methylotropic

91 yeast (*Pichia pastoris*), and was successful in inducing steroidogenesis in the gonads of
92 both sexes cultured *in vitro* [14,16,17]. *In vivo* trials were also carried out on male eels,
93 inducing testicular growth and the beginning of spermatogenesis [18]. Subsequently, with
94 the aim of improving the biological activity of rGths, new Japanese eel rGths were
95 produced using a *Drosophila* expression system [19]. Both recombinant Fsh and Lh
96 induced complete spermatogenesis in *in vitro* conditions, but small effects were observed
97 *in vivo*. In order to obtain a large-scale production of rGths, Hayakawa et al. [20] used a
98 baculovirus-silkworm larvae system to produce rGths in Japanese eel. Complete
99 spermatogenesis was accomplished *in vivo*, but no male reached spermiation [20–22].
100 rGths have also been tested in other teleosts [15]. They have been successful in inducing
101 steroidogenesis and gonad development both *in vitro* and *in vivo*, however, the *in vivo*
102 results have been variable [23]. rGths have been reported to induce androgenesis e.g. in
103 zebrafish (*Danio rerio*; [24]) and Russian sturgeon (*Acipenser gueldenstaedtii*; [25]) and
104 recombinant Fsh has triggered spermatogenesis in immature European sea bass
105 (*Dicentrarchus labrax*; [26]). In general, the hormonal treatment was unable to induce
106 spermiation *in vivo*, except in the case of goldfish (*Carassius auratus*; [27]) and European
107 sea bass [28], but both these cases the treated fish were already sexually mature at the
108 beginning of the experiment. A different approach to the administration of rGths was to
109 inject a plasmid containing the Gth sequence directly into the muscle. Mazón et al. [28]
110 reported an improvement in sperm quality of European sea bass using an Lh sequence,
111 but again the treated fish were already sexually mature at the beginning of the
112 experiments.
113 The main objective of this study was to test if new European eel recombinant
114 gonadotropins (aarFsh and aarLh) were able to induce *in vivo* eel spermatogenesis and
115 spermiation in captivity, and demonstrate the differential function of fsh and lh during
116 spermatogenesis in eel.
117

118 **2. Materials and methods**

119 **2.1 Fish maintenance**

120 Immature yellow European eel males from the Valenciana de Acuicultura, S.A fish farm.
121 (Puzol, Valencia; East coast of Spain) were moved to the aquaculture facilities at the
122 Universitat Politècnica de València (UPV, Spain). The fish (6-8 fish/aquarium) were
123 distributed into 150-L aquaria equipped with separate recirculation systems, a

4

124 temperature control system (with heaters and coolers) and completely covered to maintain

125 as maximum possible a constant dark photoperiod. Before the hormonal treatment, the

126 eels were gradually acclimatized from freshwater to sea water (37 g/L) increasing the

127 salinity 10‰ each 2 days for 8 days, and 2 days more of resting at 37‰. The temperature,

128 oxygen level and pH of rearing were 20 ºC, 7-8 mg/L (thanks to aerators) and ~8.2,

129 respectively. Finally, due to its ethology during its reproductive migration, the fish were

130 fasted throughout the treatment.

131

132 **2.2 Production of recombinant gonadotropins**

133 Homologous single-chain recombinant Fsh and Lh, containing a modified linker, were

134 produced in the ovarian cells of Chinese hamster (CHO) in suspension by Rara Avis

135 Biotec S.L. (Valencia, Spain) using in-house technology. The purity was determined by

136 western blot using specific antibodies and the final amount of rGth was quantified by

137 immunoassay using these same specific antibodies.

138 European eel β and α subunits (GenBank accession numbers: CAA43373.1 (α common

139 subunit), AAN73407.1 (*βfsh*), CAA43374.1 (*βlh*) were used to obtain the single chain

140 Gths.

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142 **2.3 Experimental setup: hormonal treatments and samplings**

143 Two experiments were carried out in order to evaluate the effect of specific rGths had on

144 inducing maturation in European eels in captivity. In experiment 1 (October to December;

145 2015), the aim was to test the effect of aarFsh and aarLh on their own, as well as the

146 optimum combination of both rGths. Meanwhile in experiment 2 (January to March;

147 2016), the objective was to improve maturation and sperm quality using the treatment that

148 yielded the best results in terms of aquaculture purposes in experiment 1.

149 *Experiment 1*

150 Sixty immature male eels were used in this trial (mean body weight = 89.21±5.44 g; mean

151 body length = 38.12±2.28 cm). Before starting the hormonal treatments, 10 eels were

152 sampled as soon as they arrived at our facilities (thus in freshwater, FW); and 10 new eels

153 were sacrificed after they had been acclimatized to sea water (SW). Subsequently,

154 intramuscular hormonal treatments were administered weekly using the protocol

155 described by Asturiano et al. [10]. The fish were anaesthetized with benzocaine (60 ppm)

156 before receiving the hormone by intramuscular injection.

157 Forty immature fish (90.04±1.92 g; 38.10±0.33 cm) underwent five hormonal treatments

158 (~8 males per treatment; Fig. 1) for 9 or 11 weeks. Two groups, group F and L, were

159 injected once per week for 9 weeks only with aarFsh (4 µg/fish; Fig. 1A) or aarLh (2

160 µg/fish; Fig. 1B) respectively. The other three groups, F/L, F/LI and FD/LI, were injected

161 with different combinations of both hormones once per week for 11 weeks. Group F/L

162 (Fig. 1C) was treated with 4 µg aarFsh/fish for the first 4 weeks, followed by 2 µg

163 aarLh/fish for the last 8 weeks. Group F/LI (Fig. 1D) was treated with 4 µg aarFsh/fish

164 throughout the experimental period, combined with increasing doses of aarLh, from 0.5

165 to 2 µg/fish (between weeks 1 and 4) and then a constant dose of 2 µg aarLh/fish from

166 week 4. Group FD/LI (Fig. 1E) received decreasing doses of aarFsh from 4 to 0.5 µg/fish

167 (from week 3 to week 8) combined with increasing doses of aarLh from 0.5 to 2 µg/fish

168 (between week 1 and week 4) and then a constant dose of 2 µg aarLh/fish from the 4th

169 week on.

170 In order to evaluate the progression of maturation, every two weeks blood samples were

171 taken for steroids analyses, and biometric parameters, such as eye index (EI = 100 p 0.25

172 $(Dh + Dv)^2/Lt$); Lt: total length; Dh: horizontal distance; Dv: vertical distance; [29]) and

173 fin colour (FC; 0 = Transparent, 1= Light grey, 2= Dark grey, 4 = Black) were registered.

174 Both biometric parameters have previously been used in eel as indicators of maturation

175 [7].

176 After beginning of spermiation, samples were collected and analysed as described by

177 Gallego et al. [30]. Once the experiment had finished, the gonadosomatic index was

178 calculated (GSI = 100 gonad weight/total body weight) and testis samples were collected

179 to determine the stage of development by histology.

180 *Experiment 2*

181 In order to improve eel maturation in captivity, new hormonal treatments were designed,

182 with the treatment that yielded the best results in experiment 1 in terms of aquaculture

183 requirements, being used as the basis. The aarFsh levels were reduced slightly, in order

184 to find the minimum effective dose, whereas the aarLh dose was increased to enhance its

185 effect on the spermatogenesis process.

186 As with experiment 1, before starting the hormonal treatments, 8 immature eels were

187 sampled after they had been acclimatized to sea water (SW). Subsequently, 72 immature

188 fish (99.98±2.01 g; 38.56±2.28 cm) underwent three intramuscular hormonal treatments

189 (24 males/treatment), administered weekly for a total of 12 weeks. The males received a

190 constant dose of aarFsh, with the Low treatment group receiving 0.7, and the Medium

191 and High treatment groups receiving 1.4 and 2.8 µg/fish respectively. This was combined

192 with an increasing dose of aarLh: 1, 2, 6 µg/fish. The aarLh dose was increased every 3

193 weeks, from the 3rd week of the treatment (Fig. 2). In order to evaluate the progression of

194 maturation, three males per treatment were sacrificed every 3 weeks to collect blood and

195 gonad samples, and to measure biometric parameters, including GSI.

196 During the weeks of spermiation, sperm samples were collected and analysed as described

197 by Gallego et al. [30].

198

199 **2.4 Gonadal histology**

200 For experiment 1, sections from the mid-part of testis were preserved in 4% buffered

201 (PBS) glutaraldehyde at 4 ℃ overnight. The tissue was dehydrated and embedded in

202 Technovit® 7100. Nevertheless, in the experiment 2, sections from the mid-part of testis

203 were preserved in 10% formalin buffered at pH 7.4, dehydrated in ethanol and embedded

204 in paraffin. In both cases, between 10-20 sections/testis (5 µm thickness) were cut with a

205 Microm HM325, and stained with 1% toluidine blue (Exp. 1) or with a Shandom

206 Hypercut manual microtome and stained with haematoxylin and eosin (Exp. 2). Two

207 slides/testis were observed with a Nikon Eclipse E-400 microscope, and pictures were

208 taken with a Nikon DS-5M camera attached to the microscope. The stages of

209 spermatogenesis were determined by the following parameters: germ cell types present

210 in the testis and their relative abundance (according to Leal et al. [31]), the degree of

211 development of the seminal tubules and the GSI by the male at the time of sacrifice [32]

212 (see supplementary Fig.1: SPG1: Dominance of A spermatogonia, with a general absence

213 of lobule lumen. Gonad in non- proliferating stage. Stage SPG2: Dominance of A

214 spermatogonia, with some B spermatogonia and presence of lobule lumen. Stage SPC1:

215 Dominance of B spermatogonia and presence of spermatocytes. Stage SPC2: Dominance

216 of spermatocytes and B spermatogonia. Stage SD: is characterized by an abundance of

217 spermatids. Stage SPZ1: Early spermiation stage. Stage SPZ2: Stage of maximum

218 spermiation, showing fusion of sperm lobules.

219

220 **2.5 Plasma steroid assays**

221 Blood was collected and centrifuged at 3500 r.p.m. for 15 min, and the blood plasma was

222 stored at -80 ℃ until steroid analysis.

223 Plasma levels of testosterone (T) and 11-ketotestosterone were analyzed by a specific

224 enzyme immunoassay (EIA) following the methods developed for European sea bass

225 [33,34]. The lower limits of detection (80% binding) were 0.00117 ng/ml for 11-KT and

226 0.019 ng/ml for T. Analysis of serial dilutions of eel plasma dilutions were shown to be

227 parallel to the standard curve. The inter-assay coefficients of variation were 9.74% (n=7)

228 for 11-KT and 12.6% (n=7) for T. The intra-assay coefficients of variation were 8.64%

229 (n=10) for 11-KT y 2.93% (n=4) for T.

