Gonadotropin induction of spermiation in Senegalese sole: effect of temperature and stripping time

François Chauvigné[a],*, Jessica Lleberia[a], Carlos Vilafranca[b], Diogo Rosado[b], Micaela Martins[b], Frederico Silva[b], Wendy González-López[c], Sandra Ramos-Júdez[c], Neil Duncan[c], Ignacio Giménez[d], Isidro Blanquet[b], Joan Cerdà[a],*

[a] *Institute of Agrifood Research and Technology (IRTA)-Institute of Biotechnology and Biomedicine (IBB), Universitat Autònoma de Barcelona, 08193 Barcelona, Spain*

[b] *Safiestela-Sustainable Aqua Farming Investments, S. A., 4570-275 Porto, Portugal*

[c] *IRTA, Sant Carles de la Ràpita, 43540 Tarragona, Spain*

[d] *Rara Avis Biotec, S. L., 46002 Valencia, Spain*

* Corresponding authors

E-mail addresses: françois.chauvigne@irta.cat (F. Chauvigné), joan.cerda@irta.cat (J. Cerdà)

Highlights

- Treatment with rFsh and rLh at 12ºC enhance spermiation in Senegalese sole F1 males.
- One batch of spermatids is recruited into spermatozoa differentiation after a single rLh injection.
- Maximum sperm production occurs 48 h after rLh injection at 12ºC.
- rFsh and rLh treatments at 12ºC and 17ºC, respectively, increase spermiation.

34   **Abstract**

35

36   Treatments with homologous recombinant follicle-stimulating and luteinizing hormones (rFsh

37   and rLh, respectively) are known to enhance spermatogenesis and sperm production in sole, but

38   the response can be highly variable depending on the dose, duration and time of the year of the

39   rFsh treatment. To further investigate the physiological effects of rFsh and rLh on sperm

40   production in sole, here we examined the pattern of spermiation of F1 males, of approximately

41   450 g, treated with rFsh and rLh under controlled temperature. In an initial trial at 12ºC, males

42   were weekly injected intramuscularly with 18 µg kg^{-1} rFsh over five weeks and subsequently

43   treated with a single injection of 18 µg kg^{-1} rLh. Histological analysis indicated that the

44   rFsh+rLh treatment increased gonad weight and stimulated spermatogenesis, and also enlarged

45   the size of the seminiferous and efferent duct (ED) tubules, resulting in a doubling of sperm

46   production with respect to the controls. Sperm counts in the ED and sequential stripping of

47   males at 24, 48 and 72 h post rLh injection further revealed that only one batch of spermatids is

48   recruited into spermatozoa (Spz) differentiation after a single rLh induction. A peak of sperm

49   accumulation in the ED occurs at 48 h, coinciding with the upregulation of genes potentially

50   involved in Spz maturation. In a second experiment, we tested the effect of two rFsh doses (10

51   or 18 µg kg^{-1}) over five weeks as previously, followed by one rLh injection at 12ºC or 17ºC.

52   The results confirmed that spermiation was the highest 48 h after rLh treatment at 12ºC, which

53   was increased in a dose-dependent manner with the dose of rFsh previously supplied (from 0.36

54   to 0.95 x 10^9 Spz kg^{-1}). However, sperm production elicited with the low rFsh dose was

55   potentiated by ~3-fold (from 0.36 to 1.06 x 10^9 Spz kg^{-1}) when the rLh treatment was given at

56   17ºC. These data suggest that in Senegalese sole sperm collection should be carried out at 48 h

57   after rLh treatment, and that a low dose of rFsh at 12ºC is highly efficient for stimulating sperm

58   production when rLh is administered at a temperature close to that occurring during maximum

59   natural spermiation.

60

61

62   **Keywords**

63

64   Flatfish, Recombinant gonadotropins, Spermatogenesis, Spermiation, Temperature

65

66

1. Introduction

The control of reproduction in aquaculture is critical to provide good quality gametes for the mass production of larvae, and to facilitate methods to preserve traits of commercial interest through genetic breeding programs (Lind et al., 2012). In the last decades, the high demand for the diversification of marine fish aquaculture has identified the Senegalese sole (*Solea senegalensis*) as one of the target species in the Southern Mediterranean because of its high commercial value (FAO, 2018). However, the domestication of this species to assure the sustainability of its culture is impaired by the lack of methods to control reproduction, particularly of the F1 offspring of wild captive broodstock, which results in the obtention of poor or none fertilization, as well as variable larval quality and high incidence of abnormalities, heterogenous growth or mortality (Morais et al., 2016). As for other species (Mylonas et al., 2017), the use of *in vitro* fertilization in Senegalese sole culture has been proposed as a more controlled method for obtaining eggs and larvae (Liu et al., 2008; Rasines et al. 2012ab; Ramos-Júdez et al., 2021b). However, the low quantity and variable quality of the sperm that the sole males typically produce (Beirão et al., 2011; Cabrita et al, 2011) impedes the transfer of these protocols to the industry.

The Senegalese sole is oligospermic (producing <130 μl of semen), as other flatfishes, and shows asynchronous and semicystic spermatogenesis, i.e. the differentiation of haploid spermatids to spermatozoa (spermiogenesis) takes place within the lumen of the seminiferous tubules (García-López et al., 2005). Due to the asynchronous nature of sole spermatogenesis consecutive batches of spermatids are recruited into spermatozoa differentiation during the year, and consequently spermiation occurs all year-round. However, sperm production is more intense during spring, when females ovulate, which coincides with a peak in the plasma levels of the gonadotropins follicle-stimulating (Fsh) and luteinizing (Lh) hormones and of the major androgen 11-ketotestosterone (11-KT) (García-López et al., 2006; Cabrita et al., 2011; Chauvigné et al., 2015, 2016). During the last years, different hormone treatments based on the administration of gonadotropin-releasing hormone analogue (GnRHa) or human chorionic gonadotropin, with or without 11-KT precursors, such as 11-ketoandrostenedione, or dopaminergic inhibitors, have been tested with the aim of increasing semen production in Senegalese sole. However, none of these treatments result in a marked increase of spermiation, although they do induce a transient elevation

101   of circulating androgens, and may increase the hydration or the motility of sperm

102   (Agulleiro et al., 2006, 2007; Cabrita et al., 2011; Guzmán et al., 2011ab).

103   Recently, however, the use of Senegalese sole recombinant Fsh and Lh (rFsh and

104   rLh, respectively), which activate specific receptors in somatic and germ cells in the

105   testis, have shown to be useful to enhance sperm production. Recombinant

106   gonadotropins can be produced as single-chain polypeptides in different heterologous

107   host systems, such as the yeast or mammalian cells, which allows continuous

108   availability of the hormones (Dalton and Balton, 2014; Molés et al., 2020). Treatment

109   with recombinant gonadotropins is effective at inducing spawning and spermiation in

110   several fish species (Sanchís-Benlloch et al., 2017; Zhang et al., 2018; Peñaranda et al.,

111   2018; Kobayashi et al., 2010; Mazón et al, 2013, 2014; Molés et al., 2020, Ramos-Júdez

112   et al., 2021a), which highlights the great potential of these hormones for aquaculture. In

113   Senegalese sole, homologous rFsh and rLh can stimulate spermatogenesis and

114   spermiogenesis *in vitro* (Chauvigné et al., 2012, 2014ab), as well as increase testicular

115   growth, spermatogenesis and spermiation *in vivo* (Chauvigné et al., 2017, 2018).

116   However, these treatments can sometimes produce results with a high variability, which

117   may be related to the duration and dose of the rFsh treatment and the time of the year

118   when this hormone is administered (Chauvigné et al., 2017, 2018). In addition, the time-

119   course effects of rLh on spermiation *in vivo*, which are crucial in order to select the best

120   time for the collection of mature and highly motile sperm, are not known. Therefore, to

121   establish reliable recombinant gonadotropin-based hormone therapies for increasing

122   semen production in the Senegalese sole it is necessary to decipher the physiological

123   effects of rFsh and rLh on spermatogenesis and spermiation.

124   In the present study, we have examined the production of sperm by pubescent sole

125   F1 males after treatment with increasing doses of rFsh at low temperature, and

126   subsequent induction of spermiation with rLh at low and high temperatures. In addition,

127   by sequential or separate stripping of males and histological analysis we have

128   investigated the pattern of sperm production at different times after rLh treatment.

129   These new data and approaches provide a significant advance towards the establishment

130   of industrial protocols for spermiation enhancement in Senegalese sole.

131

132   **2. Materials and methods**

133

134   *2.1. Animals and recombinant hormones*

135　　　　The fish employed in this study were approximately two-year pubescent

136　Senegalese sole F1 males, which were maintained at the Institute of Agrifood Research

137　and Technology (IRTA) research facilities in Sant Carles de la Ràpita (Spain), as

138　previously described (Chauvigné et al., 2017), or at the facilities of Safiestela-

139　Sustainable Aqua Farming Investments in Porto (Portugal). The experimental

140　procedures relating to the care and use of animals were approved by the Ethics

141　Committee from IRTA and the Portuguese legislation for the use of laboratory animals

142　in accordance with the guidelines of the European Directive (2010/63/EU).

143　　　　Single-chain Senegalese sole rFsh and rLh were produced in Chinese hamster

144　ovary (CHO) cells by Rara Avis Biotec (Valencia, Spain) as described previously

145　(Chauvigné et al., 2017). The biological activity of the hormones produced for the

146　present study was confirmed by intramuscular injection of male fish and measurement

147　of 11-KT plasma levels at 48 h after injection (see below).

148

149　*2.2. Experimental design*

150　*2.2.1. Experiment 1*

151　　　　Males (394 ± 12 g; mean ± SEM) were kept in 10 m^3 tanks connected to a

152　recirculation system (IRTAmar1) and acclimated to 12°C for 2 weeks (from late

153　October to mid-November) under a natural photoperiod. Based on previous studies

154　(Chauvigné et al., 2018), fish were injected intramuscularly with a dose of 18 µg kg^{-1} of

155　rFsh ($n = 25$) or saline buffer (controls, $n = 25$) once a week for 5 consecutive weeks.

