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Effects of 17α-Ethinylestradiol (EE2) exposure during early life development on the gonadotropic axis ontogenesis of the European sea bass, *Dicentrarchus labrax*

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Abstract :

Exposure of young organisms to oestrogenic endocrine disrupting chemicals (EDCs) can elicit adverse effects, particularly on the reproductive function. In fish, as in other vertebrates, reproduction is controlled by the neuroendocrine gonadotropic axis, whose components are mainly regulated by sex steroids and may then be targets for EDCs.

In the present study, we investigated the effects of a xenoestrogen exposure on the ontogenesis of the gonadotropic axis in European sea bass. After exposure of hatching larvae for 8 days to 17α-ethinylestradiol (EE2) (0.5 nM and 50 nM), gene expression for kisspeptins (kiss1, kiss2), gonadotropin-releasing hormones (gnrh1, gnrh2, gnrh3), gonadotropin beta subunits (lhβ and fshβ) and brain type aromatase (cyp19a1b) were measured using quantitative real-time PCR. Our results demonstrate that EE2 strongly stimulated the expression of brain type aromatase (cyp19a1b) in sea bass larvae. In addition, EE2 exposure also affected the mRNA levels of kiss1, gnrh1 and gnrh3 by inducing a downregulation of these genes during the early developmental stages, while no effect was seen in gnrh2, lhβ and fshβ. These results reinforce the idea that the larval development is a sensitive critical period in regard to endocrine disruption and that the gonadotropic axis in the developing sea bass is sensitive to xenoestrogen exposure.

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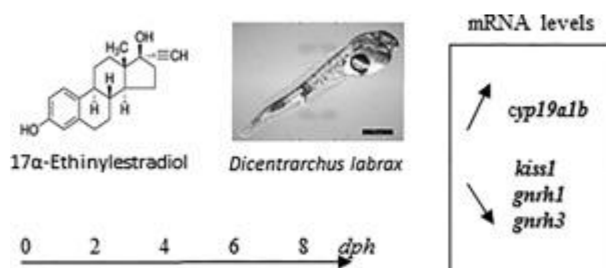
Graphical abstract



Highlights

► Gonadotropic axis components are expressed in sea bass larvae. ► 17α-ethinylestradiol modulates the expression of gonadotropic axis components. ► 17α-ethinylestradiol affects the expression of cyp19a1b, kiss1, gnrh1 and gnrh3. ► Sea bass larvae are sensitive to xenoestrogen exposure.

Keywords : Neuroendocrine disruption, Xenoestrogens, Gonadotropin-releasing hormones, Kisspeptins, Gonadotropins, Larva

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47 **Introduction**

48 Oestrogens, mostly known for their key role in the reproductive function, are also described in
49 vertebrates as important factors in several systems such as the cardiovascular system
50 (Dworatzek and Mahmoodzareh, 2017), the adipose tissue (Kim et al, 2014) or the central
51 nervous system (Diotel et al., 2013). In the brain of mammals, through interactions with their
52 specific membrane and nuclear receptors, oestrogens have been shown to act on cell
53 proliferation, migration and differentiation, on synaptic plasticity and on cell survival
54 (McCarthy, 2008; Coumailleau et al., 2015). These oestrogenic activities described on adult
55 brain are also observed in the developing brain of fish and mammals (McCarthy et al., 2008;
56 Coumailleau et al., 2015; Diotel et al., 2018).

57 Oestrogenic endocrine disrupting compounds (EDCs) are chemicals that can interfere with
58 biological processes by mimicking endogenous oestrogens through interaction with oestrogen
59 receptors (ERs) (Diamanti-Kandarakis *et al.*, 2009). Because of their habitat, fish are
60 particularly exposed to EDCs and several reports described the impact of xenoestrogens on fish
61 endocrine functions (Aris et al, 2014). The 17α-ethinylestradiol (EE_2) is a synthetic oestrogen
62 used in oral contraceptives. Due to its ubiquitous occurrence, its chronic detection in aquatic
63 environment (Aris et al, 2014; Du et al, 2020) and its high affinity to ERs (Denny et al, 2005),
64 EE2 is considered as a potent EDC. It has been used as a model molecule to evaluate the toxic
65 potential of xenoestrogen in organisms (Aris et al, 2014; Martyniuk et al, 2020). The reported
66 effects range from changes in gene expression levels to complete sex reversion in laboratory
67 fish models as well as in natural fish populations (for review: Aris *et al.*, 2014; Rutherford et
68 al, 2020). Other commonly reported disorders are vitellogenin production in males, altered egg
69 production in females or reduction of the fertilization rate (Matozzo *et al.*, 2008; Aris *et al.*,
70 2014; Volkova *et al.*, 2015). Finally, chronic exposure to an oestrogenic EDC leads to the
71 complete collapse of a fish population (Kidd et al., 2007).