230

231 **2.6 Sperm collection and evaluation**

232 Sperm samples were collected 24 h after the administration of the hormone to obtain the

233 highest quality sperm [12]. After the eels were anesthetized, the genital area was first

234 cleaned with distilled water and thoroughly dried to avoid the sperm becoming

235 contaminated with faeces, urine or sea water. The sperm was then collected by the

236 application of gentle abdominal pressure, and with the help of a small-modified aquarium

237 air pump which produced a vacuum the sperm was collected into plastic Falcon tubes.

238 The sperm was diluted (1:10) before being activated in P1 medium [35]; in mM: 125

239 NaCl, 20 NaHCO$_3$, 2.5 MgCl$_2$·6H$_2$O, 1 CaCl$_2$·2H$_2$O, 30 KCl). The sperm was activated

240 by mixing 0.5 µl of this dilution with 4 µl of artificial sea water and 2% BSA (w/v), and

241 by adjusting the pH to 8.2 [12]. All the motility analyses were performed in triplicate

242 using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain) as described by

243 Gallego et al. [30].

244 The sperm parameters considered in this study were total motility (TM, %), defined as

245 the percentage of motile cells; progressive motility (PM, %), defined as the percentage of

246 spermatozoa which swim in essentially a straight line; curvilinear velocity (VCL, µm/s),

247 defined as the time-average velocity of a sperm head along its actual curvilinear

248 trajectory; straight line velocity (VSL, µm/s), defined as the time/average velocity of a

249 sperm head along the straight line between its first detected position and its last position.

250 Spermatozoa were considered immotile if their VCL was lower than 10 µm/s.

251

252 **2.7 Statistical analysis**

253 The mean and standard errors were calculated for all the biometric indexes (GSI, EI, and

254 FI) and sperm parameters (volume, density, motility and rest of kinetic parameters).

255 Shapiro-Wilk and Levene tests were used to check the normality of data distribution and

256 variance homogeneity, respectively. One-way analyses of variance (ANOVA) and

257 Student's t-test were used to analyse data (normal distribution) between groups at the

258 same week. Significant differences between treatments were detected using the Tukey

259 multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis

260   one-way ANOVA on ranks and Mann-Whitney U-test were used. Moreover, GLM for

261   repeated measures was used for comparing data over the weeks within the same hormonal

262   treatment; and significant differences were also detected using the Tukey multiple range

263   test ($P<0.05$). All statistical analyses were performed using the statistical package SPSS

264   version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

265

266   **2.8 Human and Animal Rights**

267   This study was carried out in strict accordance with the recommendations given in the

268   Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013

269   regarding the protection of animals used for scientific purposes (BOE 2013). The protocol

270   was approved by the Experimental Animal Ethics Committee from the Universitat

271   Politècnica de València (UPV) and final permission was given by the local government

272   (Generalitat Valenciana, Permit Number: 2014/VSC/PEA/00147). The fish were

273   sacrificed using anaesthesia and all efforts were made to minimize suffering. The fish

274   were not fed throughout the experiment and were handled in accordance with the

275   European Union regulations concerning the protection of experimental animals (Dir

276   86/609/EEC).

277

278   **3. Results**

279   **3.1 Experiment 1**

280   *Biometric parameters: EI, FC and GSI*

281   Regarding Eye Index (EI; Table 1), significant differences between the FW and SW

282   values (before hormone administration), were observed in all the groups. Fin Colour (FC)

283   became progressively dark throughout the hormonal treatment, becoming almost

284   completely black around the week 5 in groups F, L, F/LI and FD/LI, and around week 7

285   in group F/L.

286   Before hormonal treatment, the eels showed a GSI of 0.20±0.05% in FW and 0.22±0.04%

287   in SW (Fig. 3). No significant differences were found at the end of the different hormonal

288   treatments except in group F/LI, which showed a mean GSI of 3.83±0.84% after eleven

289   weeks. On the other hand, eels from groups F and FD/LI showed increases in GSI (of

290   around 1%) at week 9 and 11, respectively. The difference however was not significant

291   compared to the initial values (FW and SW).

Progression of gonad development

293 Regarding testis development (Figs. 4 and 5), aarFsh alone was able to induce

294 spermatozoa stages (SPZ1, SPZ2) in 37.5% of fish, and SPD stage in 25% of fish. In

295 contrast, all aarLh treated males were less developed, with 100% being in stage SPG2,

296 with a dominance of SPGA, clusters of differentiated SPGA (Fig. 5B), and in some cases

297 the presence of SPGB. In groups F/LI and FD/LI, the treatments which combined aarFsh

298 and aarLh, 87.5% of fish reached normal spermiation stages (SPZ1 and SPZ2). However,

299 treatment F/L, also combining both hormones, induced abnormal testis development in

300 50% of fish: whilst the testis had the structure of stage SPG2, with dominance of SPGA,

301 a few small spermatozoa cysts were present. We considered this to be a regressive stage,

302 with the spermatocytes and even the SPGB having disappeared from the testis after an

303 initial production of spermatozoa. This is supported by the fact that apoptotic cells were

304 observed in this stage of development (Fig. 5D).

305 *Steroid evolution during hormonal treatment*

306 The administration of aarFsh or aarLh alone (groups F and L, respectively) was enough

307 to initiate steroidogenesis (Fig. 6), inducing the production of both 11-KT and T.

308 However, the treatment which combined both aarGths (groups F/LI and FD/LI) yielded

309 higher levels of these steroids. On the other hand, a sequential administration of aarGths

310 (group F/L) provided a steroid profile closer to group F and L than to the profiles showed

311 by aarGths combined treatments (group F/LI and FD/LI).

312 *Sperm quality analyses*

313 Eels treated with a single Gth showed the lowest percentage of spermiating males (Fig.

314 7A): in the case of aarLh (group L) non-spermiating males at all were observed, whereas

315 in group F (aarFsh) they were only registered in weeks 8 and 9. The percentage of

316 spermiating males was higher when aarFsh and aarLh were combined.

317 Group F/LI (which received constant doses of aarFsh and increasing doses of aarLh)

318 yielded the best results, with 100% of males reaching spermiation at weeks 10 and 11.

319 Sperm volumes were remarkably low in all the treatments, with no significant differences

320 observed over the course of the weeks neither within the individual groups nor between

321 the different treatments (Fig. 7B, see also supplementary data: Fig. 2A). The highest

322 density values were observed in group F/LI, with significant differences observed in

323 weeks 8 and 10 (Fig. 7C).

324 Regarding sperm quality, total (TM) and progressive (PM) motilities (Figs. 8A and 8B)

325 yielded the highest values at week 8 in group F/LI (without significant differences inter-

326 treatments but with significant differences intra-treatment; see also supplementary data:

327 Fig. 2B), with the maximum values reached being 20% TM and 2% PM. From the 8th

328 week on, males from all the treatments displayed a marked decrease in total and

329 progressive motility.

330 The kinetic parameters of the sperm cells (VCL and VSL; Figs. 8C and 8D) showed a

331 similar evolution in groups F/LI and FD/LI, with the highest peak being observed at week

332 8 followed by a progressive decrease until the end of the treatment. Groups F and F/L did

333 not show any variations over the course of the weeks and the velocity values remained

334 low and constant throughout the weeks of spermiation.

335

336 **3.2 Experiment 2**

337 *Biometric parameters: EI, FC and GSI*

338 Regarding EI (Table 2), a significant increase was observed in all the groups between the

339 initial SW values and the values recorded in the 3rd week. This was followed by a

340 continual increase in this parameter throughout the hormonal treatments, with peaks in

341 weeks 9 and 12. FC progressively darkened, but at different rates depending on the

342 treatment, becoming almost completely black in weeks 6, 9 and 12 in the High, Medium

343 and Low treatments, respectively.

344 Irrespective of the treatment, no significant differences were observed in the GSI until the

345 12th week, when the values became significantly higher than controls, reaching values of

346 0.9, 1.8 and 4.3% in the Low, Medium and High treatments, respectively (Fig. 9). In

347 addition, significantly higher GSI values were achieved in the High treatment compared

348 to the other treatments, thus indicating an interaction between factors (week and

349 treatment).

350 *Progression of gonad development*

351 Regarding testis development (Table 3), 6 weeks after the start of the treatments, the High

352 and Medium groups demonstrated the most advanced development, with 33% of fish

353 from both groups having arrived at stage SPC2, with meiosis as the dominant process.

354 The Low treatment did not induce an evident testis development until week 9, when first

355 spermatogonia B, and well developed testis lumen were observed. In all the treatments,

356 the spermiating stages (SPZ1 and SPZ2) were reached in week 12, but only high doses of

357 aarFsh resulted in 100% of eel males reaching the spermiation stages (SPZ1 and SPZ2),

358 with the Medium and Low doses only able to induce these stages in 57% (4/7) and 42%

359 (3/7) of the fish, respectively.

360 *Steroid evolution during hormonal treatment*

361 The administration of aarFsh and aarLh throughout the Low and Medium treatments was

362 able to initiate the production of 11-KT and T (Fig. 10), but without reaching significant

363 differences in comparison to the initial levels. However, the aarGths doses used in the

364 High treatment generated a progressive increase in the plasma levels of both androgens,

365 which reached their highest values in the 6th week (Fig. 10A), followed by a progressive

366 decrease until the end of the treatment.

367 *Sperm quality analyses*

368 The percentage of spermiating males was constant (around 60%) at High treatment for

369 first weeks of spermiation, but then they increased to 80% at week 11 (Fig. 11A). The

370 Medium and Low treated males did not begin to produce sperm until weeks 9 and 10,

371 respectively, reaching approximately 40% (Low group) and 70% (Medium group) of

372 spermiating males in week 12.

373 Sperm volumes were remarkably low and showed a progressive increase over the weeks

374 (Fig. 11B; see also supplementary data: Fig. 3A). The High treatment yielded the highest

375 volumes every week, with the values being significantly higher than those recorded in the

376 Medium and Low treatments at week 10. Sperm density values followed the same pattern

377 as volume, but with significantly lower values in the Low treatment at weeks 10, 11 and

378 12 (Fig. 11C).

379 Regarding the kinetic parameters, total (TM) and progressive (PM) motility (Figs. 12A

380 and 12B; see also supplementary data: Fig. 3B), yielded the best values in the High

381 treatment, with significant differences at weeks 10 and 12, and maximum values of 60

382 and 30% of TM and PM, respectively. Moreover, by analyzing these parameters an

383 interaction between factors (week and treatment) became apparent. The motility values

384 of the sperm in the Medium and Low groups did not reach more than 50% in any week.