156　One week after the last injection, only fish treated with rFsh were injected with a single

157　dose of rLh (18 µg kg^{-1}), while control males were treated again with saline. Ten fish

158　from each group were sequentially stripped at 24, 48 and 72 h after rLh treatment,

159　whereas other 5 fish were sacrificed at each time. Blood samples were taken before the

160　first injection with rFsh (time 0) as well as at 24, 48 and 72 h after rLh treatment.

161

162　*2.2.2. Experiment 2*

163　　　　Fish (517 ± 14 g) were acclimated to 12°C during approximately four months

164　(from October to mid-February) with a photoperiod of 10 h light:14 h dark. After this

165　period, fish were divided into the following experimental treatments: Groups 1 and 2 (n

166　= 12 each) were injected with saline; Groups 3 and 4 ($n =12$ each) were treated with 10

167　µg kg^{-1} rFsh; and Groups 5 and 6 ($n = 12$ and 36, respectively) were injected with18 µg

6

168   kg^{-1} rFsh. These treatments were administered for 5 consecutive weeks. After this time

169   Groups 2, 4 and 5 were acclimated to 17ºC for one week, to test the effect of

170   temperature on hormone-induced spermiation, whereas Groups 1, 3 and 6 remained at

171   12ºC. Fish from all groups including the controls were then injected with 18 µg kg^{-1}

172   rLh. Sperm was stripped at 48 h after rLh treatments in Groups 1 to 5, whereas the

173   males from Group 6 were divided into three groups ($n = 12$ each) that were stripped for

174   sperm collection at 24, 48 or 72 h. Blood samples were taken before the start of the

175   experiment (time 0), the day before rLh injection at 12ºC (day 42) and during the

176   following three days (24, 48 and 72 h, days 43, 44 and 45, respectively). For the fish

177   treated with rLh at 17ºC (Groups 2, 4 and 5) plasma samples were collected after

178   temperature acclimation to 17ºC prior to rLh injection and two days after injection (48

179   h, day 44).

180

181   *2.3. Sampling procedures*

182   Sperm and blood samples were collected as previously described (Chauvigné et

183   al., 2017). For the extraction of testis biopsies, fish were sedated before being sacrificed

184   by decapitation and the entire testis removed in order to determine the gonadosomatic

185   index (GSI; testes weight fish weight^{-1} x 100). The dorsal testis was fixed in Bouin's

186   solution (5% acetic acid, 9% formaldehyde, and 1.5% picric acid in aqueous solution)

187   overnight at room temperature for further histological analysis. The left testis was cut

188   into two pieces that were deep frozen in liquid nitrogen and kept at -80°C for

189   subsequent gene expression analysis.

190

191   *2.4. Gonadotropin and steroid determinations*

192   To determine plasma levels of both endogenous and recombinant gonadotropins

193   enzyme-linked immunosorbent assays (ELISAs) using specific antibodies against

194   Senegalese sole Fshβ and Lhβ subunits were carried out following established protocols

195   (Chauvigné et al., 2015, 2016). A commercial enzyme immunosorbent assay (EIA;

196   Cayman Chemical Company) was used to determine 11-KT levels in plasma as

197   previously described (Chauvigné et al., 2015, 2016, 2017). Plasma free steroids were

198   extracted in methanol from 3.5 µl of plasma and the resulting pellet was diluted 1:100 in

199   EIA buffer 0.1M K$_2$HPO$_4$/KH$_2$PO$_4$, 1.54 mM sodium azide, 0.4M NaCl, 1 mM EDTA,

200 and 0.1% BSA, pH 7.4). A standard curve was run for each EIA plate and all samples

201 were analysed in duplicate.

202

203 *2.5. Histological analysis*

204   Testis fixed in Bouin's solution were subsequently dehydrated and embedded in

205 paraplast (Sigma-Aldrich). The testis biopsies were oriented in the molds in a manner to

206 obtain sagittal sections. Sections of 7 μm in thickness were attached to

207 UltraStick/UltraFrost Adhesion slides (Electron Microscopy Sciences) and stained with

208 hematoxylin and eosin as previously described (Chauvigné et al., 2017). The different

209 somatic and germ cell types in the Senegalese sole testis were identified following the

210 descriptions by García-López et al. (2005). The relative amounts (%) of spermatogonia

211 type A and B (SpgA and SpgB, respectively), spermatocytes (Spc), attached and free

212 spermatids (Spd$_A$ and Spd$_F$, respectively), and spermatozoa (Spz) were scored in 10

213 tubules from different testicular areas per fish. The area of the tubules of the efferent

214 duct and the number of spermatozoa in each tubule were also scored in 3 representative

215 tubules per fish. Counting of the cell types in the testis and efferent duct tubules was

216 carried out in 5 different fish for each group at each time point using the NIS-element

217 AR 4.30.02 software (Nikon).

218

219 *2.6. RNA extraction and gene expression analysis*

220   The expression levels of selected genes, such as sperm antigen 6 (*spag6*), sperm

221 surface protein 17 (*spa17*), cilia- and flagella-associated protein 46, 54 and 61 (*cfap46*,

222 *cfap54*, *cfap61*, respectively), radial spoke head protein 1 (*rshp1*), cytochrome P450

223 family 17 subfamily A member 1 and 2 (*cyp17a1* and *cyp17a2*, respectively), 20β-

224 hydroxysteroid dehydrogenase (*cbr1*), and membrane progestin receptor alpha (*paqr7*),

225 were determined by real-time quantitative RT-PCR (qRT-PCR). Total RNA was

226 extracted from the testes using the GenElute^{TM} Mammalian Total RNA Miniprep Kit

227 (Sigma-Aldrich), treated with DNase I, and 1 μg of total RNA was reverse transcribed

228 using 0.5 μg oligo (dT)17, 1 mM dNTPs, 40 IU RNAse inhibitor, and 10 IU SuperScript

229 II (Life technologies Corp.) for 1.5 h at 42°C. The qRT-PCR was carried out in a final

230 volume of 20 μl using 5 μl of SYBR Green qPCR master mix (Life Technologies

231 Corp.), 1 μl of diluted cDNA (1:5 in sterile mQ water), and 0.5 μM of each forward and

232 reverse primer (Table 1). The reference gene was alpha actin (Table 1). Each sample

233 was assayed in duplicate on 384-well plates using the Thermal cyclers C1000 Touch in

234 combination with the optical modules CFX384 (Biorad, LLEB, UAB). The

235 amplification protocol was an initial denaturation and activation step at 50ºC for 2 min

236 and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 63ºC for 1 min. After

237 the amplification phase, a temperature-determining dissociation step was carried out at

238 95ºC for 15 s, 60ºC for 15 s, and 95ºC for 15 s. Changes in gene expression in testicular

239 samples were determined as fold-changes with respect to the saline group at each time

240 point (24, 48 or 72 h) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

241

242 *2.7. Evaluation of sperm production*

243 The total volume of milt collected from each male was recorded, and an aliquot

244 was diluted 1:10 with non-activating medium (NAM; in mM: 75 NaCl, 1.5 KCl, 12.9

245 $MgCl_2$, 2.65 $CaCl_2$, 20 $NaHCO_3$, 4.4 glucose, 0.015 BSA, pH 7.7, 290 mOsm). The

246 concentration of Spz was determined by loading the diluted sperm sample under a cover

247 slip before being video-recorded for 1 second and analysed using the Integrated Semen

248 Analysis System (ISASv1 software, Proiser, Valencia, Spain) coupled to a phase

249 contrast microscope (Nikon Eclipse 50i, Nikon) equipped with a x20 negative phase

250 contrast objective. Sperm count was performed in three different regions of the counting

251 chamber to minimize miscalculations. The total amount of Spz per ejaculate was finally

252 normalized by the weight of each fish. The measurements were carried out in duplicate

253 for each ejaculate.

254

255 *2.8. Statistical analysis*

256 Results are expressed as the means ± SEM. Comparisons between two

257 independent groups were made by the two-tailed unpaired Student's *t*-test. The

258 statistical significance among multiple groups was analyzed by one-way ANOVA,

259 followed by the Tukey's multiple comparison test, or by the non-parametric Kruskal-

260 Wallis test and further Dunn's test for nonparametric post hoc comparisons, as

261 appropriate. Percentages were square root transformed prior to analyses. Statistical

262 analyses were carried out using the GraphPad Prism v8.4.3 (686) software (GraphPad

263 Software). In all cases, statistical significance was defined as $P < 0.05$.

264

265 **3. Results**

266

3.1. Experiment 1: effect of recombinant gonadotropins on spermiogenesis

3.1.1. rFsh and rLh increase androgen plasma levels

To monitor the correct administration and bioactivity of the recombinant hormones, the plasma levels of Fsh, Lh and 11-KT were determined by specific ELISAs. Prior to the injection with rFsh, and after acclimation at 12ºC (time 0), plasma levels of Fsh in both experimental groups were relatively low (1.45 ± 0.32 and 1.47 ± 0.40 ng ml^{-1}), and in the control group they remained low (< 2 ng ml^{-1}) throughout the experiment (Fig. 1A). However, the levels in the group treated with rFsh (18 µg kg^{-1}) for 5 weeks followed by a rLh (18 µg kg^{-1}) injection reached 17.91 ± 2.40 ng ml^{-1} 24 h after the rLh induction, and these levels decreased progressively at 48 and 72 h (10.82 ± 0.41 and 9.34 ± 1.46 ng ml^{-1}, respectively) (Fig. 1A).

As for Fsh, the plasma levels of Lh were low at time 0 (6.04 ± 0.71 and 5.40 ± 0.32 ng ml^{-1}). As expected, the group treated with rFsh showed a potent increase in the circulating levels of Lh 24 h after rLh injection (82.82 ± 8.57 ng ml^{-1}), which progressively decreased at 48 and 72 h (39.34 ± 3.36 and 21.88 ± 3.45 ng ml^{-1}) (Fig. 1B).