72 In vertebrates, the reproductive function is under the control of the hypothalamus-pituitary-
73 gonadal (HPG) axis. At the brain level, the gonadotropin-releasing hormones (GnRHs) and the
74 kisspeptins (Kiss) are the key factors that controlled the reproductive axis. At the pituitary level
75 and under the control of the brain factors, the gonadotropin hormones (luteinizing hormone, LH
76 and follicle stimulating hormone, FSH) are synthetized and released into the blood circulation.
77 LH and FSH exert their actions on gonads controlling both steroidogenesis and gametogenesis.
78 Gonadal steroids exert feedback on the HPG axis (Chedrese, 2009). In fish, most of the
79 components of the HPG axis are expressed during early developmental stages (Vissio et al,

80 2021).
81 The oestrogenic EDCs might then act at the different levels of the HPG axis altering its normal
82 functioning in juvenile and adult individuals (Aris et al, 2014; León-Olea et al, 2014). They
83 may also affect the development/organization of the HPG axis when exposure occurs during
84 early life stages (i.e embryos, larvae) (Dickerson et al, 2007 ; Gore et al, 2019) which are known
85 to be highly sensitive to EDCs exposure (Hutchinson *et al.*, 1998). For example, fish exposure
86 during the period of gonadal differentiation induced sex reversal in various species (zebrafish:
87 Hill and Janz, 2003; roach: Lange et al, 2008; largemouth bass: Leet et al, 2020). Or, in
88 zebrafish, exposure of embryos to EE2 had various effects: it induced malformations such as
89 oedema or abnormal body curvature (Santos et al., 2014); it impacted the expression of genes
90 related to growth (Schiller et al., 2013) and disrupt the development of forebrain GnRH neurons
91 (Vosges et al., 2010).
92
93 The European sea bass is a species of great importance in Atlantic coastal waters and
94 Mediterranean Sea. Since the 1970's, in order to develop farming, the species has been largely
95 studied (Barnabé and Billard, 1984; Sánchez Vásquez and Muñoz-Cueto, 2014). In addition,
96 the European sea bass has been used in several ecotoxicological and toxicological studies (Conti
97 *et al.*, 2015; Della Torre *et al.*, 2015; López *et al.*, 2015; Tornambè *et al.*, 2018; Mhadhbi *et al.*,
98 2020; Soloperto et al, 2021). Nevertheless, since 2010 a continuous decline of the north Atlantic
99 fish stock is measured (ICES, 2018) conducing the European council to adopt fishing
100 regulations (EU Regulation 2015/523; 2016/72; 2017/127; 2018/1308; 2019/124). To explain
101 this decline in fish stocks, several reasons are proposed, including fishing pressure and changing
102 environmental conditions among which chemical pollution could play an important role.
103 Indeed, during its life cycle and particularly during the larval and juvenile stages, the European
104 sea bass lives in estuarine and coastal areas that are important nurseries areas (Kelley, 1988)
105 and that are largely influenced by anthropogenic chemicals and estrogenic EDCs (Tappin and
106 Millward, 2015).
107 The aim of the present study was to assess the effects of a xenoestrogen exposure on the
108 ontogenesis of the neuroendocrine system controlling reproduction. Sea bass larvae were
109 exposed to 17α-ethinylestradiol and the expression of kisspeptins (*kiss1, kiss2*), gonadotropin-
110 releasing hormones (*gnrh1, gnrh2, gnrh3*), gonadotropin subunits (*lhβ* and *fshβ*) and brain type
111 aromatase (*cyp19a1b*) were analyzed by quantitative real-time PCR.

112 # Material and Methods

113

114 *Animals*

115 Fertilized eggs of sea bass (n ≈ 5000) were obtained from a local hatchery (Ecloserie Marine
116 de Gravelines, Gravelines, France). After their arrival to the laboratory (Sebio, Le Havre,
117 France), eggs were transferred in natural filtered and aerated sea water at a temperature of 15°C
118 and a salinity of 33 psu and maintained in the dark. All procedures were in accordance with the
119 French and European legislation concerning the protection of animals used for experimentation
120 or other scientific purposes. Procedures undertaken were approved (#10263-
121 2017061911009684v3) by the regional ethical committee (Comité d'Ethique Normandie en
122 Matière d'Expérimentation Animale, CENOMEXA; agreement number 54)

123

124 *Chemicals*

125 17α-ethynilestradiol (EE2) (98% purity) and solvent control dimethyl sulfoxide (DMSO) (99%
126 purity) were purchased from Sigma-Aldrich (St-Quentin-Fallavier, France).

127

128 *Exposure conditions and sample collection*

129 Before hatching, eggs (ca. stage 18-22 S, as described by Cucchi et al. (2012)) were randomly
130 placed in 24-well plates (Greiner Bio-One, Courtaboeuf, France) and placed in an incubator
131 (Memmert BE500, France) at 15 °C in the dark (conditions adapted from Moretti et al, 1999).
132 Each well contained one egg in 2 ml of natural sea water (33psu). A stock solution of EE2 (100
133 mM) was prepared in DMSO and serially diluted in filtered seawater to obtain the experimental
134 solutions of 1 nM and 100 nM. The exposure started at hatching, half of each well content was
135 replaced by 1 ml of the corresponding experimental solutions to reach the nominal
136 concentration of 0.5 nM and 50 nM of EE2 (respectively 0.15µg/L and 15µg/L), and for control
137 groups to solvent (DMSO 0.0005% v/v) or to sea water only (14 plates, n=336 individuals
138 /group). Plates were kept in an incubator at 15°C in the dark. Test solutions were renewed every
139 48 h to allow water oxygenation and avoid EE2 degradation. Exposure lasted 8 days and the
140 experiment was repeated twice. During the experiment, larvae were not fed as mouth opening
141 occurs after ca. 8 dph (Sánchez Vázquez and Muñoz-Cueto, 2014).