385 The velocity values (VCL and VSL; Figs. 12D and 12E) were significantly higher in the

386 group treated with the High dose of aarFsh, with the highest peak being observed in week

387 10 followed by a progressive decrease until the end of the treatment. Little or no variations

388 were observed in the Medium and Low treatments over the course of the weeks, and the

389 velocity values remained low and regular during the weeks of spermiation.

4. Discussion

Experiment 1

The present study demonstrated that the aarGths produced by a CHO system are biologically active and their half-life is long enough to induce *in vivo* effects. aarFsh alone and all the combined aarFsh and aarLh treatments were able to induce spermiation, whereas aarLh alone failed to induce completion of the maturation cycle, with the fish maturation being interrupted at the pre-meiotic stage (SPG2), with testis showing only SPGA and some lumen development.

Biometric parameters: EI, FC and GSI

With the aim of monitoring the progression of maturation, different biometric parameters (EI and FC) were used. EI changed according to the maturation stage and the treatment. Similarly, an increase in EI was observed when the aarGths were combined or when aarLh alone was administered, nevertheless aarFsh alone registered a lower EI. Results from previous studies corroborate our data, since consecutive injections with eel Lh-producing implants induced a significant increase in the EI in European eel females (Ron Dirks, personal communication). Furthermore, the coexistence of duplicated Lh receptors in the European eel genome has recently been demonstrated [36]. A comparative tissue distribution study in silvering migrating females, a high expression of both Lh receptors but no Fsh receptor expression was reported in eye tissue. This therefore would indicate that aarLh is the main factor involved in inducing eye development and EI increase.

The FC became darker throughout the course of maturation, irrespective of the treatment. This could mean that both rGths stimulate this process. Rohr et al. [37] proposed that 11-KT was responsible for silvering in short-finned eel (*Anguilla australis*). This hipothesis is supported by our results, since darker fins and higher 11-KT plasma levels were registered in the same weeks.

All hormonal treatments were able to promote spermatogenesis, but only group F/LI induced a significant increase in the GSI (around 4%). This value was lower than the values previously registered using rhCG hormones with maximum values of 10%, hormonal treatment which have been demonstrated until now the most effective to mature male eel in captivity [3,38], what indicates that the treatments used in the first experiment can be improved.

The lowest GSI values were obtained in the groups L and F/L, what may indicate that the stimulation of the Fsh receptor is crucial in order to complete spermatogenesis, since a

423   total or temporary lack of Fsh has a negative impact on gonad development. On the other

424   hand, in Japanese eel, the administration of only ajrLh (produced in baculovirus-silkworm

425   larvae system) was able to induce a higher GSI than ajrFsh alone [20,22]. The differences

426   in these studies could be explained by the fact that different systems have been used to

427   produce the rGths, meaning that their bioactivity and half-life could differ [39]. In fact,

428   the level of testis development in Japanese eel reached using ajrGths was lower than that

429   obtained in this study with either aarGths (Fig. 3) or hCG [15,40], both of which resulted

430   in complete testis maturation. Therefore, further *in vivo* studies on the interaction between

431   Gths and their receptors are necessary.

432   *Progression of gonad development*

433   All the treatments, with the exception of group L (treated with only aarLh), induced

434   complete spermatogenesis, with the most advanced stages of gonad development (SPZ1

435   and 2) being reached. aarFsh was able not only to induce complete spermatogenesis, as

436   previously observed *in vitro* in Japanese eel [19,41], but even 50% of spermiating males.

437   Fish from treatment F/L, in which aarFsh administration was arrested after 4 weeks,

438   showed abnormal testis development, with a few spermatozoa cysts in small gonads (GSI

439   0.15-0.16%) showing type A spermatogonia, but no spermatocytes nor spermatids, which

440   is the common feature when spermatozoa are present. Two processes could explain this:

441   either the spermatocytes, spermatids, or most type B spermatogonia, have undergone an

442   apoptotic process and were not present in the testes, leaving some spermatozoa cysts, or

443   an abnormal restricted meiosis and spermiogenesis have happened only in a few cysts.

444   We consider the first hypothesis, a regression after Fsh cessation, to be more likely, as

445   apoptotic cells were found.

446   aarLh stimulated the onset of testis maturation, but it did not induce complete

447   spermatogenesis by itself, unlike Japanese eel rLhs [20]. In fact, in Japanese eel, ajrLh

448   (produced by silkworm larvae) resulted in a higher level of testis development than ajrFsh

449   [22]. Other studies on Japanese eel have shown that ajrFsh (produced in a *Drosophila*

450   expression system) was able to stimulate the recombinant Fsh receptor even at low doses,

451   but not the Lh receptor. Furthermore ajrLh activated both Gths receptors, although only

452   at high doses [19]. Similar results have been observed in other teleosts [42–44].

453   Therefore, one possible explanation for the differences found between the Japanese and

454   European eel results could be the fact that the minimum aarLh dose required to stimulate

455   both receptors and obtain a complete spermatogenesis is higher than the one we used in

456 the current study. Moreover, other factors, such as the number of receptors or their
457 sensitivity could also have an effect, and therefore new studies are required in order to
458 understand the mechanism.

459 *Steroid evolution during hormonal treatment*

460 In previous studies, all Japanese eel rGths were able to induce testis steroidogenesis
461 [14,17,19,22], increasing the T and 11-KT levels both *in vitro* and *in vivo*. Furthermore,
462 it is known that in Japanese eel 11-KT alone is able to induce complete spermatogenesis
463 *in vitro* [41]. In the present study, when both aarGths were administered separately they
464 were able to induce androgen steroidogenesis, and resulted in similar profiles. Thus, a
465 lack of 11-KT in aarLh-treated fish (group F, Fig. 6A) would not explain the differences
466 in maturation between aarLh and aarFsh treated fish.

467 Similar androgen production levels were observed using a sequential treatment; first
468 aarFsh and later aarLh (group F/L). But, the abnormal testis development found in the
469 F/L group would suggest that, unlike what happens in Japanese eel testis *in vitro* [41], the
470 androgen synthesis was not enough to achieve complete spermatogenesis *in vivo*. This is
471 corroborated by studies on zebrafish which have shown that Fsh has a direct effect on the
472 testis, not mediated by androgen production. Zebrafish testes cultured with rFsh and
473 trilostane (a 3β-hydroxisteroid dehydrogenase inhibitor blocking the steroid production),
474 showed an up-regulation of gene expression in Leydig cells: *insl3, cyp17a1* [24] and Igf3
475 [45]. This in turn stimulated the proliferation and differentiation of the spermatogonia, as
476 well as the beginning of meiosis and development into adult zebrafish testis [45].

477 On the other hand, 11-KT production was higher in the fish treated with both aarGths
478 (groups F/LI and FD/LI). These groups reached the last stages of maturation and induced
479 sperm production earlier than the other groups.

480 *Sperm quality parameters*

481 In previous studies, complete *in vivo* spermatogenesis of immature Japanese eel males
482 has been accomplished after treatment with rGths, but unlike the current study no
483 spermiating male has been obtained [20–22]. Treatment with rGths has resulted in
484 spermiating males in Japanese eel and other teleosts [27,28], but the treated fish were
485 already sexually mature. Therefore, as far as we know, this is the first study where
486 spermiating males have been obtained after rGths treatment using totally immature male
487 fish.

488 The sperm quality was different depending on the hormonal treatment. Although the
489 histological features from the testis in SPZ1 or SPZ2 from treatments F, F/LI and FD/LI
490 were similar, the best sperm quality was observed in group F/LI, with almost 100%
491 spermiating males and 20% sperm motility. This group also showed higher levels of testis
492 growth (GSI), sperm volume and sperm density, indicating that a sustained level of aarFsh
493 and an increasing level of aarLh is a good system for inducing the highest sperm
494 production and quality. Even so, the sperm quality was significantly lower than that
495 registered when using rGths of human origin (rhCG; [3,46]). Consequently, taking into
496 account the results observed in Experiment 1, new hormonal treatments were assayed in
497 Experiment 2.

498 *Experiment 2*

499 In this second experiment, we confirmed that the combination of both rGths is necessary
500 to induce the sex maturation of European eel in captivity. The High treatment group
501 showed similar results in terms of gonad development to the High group in experiment 1.
502 2.8 µg aarFsh/fish was established as the minimum effective dose to induce maturation
503 in male European eel. Moreover, doses higher than 2 µg aarLh/fish registered better
504 results in terms of sperm quality.

505 *Biometric parameters: EI, FC and GSI*

506 Generally, EI levels were higher in this second experiment than in the first. The aarLh
507 doses were higher in this second experiment, supporting the hypothesis that aarLh is main
508 factor contributing to the increase in EI. In terms of FC change, the results were similar
509 to those of the first experiment, with high 11-KT levels coinciding with darker fins.
510 Unlike in Experiment 1, all the treatments in Experiment 2 resulted in a significant
511 increase in the GSI, confirming that combining both rGths is the best method of inducing
512 eel maturation. No significant differences were observed in the GSI of group F/LI from
513 Experiment 1 (3.83%; 4 µg aarFsh/fish) and that of the High treatment group from
514 Experiment 2 (4.3%; 2.8 µg aarFsh/fish). This suggests that 2.8 µg aarFsh/fish is the
515 minimum effective dose for inducing gonad development.

516 *Progression of gonad development*

517 All three treatments (High, Medium and Low) induced complete spermatogenesis, but
518 the progression of gonad development was delayed and the percentage of fish observed
519 in the spermiating stages (SPZ1 and 2) was lower in the Medium and Low groups. These

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results suggest that the administration of rFsh is relevant for the advancement of spermatogenesis. On the other hand, in previous experiences, rhCG treatments reported faster progression in spermatogenesis and a higher percentage of spermiating males [3,7,38]. Thus, further studies are necessary to test if any alternative treatments are able to yield the same or better results than those observed using the traditional method with rhCG.

Steroid evolution during hormonal treatment

The androgen profiles in this second experiment were similar to those obtained in experiment 1, with higher levels (only significant in the High group) being reached at around week 6 and levels later decreasing (around week 9). However, although the androgen profiles were similar, the results in terms of gonad development were different, with the fish in the second experiment maturing further (reaching the SPD stage at least). These results support the theory that androgen levels on their own are not enough to induce complete spermatogenesis *in vivo*, and that rGths may have a direct effect on the testes. In fact, 11-KT implants in male short-finned eel (*Anguilla australis*) were not able to induce the same stage of maturation as hCG treatment [47].