The changes in the plasma levels of the androgen 11-KT exhibited a similar pattern to that of the gonadotropins. These levels were low at time 0 (5.04 ± 1.24 and 5.84 ± 0.84 ng ml^{-1} in each group), and slightly increased toward the experiment in the control group (from 2.97 ± 0.52 to $10.22 \pm 2,13$ ng ml^{-1}), thus inversely to that observed for the Lh plasma levels in this group (Fig. 1C). According to the strong increase in plasma Lh at 24 h after rLh treatment observed in the rFsh-treated males, the 11-KT plasma levels in this group were also highly stimulated (101.41 ± 13.27 ng ml^{-1}), but the levels progressively diminished thereafter (64.30 ± 18.35 and 28.19 ± 6.67 ng ml^{-1}, at 48 and 72 h, respectively) (Fig. 1C).

3.1.2. Recombinant gonadotropins stimulate gonad growth and spermatogenesis

The treatment with rFsh followed by rLh injection clearly stimulated the testis size as indicated by the GSI of the males treated with the hormones, which was higher than that of the control fish at 24, 48 and 72 h after rLh injection (Fig. 2A). However, the GSI values in the rFsh+rLh-treated males were higher at 48 h than at 24 or 72 h after injection (Fig. 2A).

299   The visual examination of the testicular histology from hormone treated and non-

300   treated males suggested that spermatogenesis and spermiogenesis was potentiated by

301   rFsh and rLh. In this group, more Spz within the cortical part of the testis were observed

302   with respect to the controls (Fig 2.B). This observation was confirmed by the

303   quantification of the different germ cell types within the seminiferous tubules of the

304   testis. The SpgA germinal stem cells represented a low percentage of the cells within

305   the tubules at all sampling times, and their number decreased with the rFsh+rLh

306   treatment at 24 h after rLh injection (Fig 2.C). In contrast, both the percentage of

307   dividing SpgB and Spc increased within the tubules at both 24 and 48 h after rLh

308   administration with respect the controls (Fig 2.C), suggesting that germ cell meiosis was

309   stimulated in the hormone-treated group. However, the highest percentage of cells

310   encountered within the testicular tubules of control and treated males were Spd (Fig

311   2.C). The majority of Spd were attached to the Sertoli cells (Spd$_A$), while some were

312   observed free within the tubule lumen (Spd$_F$) (Fig 2.B), a typical feature of the semi-

313   cystic spermatogenesis in the Senegalese sole. After rLh treatment, the percentage of

314   Spd$_A$ decreased in the rLh-treated fish with respect to the controls at all time points,

315   whereas the occurrence of Spd$_F$ increased only at 48 and 72 h after rLh injection (Fig

316   2.C). Finally, the percentage of testicular Spz was higher than the controls after 24, 48

317   or 72 h of rLh injection, although this percentage also slightly increased in the males

318   treated with saline at 72 h (Fig 2.C). Altogether these data corroborated that

319   spermiogenesis was stimulated in the males treated with the recombinant hormones.

320   To further confirm that the treatment with rFsh and rLh potentiated

321   spermiogenesis, we evaluated the number of Spz within the tubules of the testicular

322   efferent duct (ED). The histological analysis showed that the control and treated males

323   had a similar concentration of Spz within the ED tubules, although the diameter of the

324   tubules appeared to be higher in the males treated with rFsh and rLh with respect to that

325   in the control fish (Fig. 3A). Determination of the tubule area confirmed that this was 5,

326   7 and 3 times bigger in hormone-treated fish than in the controls at 24, 48 and 72 h after

327   rLh injection, respectively (Fig. 3B). Despite this, the concentration of Spz within the

328   tubule was similar in controls and treated fish (Fig. 3C), and therefore the total

329   estimated number of Spz in the ED tubules was 6-, 10- and 3-fold higher in the treated

330   males than in the controls at 24, 48 and 72 h postinjection, respectively (Fig. 3D). The

331   combined administration of rFsh and rLh thus enhanced the accumulation of Spz within

332   the ED tubules, and this tended to be higher at 48 h after rLh injection.

3.1.3. rLh modulates the expression of sperm maturation-related genes

The previous data suggested that gonadotropin treatments induced the differentiation of Spz in the testis and their fast accumulation in the tubules of the ED already at 25 h after rLh injection. However, to investigate potential differences in sperm maturation after rLh induction we evaluated by qRT-PCR the level of expression of genes typically involved in teleost spermiation, such as progestin synthesis and progestin receptors (*cyp17a1*, *cyp17a2*, *cbr1* and *paqr7*), fertilization (*spag6* and *spa17*), and Spz flagellar motility (*cfap46*, *cfap54*, *cfap61* and *rsph1*). The result showed that while the expression of *cyp17a1* did not change at 24, 48 or 72 h after rLh injection, that of *cyp17a2*, *cbr1* and *paqr7* was enhanced at 48 h (Fig. 4). The other genes studied (*spag6*, *spa17*, *cfap46*, *cfap54* and *cfap61*) were also upregulated at 48 h post rLh injection, except *rsph1* for which no significant differences were detected (Fig. 4). These data therefore suggest that full maturation of sole Spz seems to occur at 48 h after rLh injection.

3.1.4. Sequential sperm production

The amount of sperm produced by males injected with the saline solution or rFsh+rLh was subsequently studied in the remaining fish from each group. To investigate whether rLh could induce several batches of Spd differentiation to Spz, in these experiments the same males were stripped at 24, 48 and 72 h after hormone injection. All fish were spermiating. In the control group, the sperm production at 24h was of 1.89 ± 0.34 x 10^9 Spz kg^{-1} while it was of 3.73 ± 0.78 x 10^9 Spz kg^{-1} in the rFsh+rLh treated group (~2-fold increase) (Fig. 5A). The following day, at 48h post rLh injection, the same males showed much lower sperm counts (0.55 ± 0.13 and 1.50 ± 0.19 x 10^9 Spz kg^{-1} in the control and hormone-treated groups, respectively), despite the fact that the treated group exhibited 2.8-fold more sperm than the controls (Fig. 5A). The tendency of decreasing sperm counts was confirmed by the third day of stripping (72 h post rLh injection) in both groups (0.31 ± 0.08 and 0.88 ± 0.20 x 10^9 Spz kg^{-1} in the control and hormone-treated groups, respectively), with 2.9-fold more sperm collected for the rFsh+rLh group (Fig. 5A). During the three consecutive days of collection, the accumulated total amount of sperm produced in the control and treated

365   males reached 2.74 and 6.10 x 10^9 Spz kg^{-1}, respectively, thus being 2.2-fold higher in

366   the males injected with rFsh+rLh than in the controls (Fig. 5B).

367

368   *3.2. Experiment 2: effect of rFsh dose and temperature at spermiation*

369   *3.2.1. Gonadotropin and steroid plasma levels*

370   In the second trial, we tested the effect of the administration of different doses of

371   rFsh (10 or 18 µg kg^{-1}) for 5 weeks, as well as different temperatures (12º or 17ºC) at

372   the time of rLh injection, on sperm production (Fig. 6A). To confirm the observations of

373   the previous experiment, blood sampling and stripping of different males at 24, 48 and

374   72 h after rLh injection at 12ºC were only carried out in Group 6 treated with the

375   highest dose of rFsh. For the males treated with rLh at 17ºC, blood sampling and

376   stripping were performed only at 48 h after rLh injection based on the results of the first

377   experiment (Fig. 6A).

378   As observed in the previous experiment, the plasma levels of Fsh were relatively

379   low at time 0 (8.19 ± 2.49 ng ml^{-1}) and remained < 4 ng ml^{-1} in the controls (Groups 1

380   and 2) regardless of the temperature at the time of rLh injection (Fig. 6B). In contrast,

381   males treated with 10 or 18 ug kg^{-1} rFsh (Groups 3-4 and 5-6, respectively) showed a

382   dose dependent increase in plasma Fsh before rLh treatment, with levels reaching 34.31

383   ± 4.25 and 58.55 ± 3.69 ng ml^{-1} in Groups 5 (acclimated to 17ºC) and 6 (maintained at

384   12ºC), respectively (Fig. 6B). The levels of Fsh in these groups progressively decreased

385   at 48 and 72 h following rLh injection, falling to 19.43 ± 1.01 and 34.32 ± 1.81 ng ml^{-1}

386   in Groups 5 and 6, respectively (Fig. 6B).

387   The endogenous levels of plasma Lh in the males at time 0 (3.01 ± 0.50 ng ml^{-1})

388   were lower than those of Fsh and remained equally low regardless of the temperature

389   treatment until rLh was administered (Fig. 6C). After rLh injection at 12ºC, the plasma

390   levels of Lh markedly increased at 24 h (62.12 ± 3.56 ng ml^{-1}) to progressively decrease

391   thereafter at 72 h (32.13 ± 2.43 ng ml^{-1}), while at 17ºC the rLh injection promoted a

392   similar induction of plasma Lh at 48 h as at 12ºC (29.03 ± 1.36 *vs* 29.56 ± 1.74 ng ml^{-1}

393   at 12º and 17ºC, respectively) (Fig. 6C). Curiously, males treated with the highest dose

394   of rFsh showed the highest level of plasma Lh after rLh injection at 48 h and 12ºC,

395   while the opposite trend was noted at 17ºC (Fig. 6C).

396   The 11-KT plasma levels were also fairly low at time 0 (14.78 ± 3.81 ng ml^{-1}),

397   and after the rFsh treatment period, males at 12ºC showed higher levels of plasma

androgen before rLh treatment than the controls (Fig. 6D). In contrast, the concentrations of 11-KT in males acclimated to 17ºC were not different between rFsh treated and non-treated fish (Fig. 6D), which may be related to the lower levels of plasma Fsh after the rFsh treatment in fish acclimated to 17ºC (Fig. 6B). At 12ºC, males previously treated or not with rFsh showed a similar ~3.6-fold increment of the androgen levels at 48 h after rLh injection, which decreased at 72 h, whereas at 17ºC the increase of 11-KT at 48 h was similar than that at 12ºC (Fig. 6D). Interestingly, 48 h after rLh injection at either 12º or 17ºC the levels of 11-KT were not affected by the previous treatment of males with rFsh, unlike that observed for Lh (Fig. 6D).

3.2.2. rFsh and rLh-induced sperm production is enhanced at high temperature

Sperm production was evaluated at 48 h after rLh injection (Groups 1-5), or at 24, 48 and 72 h post rLh treatment using different subgroups of males from Group 6. As observed in the experiment 1 all fish were spermiating. At 12ºC, males treated with the highest dose of rFsh (18 µg kg^{-1}) produced 0.56 ± 0.04 x10^9 Spz kg^{-1} at 24 h after rLh injection, which was ~6-fold higher than that of the controls (0.09 ± 0.04 x10^9 Spz kg^{-1}) at 48 h postinjection (Fig. 7). When the rFsh-treated fish were stripped at 48 h, the sperm count was almost doubled (0.95 ± 0.18 x10^9 Spz kg^{-1}) with respect to the fish spermiated at 24 h, representing a ~11-fold increase with respect to the control group, whereas at 72 h sperm production dropped (0.44 ± 0.13 x10^9 Spz kg^{-1}) (Fig. 7). As expected, males treated with the low dose of rFsh (10 µg kg^{-1}) and injected with rLh at 12ºC were less effective in producing sperm at 48 h (0.36 ± 0.06 x10^9 Spz kg^{-1}) (Fig. 7). However, this was not the case when males were acclimated to 17ºC before rLh injection (Groups 4 and 5), since in these groups the sperm produced by fish previously treated with 10 or 18 µg kg^{-1} rFsh was similar at 48 h after rLh injection (1.06 ± 0.30 and 0.87 ± 0.21 x10^9 Spz kg^{-1}, respectively), and as high as in males treated with 18 µg kg^{-1} of rFsh and rLh at 12ºC (Fig. 7).

4. Discussion

In the present study, two different experiments were carried out in which rFsh was administered during 5 consecutive weeks under a controlled temperature of 12ºC. Such a low temperature seems to be positive to potentiate spermatogenesis in Senegalese sole

males, since it correlated with a strong increment in the GSI and the total production of sperm as found here and in previous studies (García-López et al., 2006; Chauvigné et al., 2017, 2018). In both experiments of the present study, the endogenous basal levels of Fsh and Lh in plasma before rFsh treatment were low (~5 ng ml^{-1}), suggesting that the acclimation periods of the fish to the low temperature were efficient. The administration of rFsh at 12ºC may also be beneficial to increment the stability of the hormone in plasma. Indeed, in the present study, the plasma levels of Fsh before rLh injection reached ~35 or ~60 ng ml^{-1} in males treated with 10 or 18 µg kg^{-1} rFsh at 12ºC, respectively, while they dropped to ~17 or ~35 ng ml^{-1} when fish were acclimated to 17°C.

The plasma levels of Lh in males treated with 10 or 18 µg kg^{-1} rFsh showed however a different trend depending on the temperature, which has not been previously observed. Thus, after rLh injection at 12ºC the plasma levels of Lh in males increased in a dose dependent manner with the previous dose of rFsh received, whereas at 17ºC a decrease of the Lh levels with the rFsh dose was noted. These data could reveal differences in hormone kinetics at the temperatures tested, or a possible feedback regulation on Lhβ expression and secretion by the pituitary induced indirectly by testicular steroids produced in response to rFsh, or through dopamine regulatory mechanisms in the brain triggered by the hormone (Yaron and Levavi-Sivan, 2011). Future studies will be necessary to investigate whether these mechanisms can modulate the rLh induction of spermiation in Senegalese sole.

The combined treatment of rFsh and rLh raised the plasma levels of the androgen 11-KT, which confirmed the strong bioactivity of the recombinant gonadotropins (Chauvigné et al., 2017, 2018). However, the rLh treatment appeared to be more potent than rFsh at inducing androgen secretion, as observed in other fish species (Kazeto et al., 2008; Yom-Din et al., 2016). The rLh-stimulated 11-KT synthesis also resulted in an increase of the GSI after 48 h of rLh injection, reflecting the growth of the testis during the treatment. In the present work, the GSI approximately doubled with respect to the controls after 5 weeks of rFsh treatment and a single rLh injection, a result comparable to that found in F1 pubescent sole males treated with the same dose of rFsh for 9 weeks under natural temperature (from 15º to 11ºC) (Chauvigné et al., 2017). This again suggests that an acclimation to low temperature favours testis growth and spermatogenesis in sole.

464   Histological analysis of the different cell types in the testis revealed an

465   accumulation of SpgB in the seminiferous tubules at 24 h after rLh injection, which was

466   concomitant with a decreased percentage of SpgA, which is in agreement with the

467   differentiation and proliferation of SpgB at the onset of spermatogenesis in teleosts

468   (Schulz et al., 2010). A higher occurrence of Spc was also found in the hormone treated

469   fish, indicating that the treatment with rFsh and rLh induced an entry of Spg into

470   meiosis. No effect was observed on the number of SpgA, SpgB or Spc after 72 h,

471   suggesting that cells already differentiated to haploid spermatids. Similar results were

472   previously described for Senegalese sole (Chauvigné et al., 2017), as well as other in

473   other teleosts in which species-specific recombinant gonadotropins have been employed

474   (Peñaranda et al., 2018, Molés et al., 2020). At all sampling times, the percentage of

475   immature Spd_A was decreasing while that of mature Spd_F, as well as the number of Spz,

476   increased in the tubules at 48 and 72 h after rLh induction, as observed in our previous

477   study (Chauvigné et al., 2017). These data thus reveal an active spermiogenesis

478   controlled by gonadotropins, which was corroborated by the increment in the number of

479   Spz in the ED. Therefore, as previously reported in Senegalese sole males (Chauvigné

480   et al., 2017), recombinant gonadotropin-based hormone therapies appear to be effective

481   to promote spermatogenesis and spermiation in this species.

482   It is known that C_{21} steroids (progestins) are active players in the process of

483   spermiation in teleosts (Scott et al., 2010). Progestins, such as 17α,20β-dihydroxypregn-

484   4-en-3-one (17,20βP) or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) are known

485   maturation inducing steroids in male and female teleost gametes (Scott et al., 2010).

486   Progestins can induce spermiation, increase milt production under the control of Lh, and

487   stimulate Spz motility (Scott et al., 2010; Vizziano et al., 1996; Yueh and Chang, 1997;

488   Tubbs and Thomas, 2008; Tenegu et al., 2020). In Senegalese sole, however, previous

489   studies have reported that the plasma levels of 17,20βP are almost undetectable at the

490   time of spermiation (Garcia-López et al., 2006; Agulleiro et al., 2007). In contrast, free

491   and sulphated 17,20βP and its metabolites are readily detectable in males in which

492   spermatogenesis is enhanced by treatment with GnRHa in combination with 11-

493   ketoandrostenedione (Agulleiro et al., 2007). This suggests that sulphated or

494   glucuronidated and/or 5β-reduced 17,20βP metabolites may be the active 'spermiation-

495   inducing' hormones in Senegalese sole as in other flatfishes (Agulleiro et al., 2007;

496   Scott et al., 2010).

16

497   Therefore, as a proxy to monitor the process of sperm maturation after rLh

498   treatment, we investigated the expression of various genes related to progestin synthesis

499   and function at 24, 48 and 72 h after rLh injection. In the testis, progestins are

500   synthetized in the interstitial Leydig cells from their precursor progesterone, which is

501   metabolised to 17-hydroxyprogesterone (17-P) by the Cyp17a1 enzyme through its 17α-

502   hydroxylase activity (Kazeto et al., 2000). The Cyp17a1 has also lyase activity,

503   converting 17-P to androstenedione, the immediate precursor of testosterone, which is

504   also the precursor of estrogens and 11-KT in male fish. In salmonids and possibly in

505   other teleosts, another Cyp17a1-related enzyme, termed Cyp17a2, which exhibits

506   hydroxylase activity only, as well as the Cbr1, are upregulated during spermiation, thus

507   driving the accumulation of 17-P and further conversion to 17,20βP (Zhou et al., 2007;

508   Sreenivasulu et al., 2012). Therefore, a shift in the ratio between the two Cyp17a

509   enzymes, or alternatively the inhibition of the Cyp17a1 lyase activity by progestins

510   themselves, may lead to the synthesis of progestins rather than androgens (Barry et al.,

511   1990; Tenugu et al., 2020). According to this model, we observed that the *cyp17a1*

512   expression levels did not vary following rLh induction, while those of *cyp17a2* and *cbr1*

513   increased more at 48 h after rLh injection, suggesting a shift to progestin synthesis in

514   the testis at the time of maximum spermiation. Progestins can act on Spz through the

515   membrane progestin receptors, such as Paqr7 (Thomas et al., 2009), and in our study we

516   also detected the highest level of the corresponding *paqr7* transcripts at 48 h after rLh

517   treatment. These data therefore suggest that full maturation of Spz in the ED of the

518   testis possibly occurs at 48 h post rLh induction. This conclusion is supported by the

519   expression of other genes potentially involved in sperm motility, such as *cfap46*, *cfap54*

520   and *cfap61* (Linck et al., 2016; McKenzie et al., 2020; Huang et al., 2020; Liu et al.,

521   2021), and sperm fertilization competence, such as *spag6* and *spa17* (Liu et al., 2019;

522   Instaqui et al., 2017), which were also upregulated in the testis at 48 h after rLh

523   injection.