Sperm quality parameters

Motilities close to 60% were registered in the High treatment group. These values are 3 times higher than the best values obtained in experiment 1, and similar to those reported with the use of rhCG [3,46,48]. With regards to other sperm parameters, such as density, progressive motility and kinetic parameters (VCL and VSL), the values observed were similar to those reported in experiments using rhCG [3]. Nevertheless, the volume was lower than that achieved by hCG treatments [7]. Moreover, after a progressive increase in sperm quality up to week 10, a subsequent decrease was observed, while longer spermiation periods were found with rhCG treatments [3].

In this second experiment, the combination of both aarGths and higher levels of aarLh resulted in an improvement in sperm quality compared to experiment 1, but without reaching the values reported with rhCG treatments. In consequence, further studies are necessary in order to find out the proper aarGth amount and/or timing of administration.

In conclusion, this study has demonstrated that aarGths are able to induce the spermiation in European eel, and confirmed that the half-life of these rGths is long enough to induce *in vivo* effects. Nevertheless, due to the fact that a decrease in sperm quality was observed

552 at the end of the treatment, further experiments combining these recombinant hormones
553 are required in order to improve hormonal treatments.
554

Acknowledgements

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713

Figure legends

Figure 1. Descriptive diagram of the hormonal treatments for the five experimental
groups in the experiment 1.

Figure 2. Descriptive diagram of hormonal treatments for the three experimental groups
in the experiment 2.

Figure 3. Gonadosomatic indexes before hormone administration (freshwater (FW) and
seawater (SW) conditions) and at the end of hormonal treatments (groups F and L until
9th week; groups F/L, F/LI and FD/LI until 11th week) in the experiment 1. Data are
expressed as mean ± SEM (n = 6-8 per sampling and treatment). Asterisk indicates
significant differences between treatments at the end of the trial.

Figure 4. Relative percentages of the stages of testis development (SPG1-SPZ2) at the
end of the hormonal treatments in the experiment 1 (groups F and L until 9th week; groups
F/L, F/LI and FD/LI until 11th week; n= 6-8). See main text for definition of gonad
developmental stages: Stages: SPG1: Spermatogonia 1; SPG2: Spermatogonia 2; SPC1:
Spermatocyte 1; SPC2: Spermatocyte 2; SD: Spermatid; SPZ1: Spermatozoa 1; SPZ2:
Spermatozoa 2; Regression.

Figure 5. Histological sections of testis from different treatments (experiment 1). A)
aarFsh alone, 9 weeks; stage SPZ1; B) aarLh alone, 9 weeks, SPG2 stage; C)
aarFsh+aarLh, 12 weeks, SPZ2 stage; D) aarFsh+aarLh 3, 12 weeks, regression. Scale
bar: A=100 µm; B, C, D= 10 µm. See main text for definition of gonad developmental
stages: SPGA (spermatogonia A); SPGAdiff (spermatogonia A differenciated); SPGB
(spermatogonia B); SPC I (spermatocyte I); SPD (spermatid), SPZ (spermatozoa); Ap
(apoptotic cells).

Figure 6. Profile plasma levels of testosterone (T) and 11-ketotestosterone (11-KT)
before hormone administration (sea water conditions, SW) and throughout the hormonal
treatments (groups F and L until 9th week; groups F/L, F/LI and FD/LI until 11th week) in
the experiment 1. Steroid levels (T and 11-KT) are represented as fold change with respect

747 to the mean value of samples from freshwater fish (1.80 ± 0.14 ng T/ml and 0.29 ± 0.05
748 ng 11-KT/ml). Data are expressed as mean ± SEM (n = 6-8 per sampling and treatment).
749 Different letters indicate significant differences over the weeks within the same hormonal
750 treatment.

751

752 **Figure 7.** Evolution of sperm production parameters throughout the recombinant
753 gonadotropin treatments (groups F and L until 9th week; groups F/L, F/LI and FD/LI until
754 11th week) in the experiment 1. A) Percentage of spermiating males; B) Sperm volume;
755 and C) Sperm density. Data are expressed as mean ± SEM (n = 6-8 per sampling and
756 treatment). Capital letters indicate statistical differences between groups (treatments), and
757 lowercase letters indicate statistical differences over the time in the same treatment.

758

759 **Figure 8.** Evolution of sperm kinetic parameters throughout the recombinant
760 gonadotropin treatments (groups F and L until 9th week; groups F/L, F/LI and FD/LI until
761 11th week) in the experiment 1. A) Total motility; B) Progressive motility; C) Curvilinear
762 velocity (VCL); and D) Rectilinear velocity (VSL). Data are expressed as mean ± SEM
763 (n = 6-8 per sampling and treatment). Capital letters indicate statistical differences
764 between groups (treatments), and lowercase letters indicate statistical differences over the
765 time in the same treatment.

766

767 **Figure 9.** Gonadosomatic indexes of three experimental groups (High, Medium and Low
768 treatments) over the weeks in the experiment 2. Data are expressed as mean ± SEM (n =
769 3-7). Asterisk indicates significant differences between groups at the same week and
770 different letters indicate significant differences between weeks for the same hormonal
771 treatment.

772

773 **Figure 10.** Profile plasma levels of testosterone (T) and 11-ketotestosterone (11-KT)
774 before hormone administration (sea water conditions, SW) and throughout the hormonal
775 treatments (High, Medium and Low) in the experiment 2. Steroid levels (T and 11-KT)
776 are represented as fold change with respect to the mean value of samples from freshwater
777 fish (2.12 ± 0.22 ng T/ml and 1.80 ± 0.21 ng 11-KT/ml). Data are expressed as mean ±
778 SEM (n = 3-7 per sampling and treatment). Different letters indicate significant
779 differences over the weeks for the same hormonal treatment.

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781   **Figure 11.** Evolution of sperm production parameters throughout the recombinant
782   gonadotropin treatments (High, Medium and Low) in the experiment 2. A) Percentage of
783   spermiating males; B) Sperm volume; and C) Sperm density. Data are expressed as mean
784   ± SEM (n = 3-7 per sampling and treatment). Capital letters indicate statistical differences
785   between groups (treatments), and lowercase letters indicate statistical differences over the
786   time in the same treatment.

787

788   **Figure 12.** Evolution of sperm kinetic parameters throughout the recombinant
789   gonadotropin treatments (High, Medium and Low) in the experiment 2. A) Total motility;
790   B) Progressive motility; C) Curvilinear velocity (VCL); and D) Rectilinear velocity
791   (VSL). Data are expressed as mean ± SEM (n = 3-7 per sampling and treatment). Capital
792   letters indicate statistical differences between groups (treatments), and lowercase letters
793   indicate statistical differences over the time in the same treatment.

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800   **Supplementary Figure 1** Histological sections of eel testis at different developmental
801   stages. A) Testis at SPG1. B) Testis at SPG2. C) Testis at SPC1. D) Testis at SPC2. E)
802   Testis at SPD. F) Testis at SZ1. G, H) Testis at SZ2. See main text for definition of gonad
803   developmental stages. SPGA= Spermatogonia type A; SPGB= Spermatogonia type B;
804   SPC: Spermatocytes; SPD: Spermatids; SPZ: Spermatozoa. Scale bars, 25 μm (A, B, C,
805   D, E, F, H) and 100 μm (G).

806

Table legends

Table 1. Biometric parameters (eye index and fin colour) before hormone administration
(freshwater, FW; and sea water, SW) and over the weeks of hormonal treatments on the
five experimental groups in the experiment 1 (groups F and L until 9th week; groups F/L,
F/LI and FD/LI until 11th week) 6-8. Data are expressed as mean (SEM); n= 6-8. Different
letters indicates significant differences between groups at the same week and different
letters indicate significant differences among weeks for the same hormonal treatment.

Table 2. Biometric parameters (eye index and fin colour) over the weeks in the three
experimental groups (High, Medium and Low) in the experiment 2. Data are expressed
as mean (SEM); n= 3-7. Different letters indicates significant differences between groups
at the same week and different letters indicate significant differences among weeks for
the same hormonal treatment.