524   The sequential stripping of males at 24, 48 and 72 h following rLh treatment

525   revealed that sperm counts, while remaining higher than in the controls, were

526   progressively decreased from 24 to 72 h post injection, suggesting that the rLh induced

527   the recruitment of only one batch of Spd into Spz differentiation and maturation. This

528   observation was confirmed in the second experiment, in which males stripped at 48 h

529   showed more ejaculated sperm than males sampled at 24 or 72 h after rLh injection.

530   These data agree with the asynchronous type of spermatogenesis described in

17

Senegalese sole (García-López et al., 2005, 2006), and could be the result of a negative feedback mechanism on Spz differentiation occurring in the testis. Although the nature of these mechanisms are yet unknown, previous studies in sole have identified that the maturation of Spds is associated with the translation of the Lh receptor in these cells and their release to the lumen of the seminiferous tubules, where they will differentiate to Spz in response to Lh (Chauvigné at al., 2014ab). The investigation of the molecular regulation of the Lh receptor in immature Spd_A will therefore be of interest to elucidate the endocrine mechanisms controlling spermiation in Senegalese sole.

Finally, we investigated the effect of a rise in temperature prior to the rLh administration on sperm production. The aim of this experiment was to replicate the conditions of maximum spermiation in the wild, which occurs in spring when temperature is around 16-18°C (Cerdà et al., 2008, García-López et al, 2006, Guzmán et al., 2009). At 12°C, sperm production at 48 h after rLh treatment was increased in a dose-dependent manner with the dose of rFsh previously administered, but at 17°C we found that rLh injection of males previously treated with a low dose of rFsh resulted in a similar production of sperm at 48 h than that observed in males treated with a high dose of rFsh and rLh at 12°C. Whether this observation is the result of more Spd_A being recruited into maturation at 17°C, or of a faster process of Spd_F differentiation to Spz, remains to be studied. In addition, in the present study we did not measure sperm production at 24 h after rLh injection at 17°C, and therefore it is possible that under these conditions production of sperm at this time can be similar to that at 48 h. Nevertheless, our results suggest that maximum spermiation was obtained under the hormone doses and conditions employed in the present study. Future research should be conducted to synchronize the testis to accumulate more SpdF in the testis prior to the rLh injection, which might increase the quantity of sperm collected.

In conclusion, we confirm the efficiency of a dual rFsh and rLh hormone therapy to enhance testis growth, spermatogenesis and spermiation in Senegalese sole. Our data also suggest that 48 h after rLh injection is the time that assures maximum production of mature Spz. In addition, we report that using a low dose of rFsh for 5 consecutive weeks at 12°C is very efficient in terms of sperm production if further rLh treatment is given at 17°C, which could be of interest to reduce the economic cost of using rFsh and rLh in a commercial hatchery. Altogether, the present study therefore proposes a more refined protocol for recombinant gonadotropin-based hormone therapies to promote sperm production in F1 Senegalese sole males.

Declaration of Competing Interest

580

581   The recombinant gonadotropins employed in this study were produced by the

582 biotech commercial company Rara Avis Biotec, S. L. (Valencia, Spain). There are no

583 other competing interest to declare.

584

597

598 **References**

19

599

600 Agulleiro, M.J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdà, J.,
601   2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using
602   different administration methods for gonadotropin-releasing hormone agonist.
603   Aquaculture. 257, 511-524. https://doi.org/10.1016/j.aquaculture.2006.02.001

604 Agulleiro, M.J., Scott, A.P., Duncan, N., Mylonas, C.C., Cerdà, J. 2007. Treatment of GnRHa-
605   implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates
606   spermatogenesis and increases sperm motility. Comp Biochem Physiol A Mol Integr
607   Physiol., 147, 885-892. https://doi.org/10.1016/j.cbpa.2007.02.008

608 Barry, T.P., Aida, K., Okumura, T., Hanyu, I. 1990. The shift from C-19 to C-21 steroid
609   synthesis in spawning male common carp, *Cyprinus carpio*, is regulated by the inhibition
610   of androgen production by progestogens produced by spermatozoa. Biol reprod, 43(1),
611   105–112. https://doi.org/10.1095/biolreprod43.1.105

612 Beirão, J., Soares, F., Herraez, M.P., Dinis, M.T., Cabrita, E., 2011. Changes in *Solea*
613   *senegalensis* sperm quality throughout the year. Anim Reprod Sci. 126, 122-129.
614   https://doi.org/10.1016/j.anireprosci.2011.04.009

615 Cabrita, E., Soares, F., Beirão, J., García-López, A., Martínez-Rodríguez, G., Dinis, M.T. 2011.
616   Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in
617   captivity. Theriogenology 75, 1-9. https://doi.org/10.1016/j.theriogenology.2010.07.003

618 Cerdà, J., Chauvigné, F., Agulleiro, M.J., Marin, E., Halm, S., Martínez-Rodríguez, G., Prat, F.
619   2008. Molecular cloning of Senegalese sole (*Solea senegalensis*) follicle-stimulating
620   hormone and luteinizing hormone subunits and expression pattern during
621   spermatogenesis. Gen Comp Endocrinol, 156(3), 470-481.
622   https://doi.org/10.1016/j.ygcen.2008.02.006

623 Chauvigné, F., González, W., Ramos, S., Ducat, C., Duncan, N., Giménez, I., Cerdà, J. 2018.
624   Seasonal-and dose-dependent effects of recombinant gonadotropins on sperm production
625   and quality in the flatfish *Solea senegalensis*. Comp Biochem Physiol A Mol Integr
626   Physiol., 225, 59–64. https://doi.org/10.1016/j.cbpa.2018.06.022

627 Chauvigné, F, Ollé, J., González, W., Duncan, N., Giménez, I., Cerdà, J. 2017. Toward
628   developing recombinant gonadotropin-based hormone therapies for increasing fertility in
629   the flatfish Senegalese sole. PLoS ONE 12(3): e0174387.
630   https://doi.org/10.1371/journal.pone.0174387

631 Chauvigné, F., Fatsini, E., Duncan, N., Ollé, J., Zanuy, S., Gómez, A., Cerdà, J. 2016. Plasma
632   levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of
633   wild and cultured Senegalese sole (*Solea senegalensis*). Comp Biochem Physiol A Mol
634   Integr Physiol., 191, 35–43. https://doi.org/10.1016/j.cbpa.2015.09.015

635 Chauvigné, F., Verdura, S., Mazón, M.J., Boj, M., Zanuy, S., Gómez, A., Cerdà, J. 2015.
636   Development of a flatfish-specific enzyme-linked immunosorbent assay for Fsh using a
637   recombinant chimeric gonadotropin. Gen. Comp. Endocrinol. 221, 75–85.
638   https://doi.org/10.1016/j.ygcen.2014.10.009

639 Chauvigné, F., Zapater, C., Crespo, D., Planas, J.V., Cerdà, J. 2014a. Fsh and Lh direct
640   conserved and specific pathways during flatfish semicystic spermatogenesis. J. Mol.
641   Endocrinol., 53(2), 175-190. https://doi.org/10.1530/JME-14-0087

642 Chauvigné, F., Zapater, C., Gasol, J.M., Cerdà, J. 2014b. Germ-line activation of the luteinizing
643   hormone receptor directly drives spermiogenesis in a nonmammalian vertebrate. Proc
644   Natl Acad Sci U S A, 111(4), 1427-1432. https://doi.org/10.1073/pnas.1317838111

645 Chauvigné, F., Verdura, S., Mazón, M.J., Duncan, N., Zanuy, S., Gómez, A., Cerdà, J. 2012.
646   Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in
647   Leydig cells of an evolutionary advanced teleost. Biol reprod, 87(2), 35.
648   https://doi.org/10.1095/biolreprod.112.100784

649 Dalton, A.C., Barton, W.A. 2014. Over-expression of secreted proteins from mammalian cell
650   lines. Protein Sci., 23(5), 517–525. https://doi.org/10.1002/pro.2439

651 FAO, 2018. The State of World Fisheries and Aquaculture 2018, FAO Fisheries Department,
652   Rome. http://www.fao.org/documents/card/fr/c/I9540EN/

653 García-López, A., Fernández-Pasquier, V., Couto, E., Canario, A.V., Sarasquete, C., Martínez-
654   Rodríguez, G. 2006. Testicular development and plasma sex steroid levels in cultured
655   male Senegalese sole *Solea senegalensis* Kaup. Gen comp endocrinol,147(3):343-51.
656   https://doi.org/10.1016/j.ygcen.2006.02.003

657 García-López, A., Martínez-Rodríguez, G., Sarasquete, C. 2005. Male reproductive system in
658   Senegalese sole *Solea senegalensis* (Kaup): Anatomy, histology and histochemistry.
659   Histol Histopathol 20(4): 1179-1189. https://doi.org/10.14670/HH-20.1179

660 Guzmán, J.M., Cal, R., García-López, A., Chereguini, O., Kight, K., Olmedo, M., Sarasquete,
661   C., Mylonas, C.C., Peleteiro, J.B., Zohar, Y., Mañanós, E.L. 2011a. Effects of in vivo
662   treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone
663   agonist (GnRHa) on the reproductive axis of Senegalese sole (*Solea senegalensis*). Comp
664   Biochem Physiol A Mol Integr Physiol., 158(2), 235–245.
665   https://doi.org/10.1016/j.cbpa.2010.11.016

666 Guzmán, J.M., Ramos, J., Mylonas, C.C., Mañanós, E.L.. 2011b. Comparative effects of human
667   chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa)
668   treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction.
669   Aquaculture 316, 121-128. https://doi.org/10.1016/j.aquaculture.2011.03.014

670 Guzmán, J.M., Rubio, M., Ortiz-Delgado, J.B., Klenke, U., Kight, K., Cross, I., Sánchez-
671   Ramos, I., Riaza, A., Rebordinos, L., Sarasquete, C., Zohar, Y., Mañanós, E.L. 2009.

672   Comparative gene expression of gonadotropins (FSH and LH) and peptide levels of

673   gonadotropin-releasing hormones (GnRHs) in the pituitary of wild and cultured

674   Senegalese sole (*Solea senegalensis*) broodstocks. Comp Biochem Physiol A Mol Integr

675   Physiol., 153(3), 266–277. https://doi.org/10.1016/j.cbpa.2009.02.032

676 Huang, T., Yin, Y., Liu, C., Li, M., Yu, X., Wang, X., Zhang, H., Muhammad, T., Gao, F., Li,

677   W., Chen, Z.J., Liu, H., Ma, J. 2020. Absence of murine CFAP61 causes male infertility

678   due to multiple morphological abnormalities of the flagella. Sci Bull. 65 (10), 854-864.

679   https://doi.org/10.1016/j.scib.2020.01.023

680 Intasqui, P., Agarwal, A., Sharma, R., Samanta, L., Bertolla, R.P. 2018. Towards the

681   identification of reliable sperm biomarkers for male infertility: A sperm proteomic

682   approach. Andrologia, 50(3), 10.1111/and.12919. https://doi.org/10.1111/and.12919

683 Kazeto, Y., Ijiri, S., Todo, T., Adachi, S., Yamauchi, K. 2000. Molecular cloning and

684   characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. Gen Comp

685   Endocrinol, 118,123-133. https://doi.org/10.1006/gcen.1999.7449

686 Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi. S.,

687   Yamauchi, K. 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing

688   hormone (Lh): production of biologically active recombinant Fsh and Lh by *Drosophila*

689   S2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938-

690   46. https://doi.org/10.1095/biolreprod.108.070052

691 Kobayashi, M., Hayakawa, Y., Park, W., Banba, A., Yoshizaki, G., Kumamaru, K., Kagawa,

692   H., Kaki, H., Nagaya, H., Sohn, Y.C. 2010. Production of recombinant Japanese eel

693   gonadotropins by baculovirus in silkworm larvae. Gen. Comp. Endocrinol. 167(3), 379-

694   386. https://doi.org/10.1016/j.ygcen.2010.01.003

695 Linck, R.W., Chemes, H., Albertini, D.F. 2016. The axoneme: the propulsive engine of

696   spermatozoa and cilia and associated ciliopathies leading to infertility. J Assist Reprod

697   Genet., 33(2), 141–156. https://doi.org/10.1007/s10815-016-0652-1

698 Lind, C., Ponzoni, R., Nguyen, N., Khaw, H. 2012. Selective breeding in fish and conservation

699   of genetic resources for aquaculture. Reprod Domest Anim. 47, 255-263.

700   https://doi.org/10.1111/j.1439-0531.2012.02084.x

701 Liu, S., Zhang, J., Kherraf, Z.E., Sun, S., Zhang, X., Cazin, C., Coutton, C., Zouari, R., Zhao,

702   S., Hu, F., Fourati Ben Mustapha, S., Arnoult, C., Ray, P.F. 2021. CFAP61 is required for

703   sperm flagellum formation and male fertility in human and mouse. bioRxiv

704   2021.03.04.433881; doi: https://doi.org/10.1101/2021.03.04.433881

705 Liu, X., Liu, X., Lian, J., Wang, Y., Zhang, F., Yu, H. 2008. Large scale artificial reproduction

706   and rearing of senegal sole, *Solea senegalensis* (Kaup). Mar Fish Res. 29, 10-16.

707 Liu, Y., Zhang, L., Li, W., Huang, Q., Yuan, S., Li, Y., Liu, J., Zhang, S., Pin, G., Song, S.,

708   Ray, P.F., Arnoult, C., Cho, C., Garcia-Reyes, B., Knippschild, U., Strauss, J.F., Zhang,

709   Z. 2019. The sperm-associated antigen 6 interactome and its role in spermatogenesis.

710   Reproduction 158(2), 181–197. https://doi.org/10.1530/REP-18-0522

711   Livak, K. J., Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time

712   quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408.

713   https://doi.org/10.1006/meth.2001.1262

714   Mazón, M.J., Gómez, A., Yilmaz, O., Carrillo, M., Zanuy, S. 2014. Administration of follicle-

715   stimulating hormone in vivo triggers testicular recrudescence of juvenile European sea

716   bass (*Dicentrarchus labrax*). Biol. Reprod. 90(1), 6.

717   https://doi.org/10.1095/biolreprod.113.110569

718   Mazón, M.J., Zanuy, S., Muñoz, I., Carrillo, M., Gómez, A. 2013. Luteinizing hormone plasmid

719   therapy results in long-lasting high circulating Lh and increased sperm production in

720   European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 88(2), 32.

721   https://doi.org/10.1095/biolreprod.112.102640

722   McKenzie, C.W., Lee, L. 2020. Genetic interaction between central pair apparatus genes

723   CFAP221, CFAP54, and SPEF2 in mouse models of primary ciliary dyskinesia. Sci. Rep.

724   10(1), 12337. https://doi.org/10.1038/s41598-020-69359-3

725   Molés, G., Hausken, K., Carrillo, M., Zanuy, S., Levavi-Sivan, B., Gómez, A. 2020. Generation

726   and use of recombinant gonadotropins in fish. Gen. Comp. Endocrinol. 299, 113555.

727   https://doi.org/10.1016/j.ygcen.2020.113555

728   Morais, S., Aragão, C., Cabrita, E., Conceição, L.E., Constenla, M., Costas, B., Dias, J.,

729   Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L.M.P., Yúfera,

730   M., Dinis, M.T. 2016. New developments and biological insights into the farming of

731   *Solea senegalensis* reinforcing its aquaculture potential. Rev. Aquaculture 8: 227-263.

732   https://doi.org/10.1111/raq.12091

733   Mylonas, C.C., Duncan, N.J., Asturiano, J.F. 2017. Hormonal manipulations for the

734   enhancement of sperm production in cultured fish and evaluation of sperm quality

735   Aquaculture 472, 21-44, https://doi.org/10.1016/j.aquaculture.2016.04.021

736   Peñaranda, D.S., Gallego, V., Rozenfeld, C., Herranz-Jusdado, J.G., Pérez, L., Gómez, A.,

737   Giménez, I., Asturiano, J.F. 2018. Using specific recombinant gonadotropins to induce

738   spermatogenesis and spermiation in the European eel (*Anguilla anguilla*).

739   Theriogenology, 107, 6–20. https://doi.org/10.1016/j.theriogenology.2017.11.002

740   Ramos-Júdez, S., Chauvigné, F., González-López, W.Á., Rosenfeld, H., Cerdà, J., Giménez, I.,

741   Duncan, N., 2021a. Providing recombinant gonadotropin-based therapies that induce

742   oogenesis from previtellogenic oocytes to produce viable larvae in a teleost, the flathead

743   grey mullet (*Mugil cephalus*). Aquaculture 536, 736418.

744   https://doi.org/https://doi.org/10.1016/j.aquaculture.2021.736418

23

745 Ramos-Júdez, S., González-López, W.A., Huayanay, O.J., Cota Mamani, N., Marrero Alemán,
746 C., Beirão, J., Duncan, N. 2021b. Low sperm to egg ratio required for successful in vitro
747 fertilization in a pair-spawning teleost, Senegalese sole (*Solea senegalensis*). R Soc Open
748 Sci., 8:201718.201718, https://doi.org/10.1098/rsos.201718

749 Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O. 2012a. Artificial
750 fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration
751 methods, timing of ovulation and viability of eggs retained in the ovarian cavity.
752 Aquaculture 326-329, 129-135. https://doi.org/10.1016/j.aquaculture.2011.11.021

753 Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O. 2012b. Artificial
754 fertilization of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of
755 hormonal treatment on inducing ovulation. Aquaculture 392-395, 94-97.
756 https://doi.org/10.1016/j.aquaculture.2013.02.011

757 Sanchís-Benlloch, P.J., Nocillado, J., Ladisa, C., Aizen, J., Miller, A., Shpilman, M., Levavi-
758 Sivan, B., Ventura, T., Elizur, A. 2017. In-vitro and in-vivo biological activity of
759 recombinant yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone. Gen comp
760 endocrinol, 241, 41–49. https://doi.org/10.1016/j.ygcen.2016.03.001

761 Schulz, R.W., de França, L.R., Lareyre, J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H.,
762 Miura, T. 2010. Spermatogenesis in fish. Gen comp endocrinol, 165(3), 390–411.
763 https://doi.org/10.1016/j.ygcen.2009.02.013

764 Scott, A.P., Sumpter, J.P., Stacey, N. 2010. The role of the maturation-inducing steroid,
765 17,20beta-dihydroxypregn-4-en-3-one, in male fishes: a review. J fish biol, 76(1), 183–
766 224. https://doi.org/10.1111/j.1095-8649.2009.02483.x

767 Sreenivasulu, G., Senthilkumaran, B., Sridevi, P., Rajakumar, A., Rasheeda, M.K. 2012.
768 Expression and immunolocalization of 20β-hydroxysteroid dehydrogenase during
769 testicular cycle and after hCG induction, in vivo in the catfish, *Clarias gariepinus*. Gen
770 comp endocrinol, 175(1), 48-54. https://doi.org/10.1016/j.ygcen.2011.09.002

771 Tenugu, S., Pranoty, A., Mamta, S.K., Senthilkumaran, B. 2020. Development and organisation
772 of gonadal steroidogenesis in bony fishes - A review. Aquaculture and Fisheries, 6 (3),
773 223-246. https://doi.org/10.1016/j.aaf.2020.09.004

774 Thomas, P., Tubbs, C., Garry, V.F. 2009. Progestin functions in vertebrate gametes mediated by
775 membrane progestin receptors (mPRs): Identification of mPRalpha on human sperm and
776 its association with sperm motility. Steroids 74(7), 614–621.
777 https://doi.org/10.1016/j.steroids.2008.10.020