Table 3. Distribution of stages of testis development reached by the different males
through the samplings (W3-12) in the three experimental groups: (●) High (2.8 µg
aarFsh/fish); (◐) Medium (1.4 µg aarFsh/fish); and (○) Low (0.7 µg aarFsh/fish). Stages:
SPG1: Spermatogonia 1; SPG2: Spermatogonia 2; SPC1: Spermatocyte 1; SPC2:
Spermatocyte 2; SD: Spermatid; SPZ1: Spermatozoa 1; SPZ2: Spermatozoa 2.
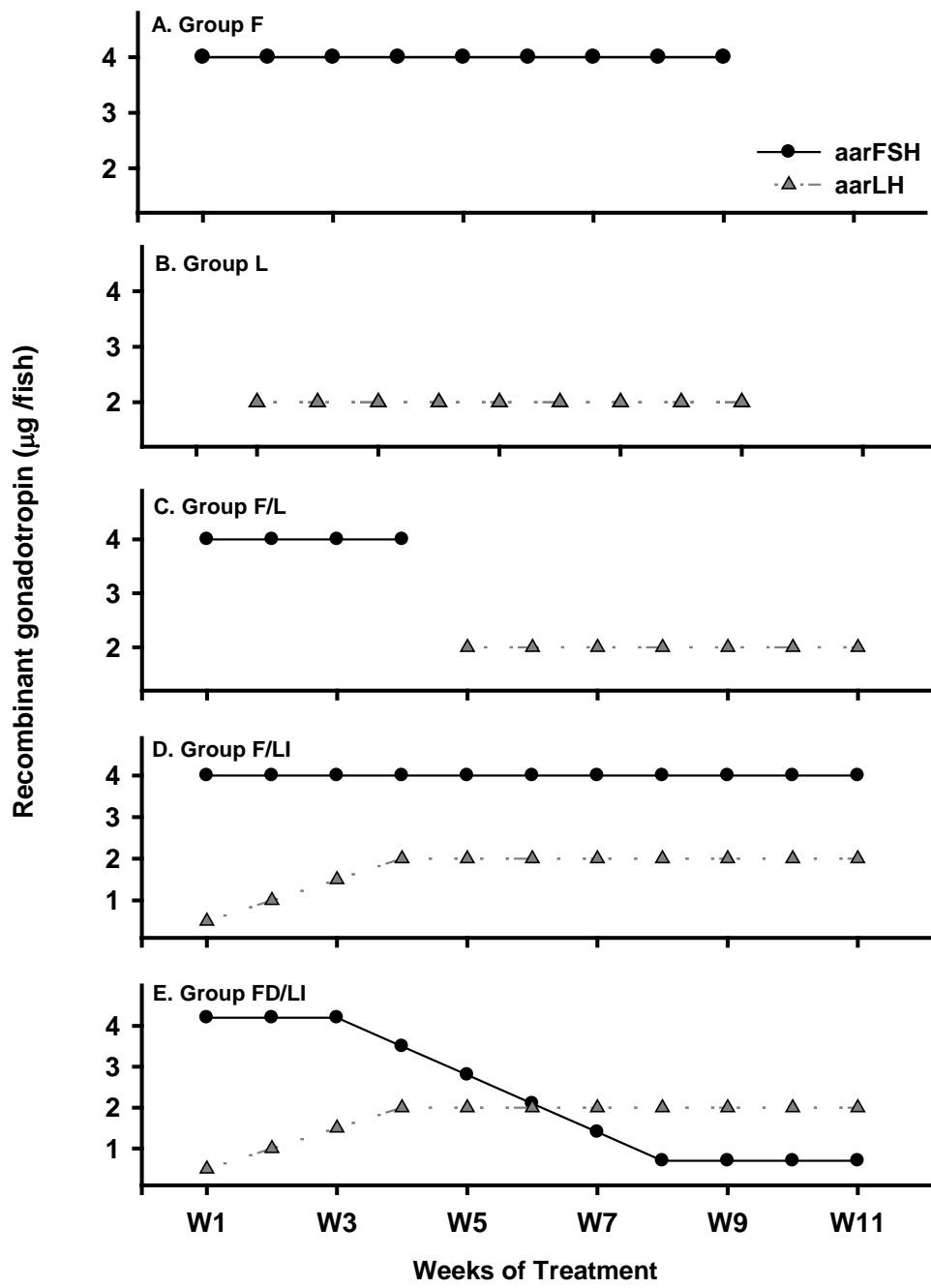
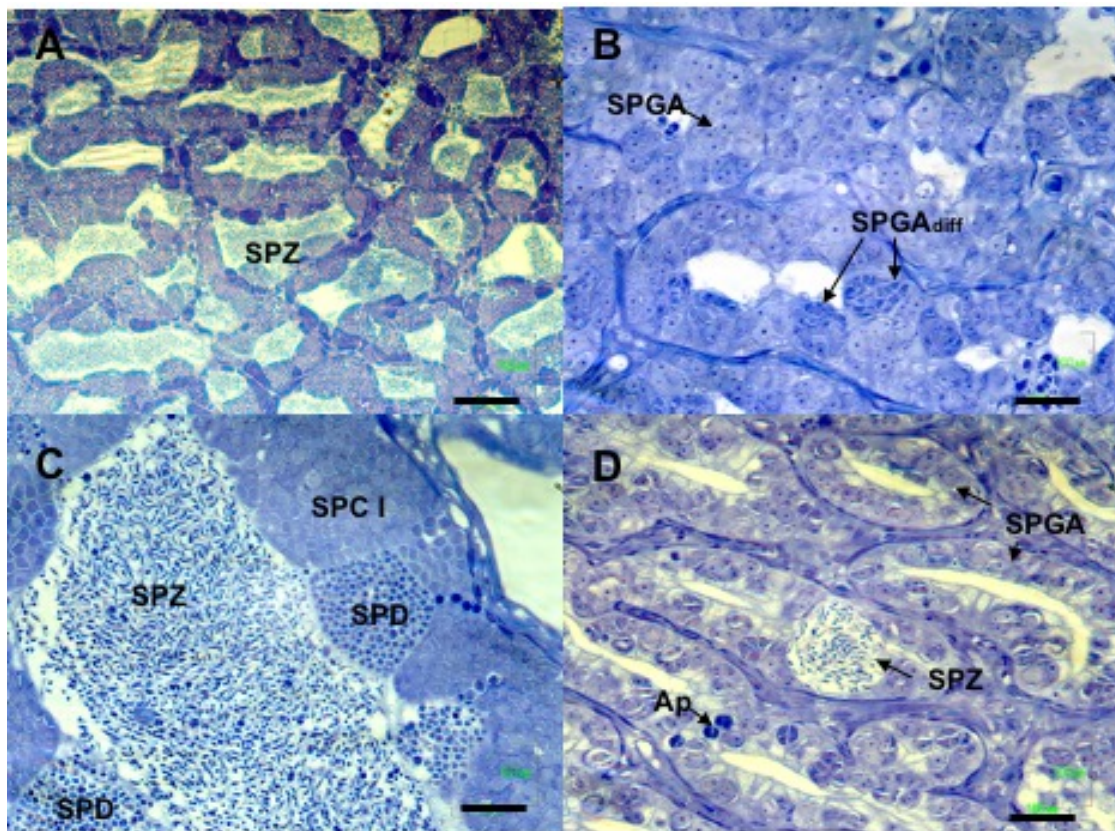**Figure 1**

829 **Figure 2**

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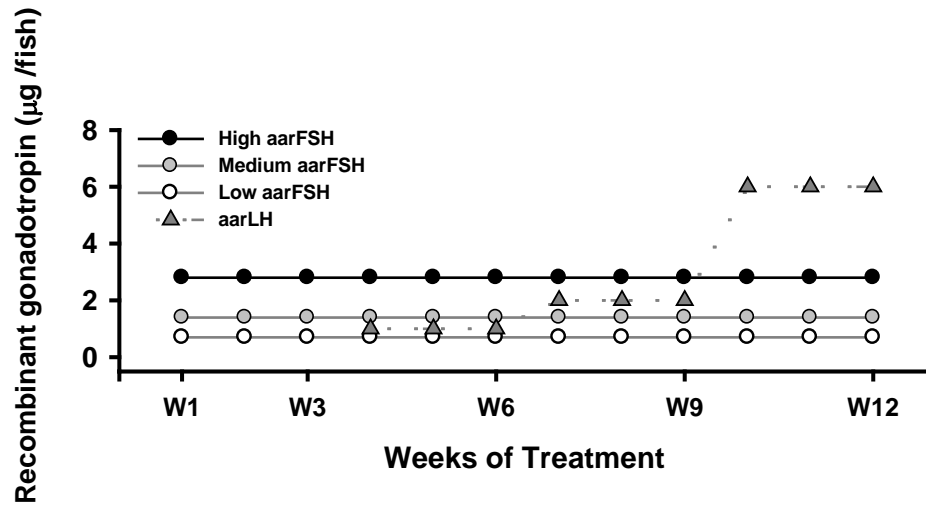
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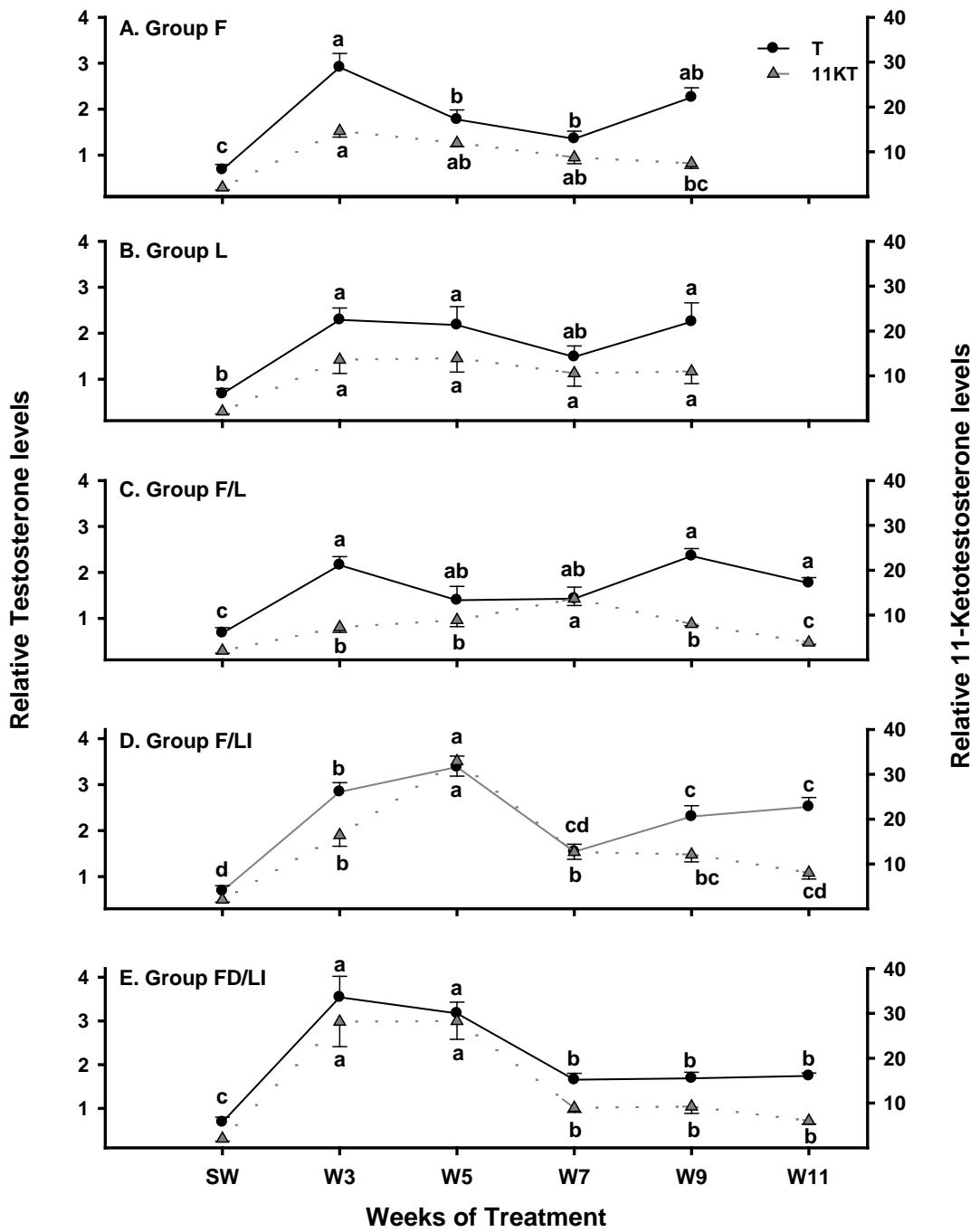832   **Figure 3**
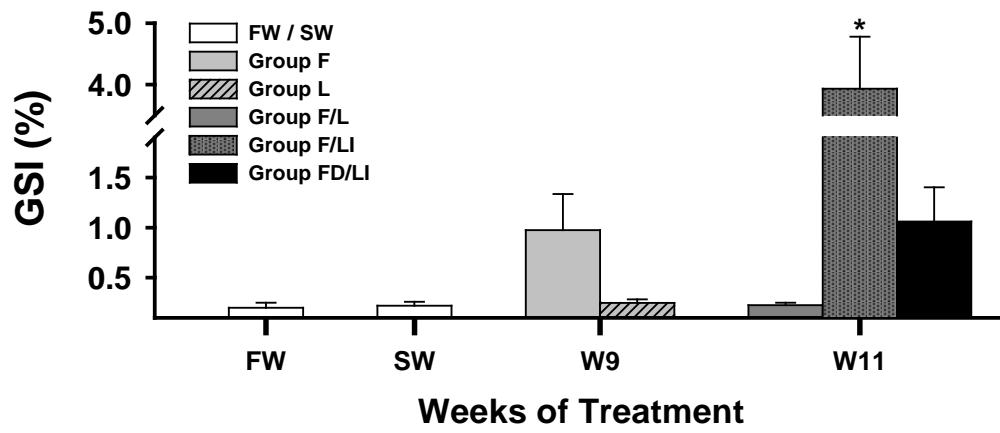
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835   **Figure 4**