778 Tubbs, C., Thomas, P. 2008. Functional characteristics of membrane progestin receptor alpha
779 (mPRα) subtypes: a review with new data showing mPRα expression in seatrout sperm
780 and its association with sperm motility. Steroids 73(9-10), 935-941.
781 https://doi.org/10.1016/j.steroids.2007.12.022

782   Vizziano, D., Le Gac, F., Fostier, A. 1996. Effect of 17β-estradiol, testosterone, and 11-
783   ketotestosterone on 17, 20β-dihydroxy-4-pregnen-3-one production in the rainbow trout
784   testis. Gen. Comp Endocrinol, 104(2), 179-188. https://doi.org/10.1006/gcen.1996.0160

785   Yaron, Z., Levavi-Sivan, B. 2011. Endocrine regulation of fish reproduction. Encyclopedia of
786   fish physiology: from genome to environment, 2, 1500-1508.
787   https://doi.org/10.1016/B978-0-12-374553-8.00058-7

788   Yom-Din, S., Hollander-Cohen, L., Aizen, J., Boehm, B., Shpilman, M., Golan, M., Hurvitz, A.,
789   Degani, G., Levavi-Sivan. B. 2016. Gonadotropins in the Russian sturgeon: their role in
790   steroid secretion and the effect of hormonal treatment on their secretion. PLoS One
791   11(9):e0162344. https://doi.org/10.1371/journal.pone.0162344

792   Yueh, W., Chang, C. 1997. 17α,20β,21-trihydroxy-4-pregnen-3-one and 17α,20β-dihydroxy-4-
793   pregnen-3-one stimulated spermiation in protandrous black porgy, *Acanthopagrus*
794   *schlegeli*. Fish Physiol. Biochem. 17, 187–193.
795   https://doi.org/10.1023/A:1007774628330

796   Zhang, G., Wang, W., Su, M., Zhang, J. 2018 Effects of recombinant gonadotropin hormones
797   on the gonadal maturation in the spotted scat, *Scatophagus argus*. Aquaculture 483, 263-
798   272. https://doi.org/10.1016/j.aquaculture.2017.10.01

799   Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F.,
800   Nagahama, Y. 2007. A novel type of P450c17 lacking the lyase activity is responsible for
801   C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology 48, 4282-
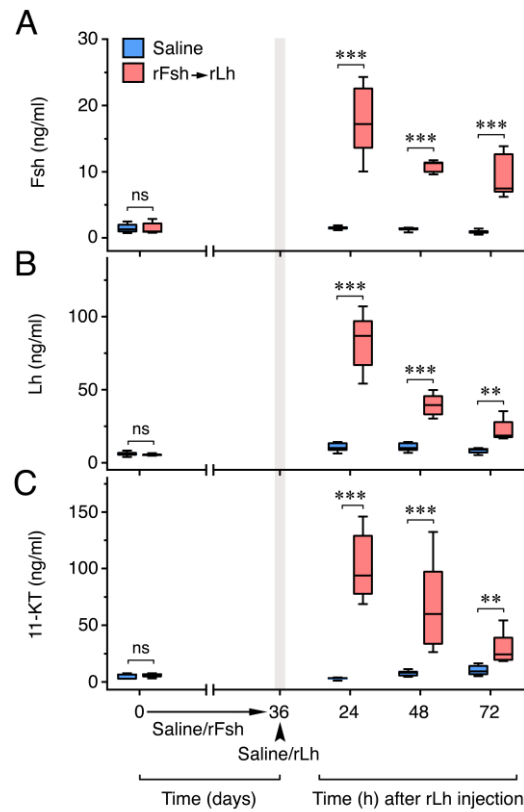802   4291. https://doi.org/10.1210/en.2007-0487

803

Fig. 1. Gonadotropin and androgen circulating levels in Senegalese sole males treated with rFsh and rLh. Plasma levels of Fsh (A), Lh (B) and 11-KT (C) in males before rFsh (18 µg kg^{-1}) treatment (day 0), and at 24, 48 and 72 h after saline (control) or rLh (18 µg kg^{-1}) intramuscular injection following a weekly treatment with saline or rFsh for 5 weeks. Data ($n = 5$ fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point as indicated in brackets (**, $P < 0.01$; ***, $P < 0.001$).
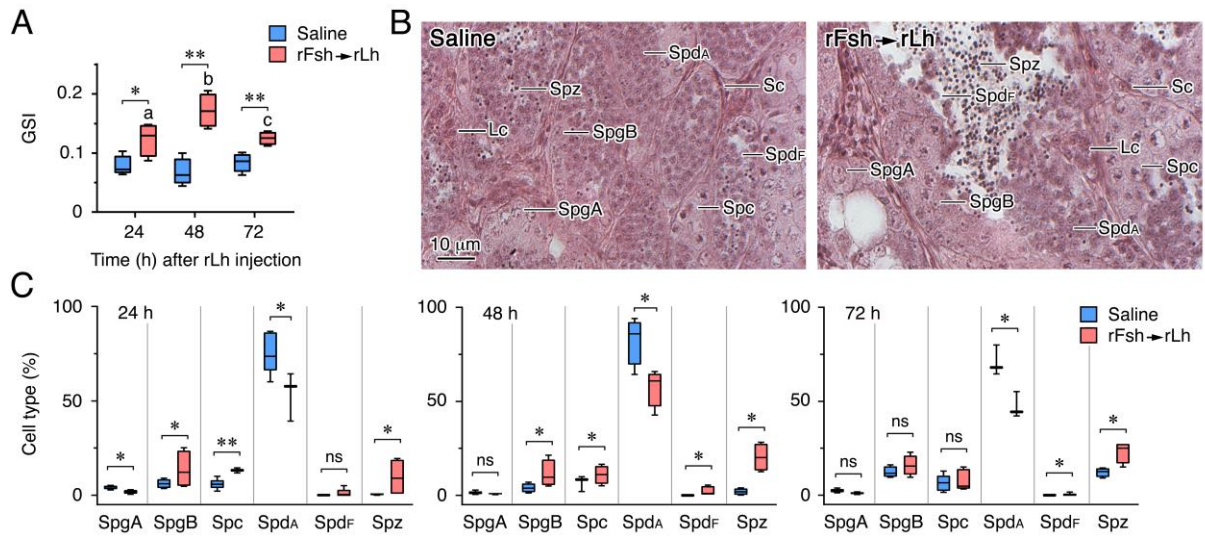
Fig. 2. Gonad weight and testicular development of males treated with rFsh and rLh. (A) GSI of males, previously treated with rFsh (18 µg kg^{-1}) for 5 weeks at 12ºC, at 24, 48 and 72 h after saline or rLh (18 µg kg^{-1}) injection at the same temperature. (B) Representative photomicrographs of histological sections from the cortical region of the testis stained with hematoxylin and eosin after 48 h of treatment with saline or rLh. (C) Percentage of germ cells in the seminiferous tubules in the testis of fish at 24, 48 and 72 h after treatment with saline or rLh. SpgA, spermatogonia type A; SpgB, spermatogonia type B; Spc, spermatocyte; Spd$_A$, spermatid attached to Sertoli cells; Spd$_F$, spermatid free in the tubule lumen; Spz, spermatozoa. In A and C, data (n = 5 fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point or cell type, or as indicated in brackets (*, $P < 0.05$; **, $P < 0.01$). In A, statistically significant data among the rFsh+rLh-treated males at different times are indicated by a different superscript on top of each box (one-way ANOVA, $P < 0.05$).
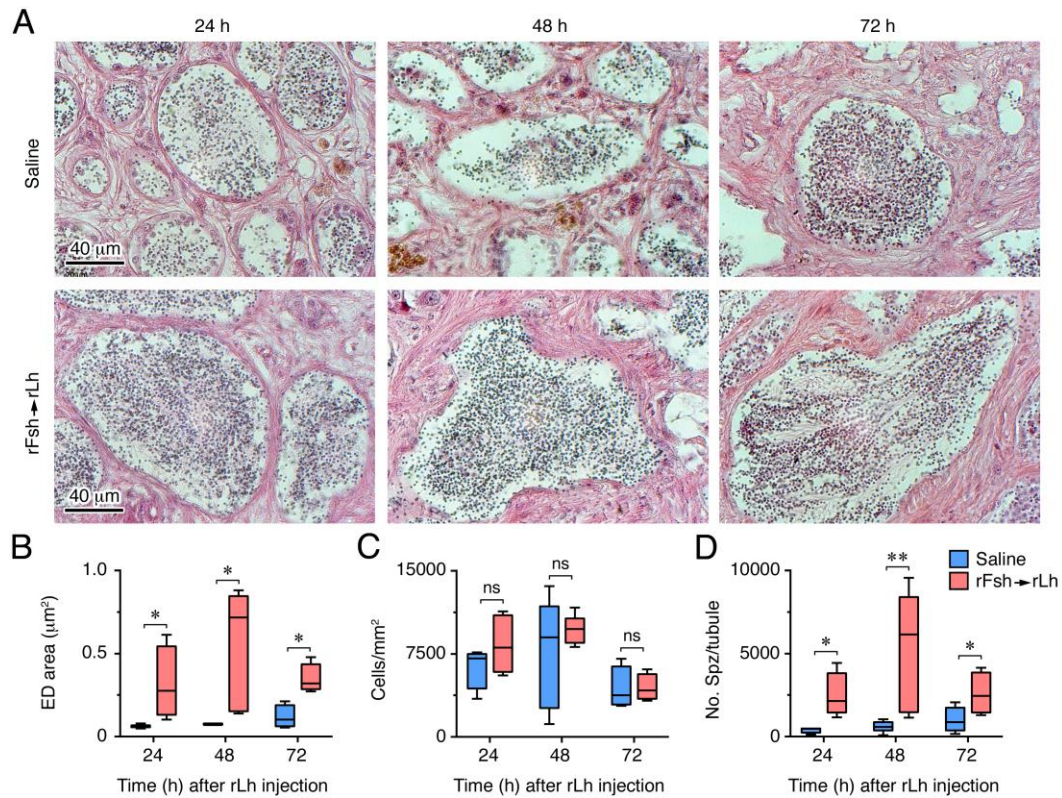
Fig. 3. Accumulation of spermatozoa in the testicular efferent duct (ED) of males treated with rFsh and rLh at 12ºC. (A) Photomicrographs of histological sections from the ED stained with hematoxylin and eosin from males at 24, 48 and 72 h after saline or rLh treatment. (B-D) Area of the ED lumen (B), density of spermatozoa (C) and number of spermatozoa per ED tubule (D) after saline or hormone treatment. In B-D, data ($n = 5$ fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point (*, $P < 0.05$; **, $P < 0.01$).
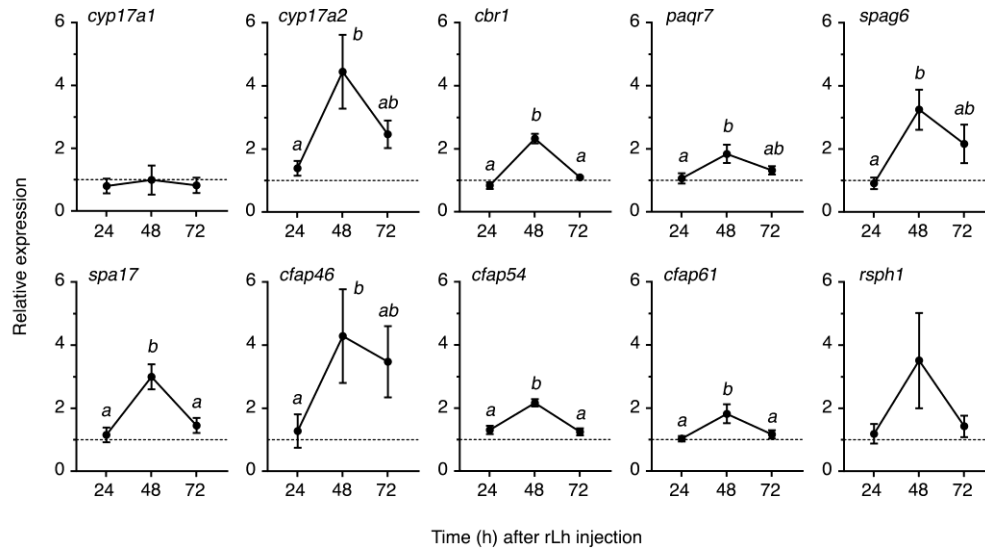
Fig. 4. Changes in the expression of testicular genes related to progestin function, flagellar motility and fertilization, in males treated with rFsh and rLh at 12ºC. Values are the relative mean expression levels of different genes normalized to the β-actin gene after 24, 48 and 72 h of rLh treatment expressed as fold-changes with respect to the control group (saline injected) at each time point. Dashed line at 1 indicates no change with respect to the controls. Data are the mean ± SEM (n = 4 fish), and values with different superscript are significantly different (one-way ANOVA, $P < 0.05$).
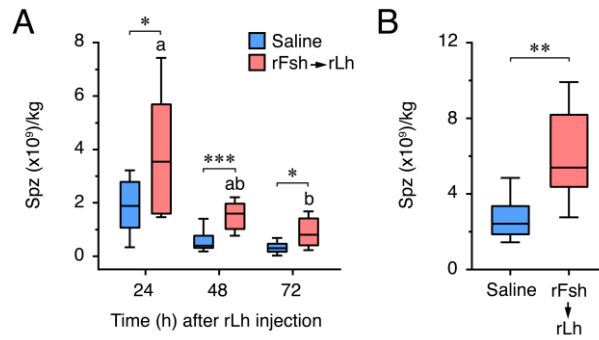
Fig. 5. Sperm production by males treated with rFsh and rLh at 12ºC. (A) Mean amount of sperm, normalized to the weight of fish, produced by the same males at 24, 48 and 72 h after saline or rLh injection. (B) Total sperm produced by each group during three days after the treatments. Data ($n = 10$ fish) are box and whisker plots and were statistically analyzed by the Student's t-test as indicatd in brackets (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In A, statistically significant data among the rFsh+rLh-treated males at different times are indicated by a different superscript on top of each box (one-way ANOVA, $P < 0.05$).
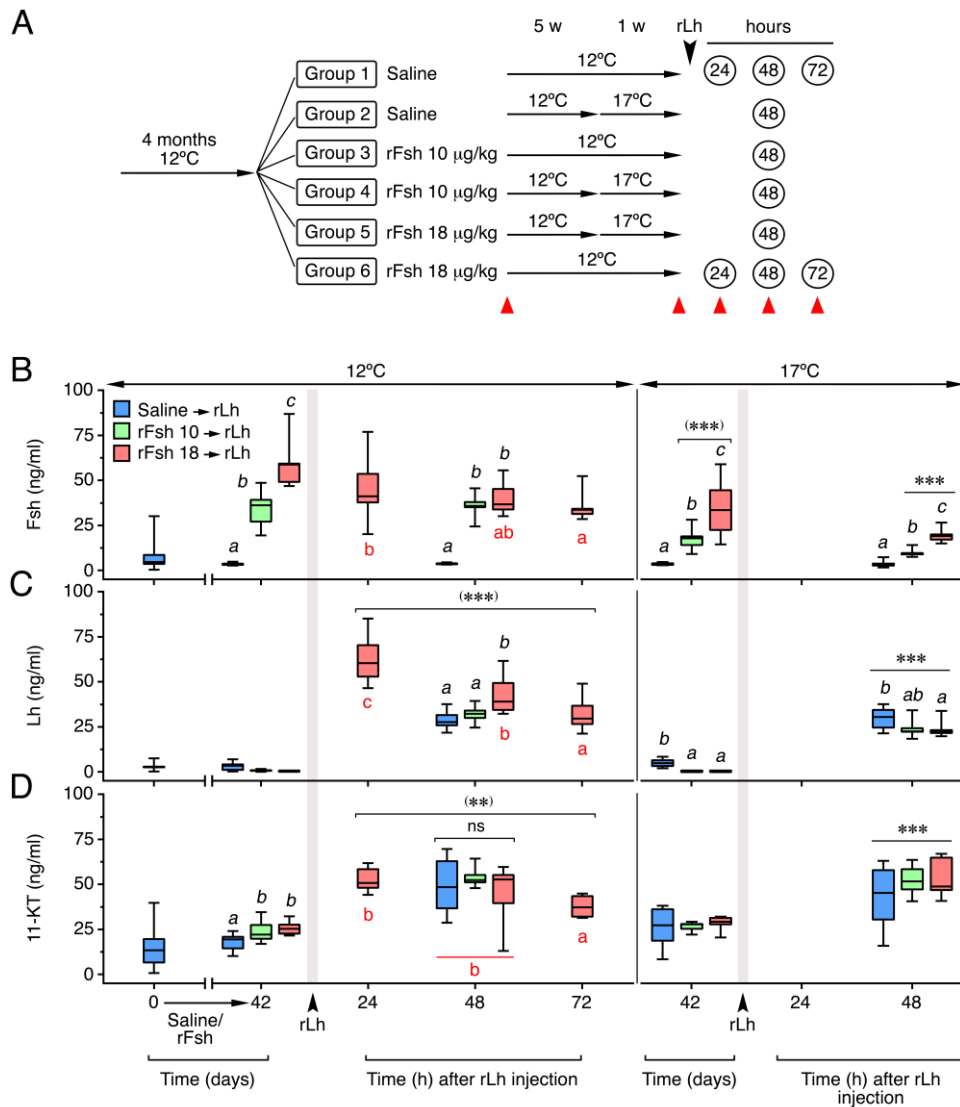
Fig. 6. Gonadotropin and androgen plasma levels in Senegalese sole males treated with two doses of rFsh and with one rLh dose at two different temperatures. (A) Schematic representation of the experimental setup. (B-D) Concentration of Fsh (B), Lh (C) and 11-KT (D) were measured at day 0 (before rFsh treatment), after the saline or rFsh treatment with 10 or 18 µg kg^{-1} (rFsh 10 and rFsh 18, respectively) for 5 weeks plus one more week at 12 or 17ºC (day 42), and at 24, 48 and 72 h after rLh (18 µg kg^{-1}) injection at 12 or 17ºC. Note that in this case control fish were also treated with rLh at day 42. Data ($n = 12$ fish) are box and whisker plots and were statistically analyzed by one-way ANOVA. Bars with different superscript within a time point (black color) or amongst the times after rLh injection (red color) at 12ºC are significantly different ($P < 0.05$). The asterisks in parenthesis indicate data significantly different with respect to groups treated with rFsh at 12ºC before rLh injection, whereas asterisks without parenthesis indicate differences with respect to groups maintained at 17ºC before rLh treatment (**, $P < 0.01$; ***, $P < 0.001$).
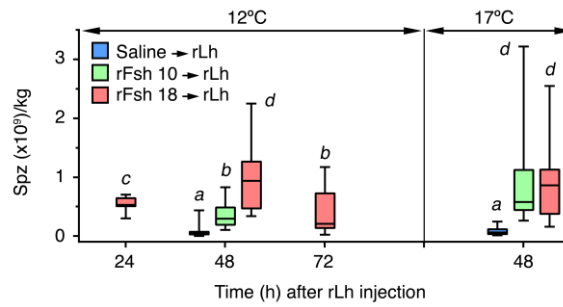
31

Fig. 7. Sperm production by males treated with two doses of rFsh and with one rLh dose at two different temperatures. Data represent the mean amount of sperm, normalized to the weight of fish, produced by different males at 24, 48 and 72 h after rLh injection. Note that control and 10 µg/kg rFsh treated fish were spermiated only at 48 h. Data ($n = 12$ fish) are box and whisker plots and were statistically analyzed by one-way ANOVA. Bars with different superscript are significantly different ($P < 0.05$).