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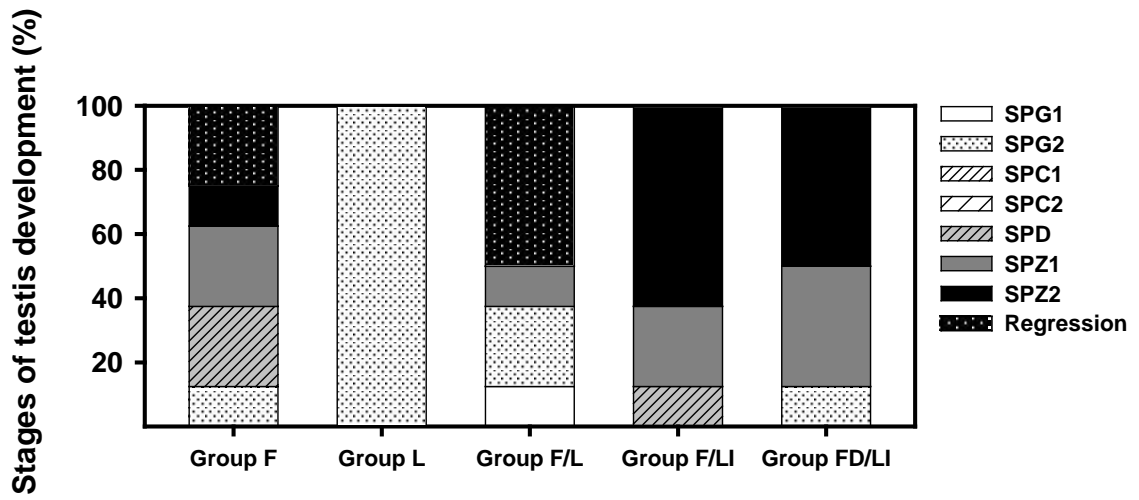
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Figure 5

Figure 6

Figure 7

853 **Figure 8**
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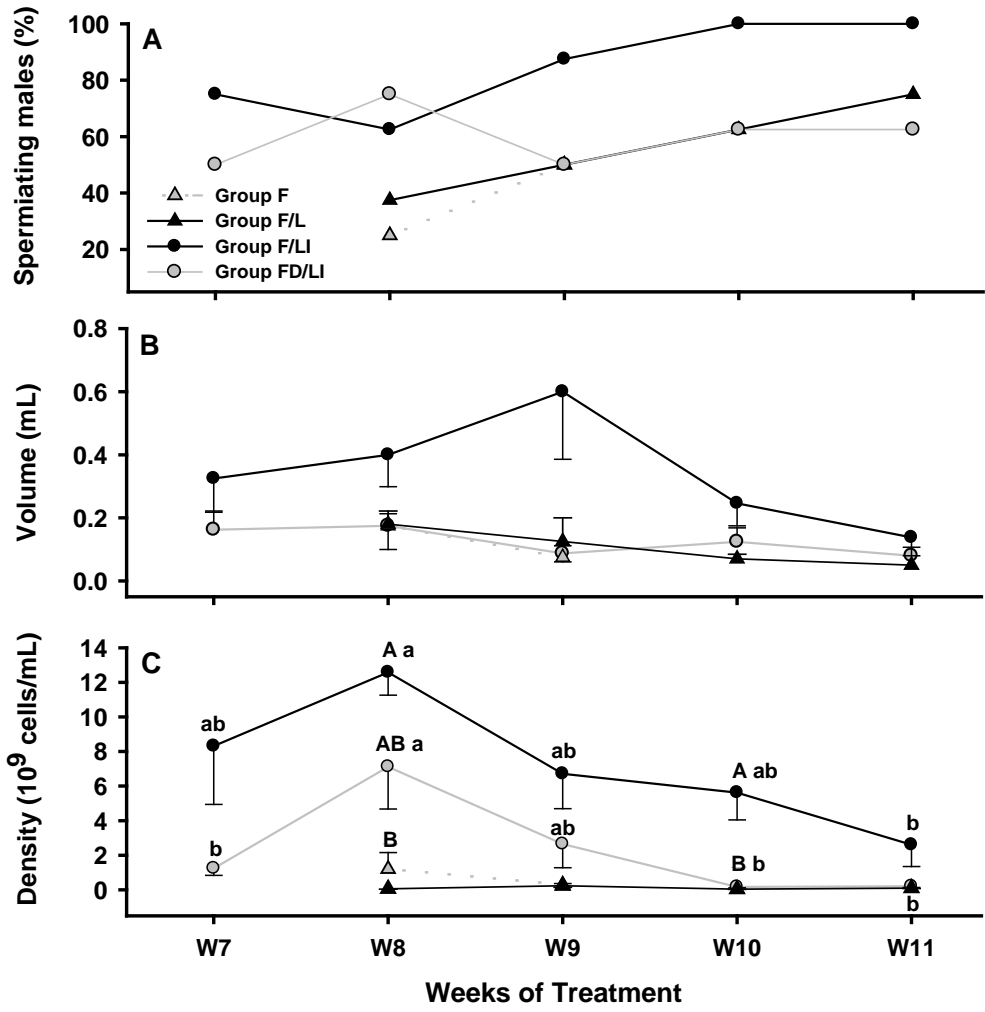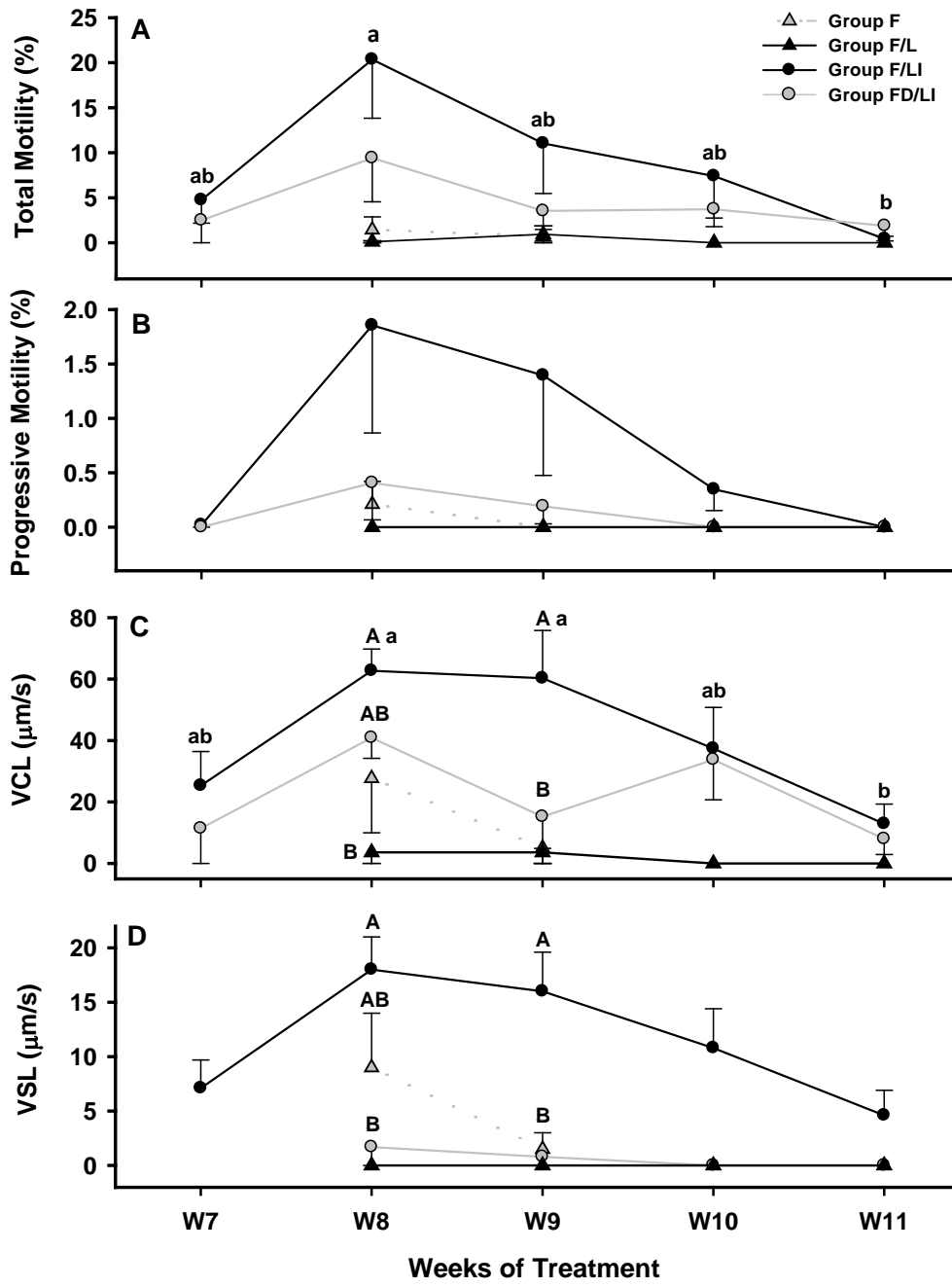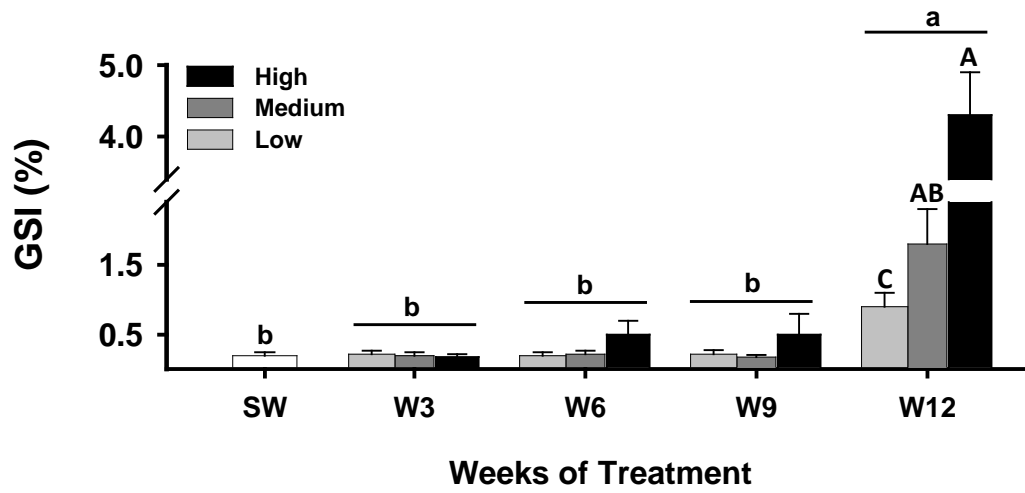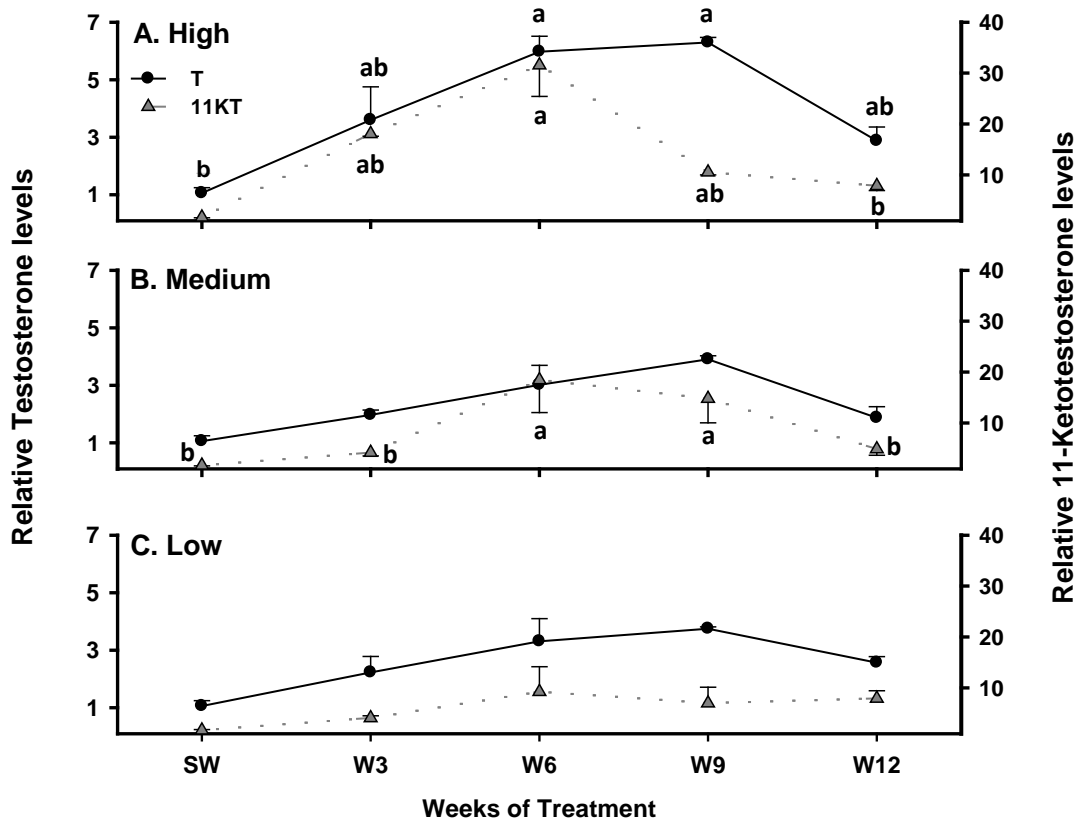
Figure 9

Figure 10

864 **Figure 11**
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Figure 12
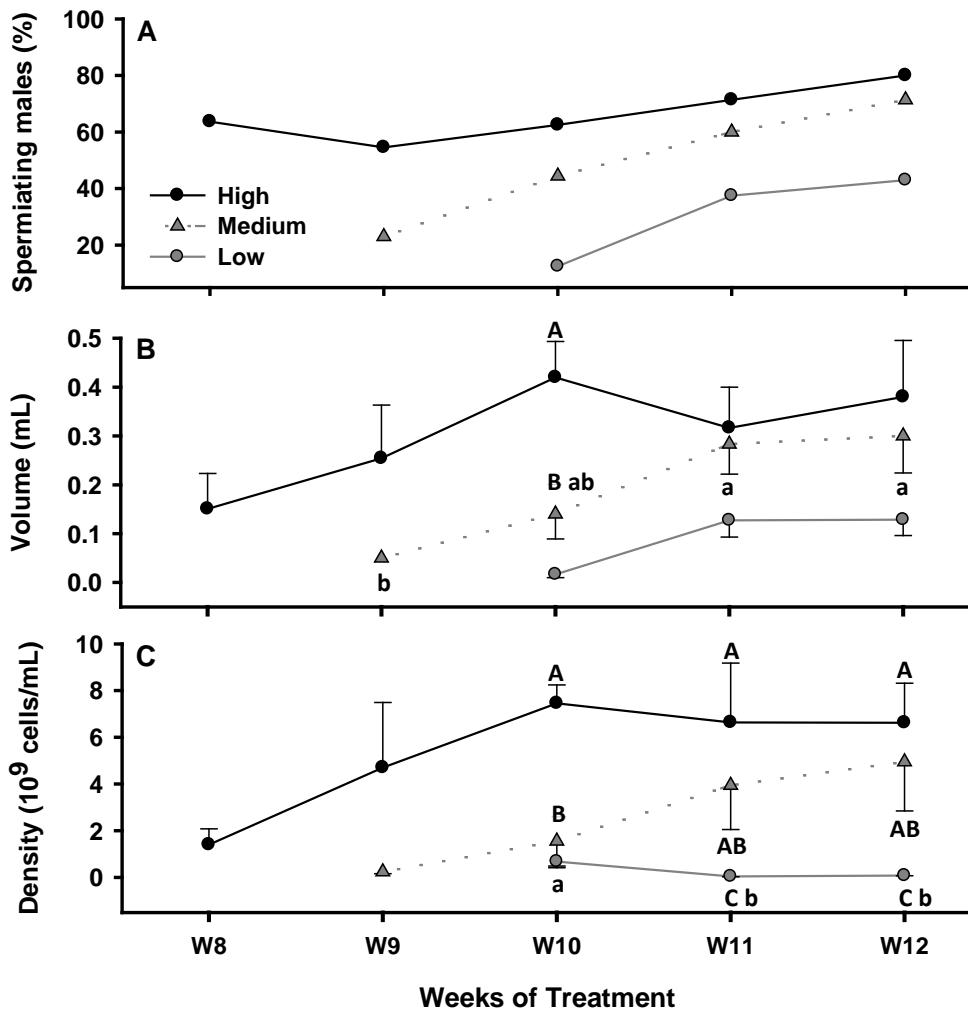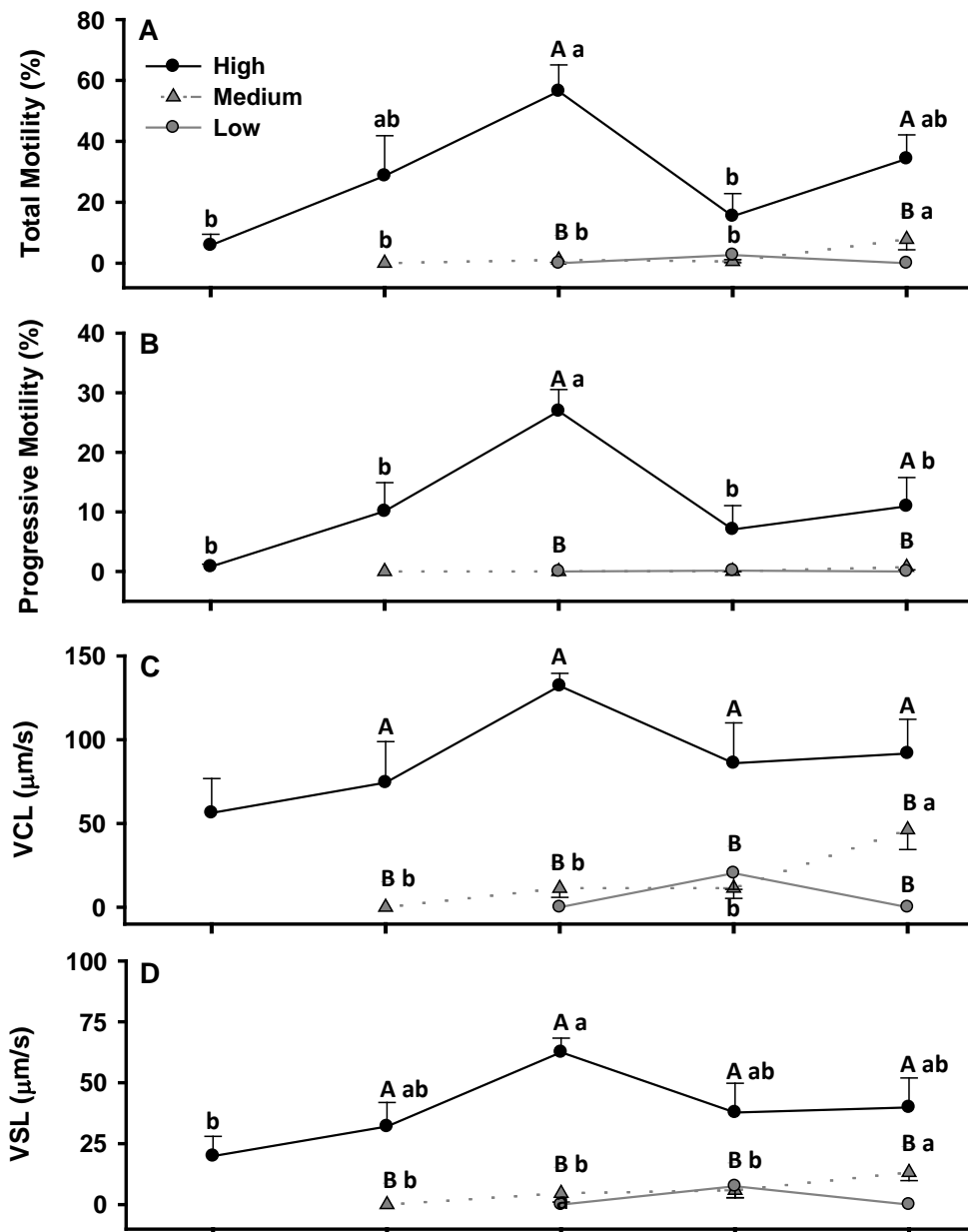
Table 1

	Eye Index					Fin Colour				
	Group F	**Group L**	**Group F/L**	**Group F/LI**	**Group FD/LI**	**Group F**	**Group L**	**Group F/L**	**Group F/LI**	**Group FD/LI**
FW	3.8 (0.1) c	3.8 (0.1) c	3.8 (0.1) c	3.8 (0.1) d	3.8 (0.1) c	1.8 (0.3)	1.8 (0.3)	1.8 (0.3)	1.8 (0.3)	1.8 (0.3)
SW	4.7 (0.1) ab	4.7 (0.1) b	4.7 (0.1) ab	4.7 (0.1) bc	4.7 (0.1) ab	2.7 (0.4)	2.7 (0.4)	2.7 (0.4)	2.7 (0.4)	2.7 (0.4)
W3	4.4 (0.2) abc	4.8 (0.2) b	4.4 (0.2) bc	5.3 (0.3) ab	3.9 (0.1) c	3.3 (0.3)	3.3 (0.2)	2.1 (0.1)	3.4 (0.4)	3.1 (0.3)
W5	4.1 (0.1) bc	4.5 (0.1) b	4.3 (0.2) bc	4.5 (0.2) c	4.5 (0.2) b	4.0 (0.0)	3.9 (0.1)	3.0 (0.3)	3.8 (0.3)	3.9 (0.1)
W7	4.2 (0.2) bc	5.1 (0.2) ab	4.8 (0.2) ab	4.8 (0.1) bc	4.4 (0.2) b	4.0 (0.0)	3.8 (0.2)	4.0 (0.0)	4.0 (0.0)	4.0 (0.0)
W9	4.9 (0.2) a	5.6 (0.3) a	5.2 (0.1) a	5.3 (0.2) ab	4.8 (0.1) ab	4.0 (0.0)	3.8 (0.2)	4.0 (0.0)	4.0 (0.0)	4.0 (0.0)
W11			5.3 (0.3) a	5.7 (0.2) a	5.1 (0.2) a			3.6 (0.2)	4.0 (0.0)	4.0 (0.0)

872 **Table 2**

873

	Eye Index			Fin Colour		
	High	**Medium**	**Low**	**High**	**Medium**	**Low**
SW	3.3 (0.2) c	3.3 (0.2) c	3.3 (0.2) c	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)
W3	4.6 (0.1) b	4.2 (0.2) b	4.0 (0.2) bc	2.7 (0.3)	1.3 (0.3)	2.0 (0.0)
W6	5.0 (0.2) b	4.4 (0.2) b	3.8 (0.4) bc	4.0 (0.0)	2.7 (0.3)	3.0 (0.6)
W9	5.4 (0.5) a	6.5 (0.7) ab	4.7 (0.4) ab	4.0 (0.0)	4.0 (0.0)	3.3 (0.3)
W12	7.0 (0.6) a	5.8 (0.3) a	5.6 (0.2) a	3.7 (0.3)	3.1 (0.3)	3.7 (0.2)

875　**Table 3**

876

|	SPG1	SPG2	SPC1	SPC2	SD	SPZ1	SPZ2
W3	○○○ ●●● ●●●						
W6	○○○ ●	● ●	●	● ●			
W9	○	○○ ● ●		●	●		●
W12		○○ ● ●		○○	●	○○○ ● ● ●●●	● ● ●●●

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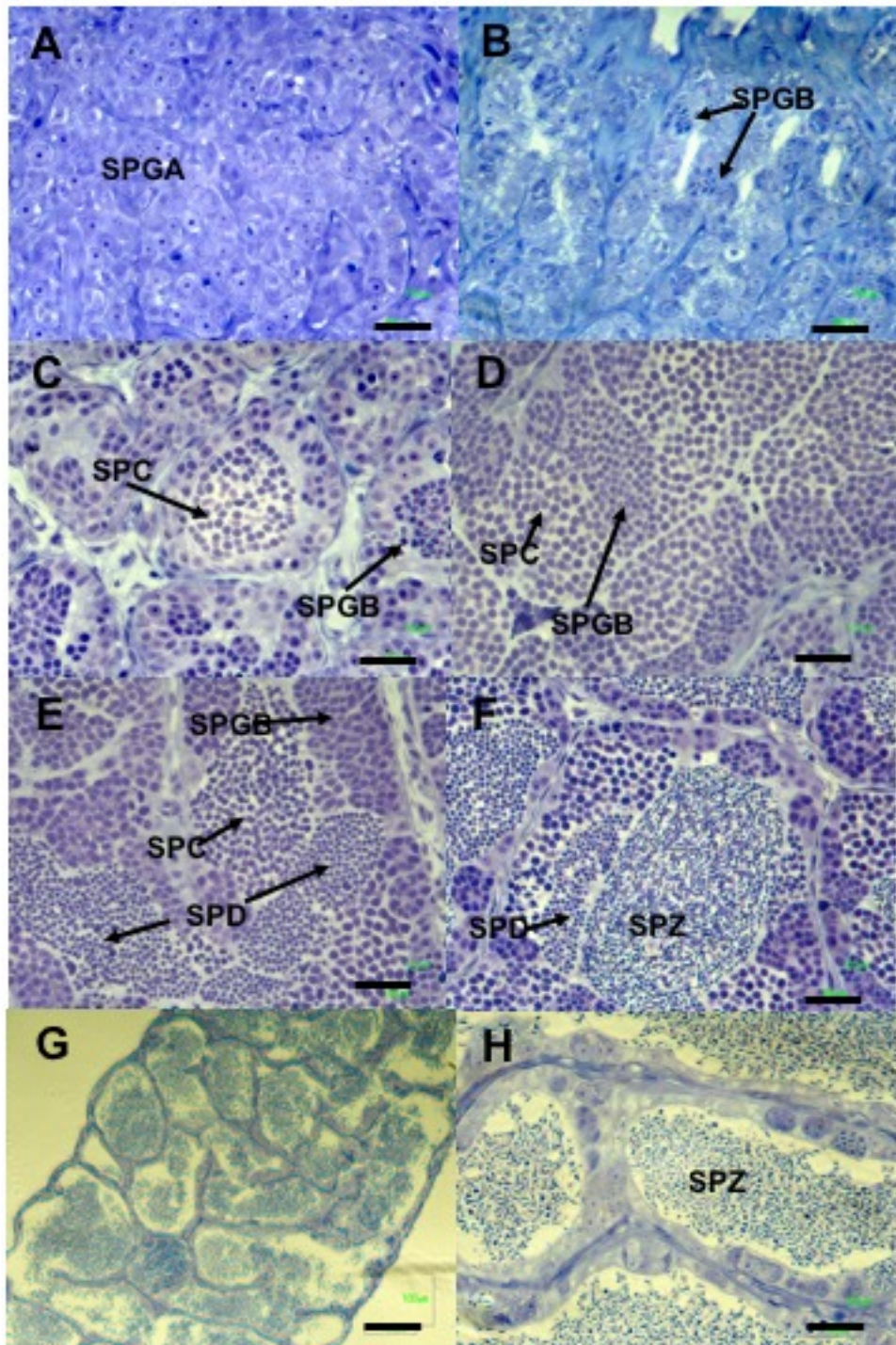
878 **Appendix A**

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881 **Figure 1** Histological sections of eel testis at different developmental stages. A) Testis at
882 SPG1. B) Testis at SPG2. C) Testis at SPC1. D) Testis at SPC2. E) Testis at SPD. F)
883 Testis at SZ1. G, H) Testis at SZ2. See main text for definition of gonad developmental
884 stages. SPGA= Spermatogonia type A; SPGB= Spermatogonia type B; SPC:
885 Spermatocytes; SPD: Spermatids; SPZ: Spermatozoa. Scale bars, 25 μm (A, B, C, D, E,
886 F, H) and 100 μm (G).
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889
890 **Figure 2.** Evolution total motility throughout the recombinant gonadotropin treatments
891 in the experiment 1: Group F (△), Group F/L (▲), Group F/LI (●), and Group FD/LI (◐)
892 treatment. Each point means a data from an individual male.
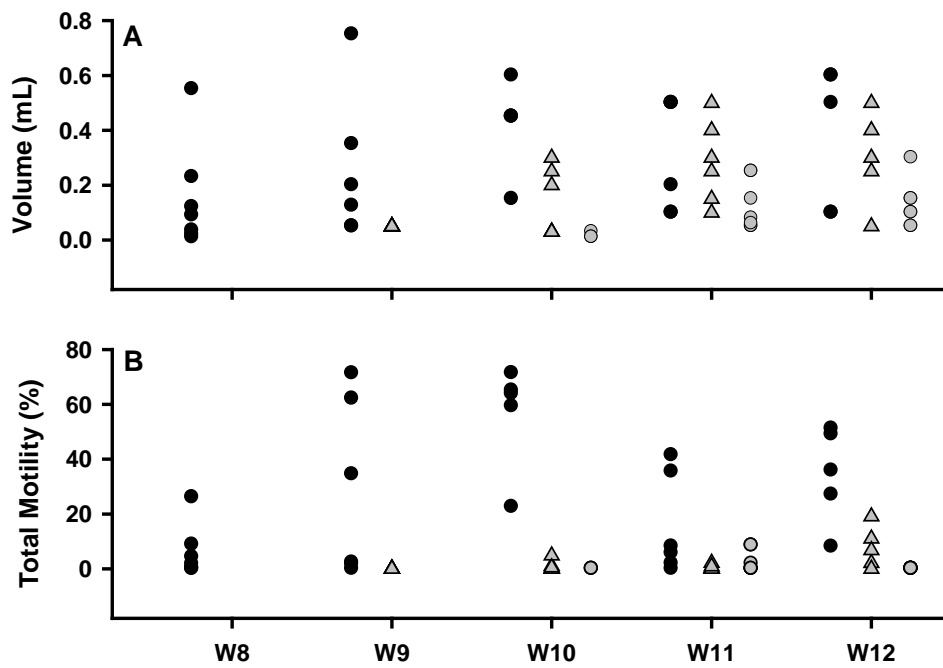
Figure 3. Evolution total motility throughout the recombinant gonadotropin treatments in the experiment 2: High (●), Medium (△), and Low (○) treatment. Each point means a data from an individual male.