142 At 0, 2-, 4-, 6- and 8-days post hatching (dph), 40 individuals per condition were sampled for
143 the qPCR assays. According to González-Martínez et al (2002b), during this period the
144 developing brain starts to express some targeted genes. Larvae were euthanized by immersion

145 in buffered tricaine methanesulfonate (200mg/L) (MS-222, Sigma-Aldricht, France), quickly
146 frozen into liquid nitrogen and kept at -80°C until analysis.
147 To confirm the oestrogenicity of the medium of exposure, 10 mL of exposure medium were
148 collected for each condition on days 2 and 8 when renewing test solutions, and stored at -20°C
149 until analysis by yeast oestradiol sensitive (YES) assay (Toxem, Le Havre, France) after a solid-
150 phase extraction procedure (SPE procedure).
151

152 *RNA isolation and reverse transcription*
153 Larvae (40 larvae per condition and sampling time) were pooled by 5 allowing 8 replicates to
154 be obtained by condition and sampling time. Samples were then homogenized in a Soft Tissue
155 Homogenizer CK14 (Bertin, Montigny-le-Bretonneux, France). Total RNA was extracted in
156 Tri-Reagent (Ambion Inc., Austin, TX) according to the manufacturer's instructions, then
157 samples were submitted to a deoxyribonuclease I treatment (Roche, Indianapolis, IN). Total
158 RNA quality and quantity was determined by measuring the absorbance at 260 nm and 280 nm
159 using a Nanodrop One (ThermoFisher). Absorbance ratios 260/280 greater than 1.8 were
160 selected for analyses. First strand cDNA was synthesized from 1µg of total RNA using
161 Superscript Reverse Transcriptase III and random hexamers (Invitrogen, Cergy Pontoise,
162 France). cDNAs were stored at -20°C until analysis.
163

164 *Quantitative Real Time PCR*
165 Selected primers and the amplicon size are shown in Table 1. qPCR analyses were performed
166 by using a Rotor Gene Q device (Qiagen, France). The mix consisted of 5 µl of QuantiTect
167 SYBR Green Master Mix (Qiagen), 1.5 µl of diluted cDNA template (1:10) and forward/reverse
168 primers (500 nM each) in a final volume of 10µl. The following conditions were chosen:
169 denaturation at 95 °C for 15min, followed by 45 cycles of 15s at 94°C, 25s at a temperature
170 ranging from 57 to 60°C according to the target gene, 25s at 72°C. The first derivative of the
171 melting curve (from 55°C to 95°C) was systematically plotted at the end of assay to control the
172 amplification of a single product.
173 For each target gene, to determine the efficiency of the qPCR reaction, serial dilutions of cDNA
174 prepared from a pool of samples were assayed in duplicate. In each run, samples were analyzed
175 in duplicate and a non-template control was added to confirm the absence of contamination.
176 Different reference genes were tested (*ef1*, *L13* and *fau*) and their stability confirmed by
177 Bestkeeper software (© 2004 M. W. Pfaffl). The relative expression levels were calculated by

178 Pfaffl method and normalized by the geometric mean of the three reference genes (Pfaffl, 2001).

179

180 *Statistical analysis*

181 Statistical analyses were performed using GraphPad Prism8.0.2 (GraphPad Inc., San Diego,
182 CA). Data are presented as mean ± SEM. For mortality, curves were compared among
183 experimental groups using the Log rank test for trend and the effect of time and treatment on
184 daily mortality was compared with two-way ANOVA. For gene expression levels, comparison
185 of means was performed using an ordinary one-way ANOVA for data that met the assumptions
186 of normality (Shapiro-Wilk test) and equal variance (Brown-Forsythe test). Tukey's multiple
187 comparisons test was used as post-hoc. Data that did not meet the assumption of normality were
188 log-transformed. For data that did not meet the assumption of equal variance (Kiss1:2dph,
189 Kiss2:8dph) the Welch ANOVA tests was used, with Games-Howell's multiple comparisons
190 test as post-hoc.

191

192 Results

193

194 *Measurement of estrogenic activity in the exposure medium*

195 In order to ensure the presence of EE2 in exposure media, samples collected on day 2 and 8
196 (corresponding to 2 days after the introduction of the contaminant) were sent for YES assay
197 (Toxem, Le Havre, France). In water samples from control solvent and 0.5nM EE2, no
198 oestrogenic activity has been detected by the YES assay. In contrast, water samples from 50nM
199 EE2 treatment have an oestrogenic activity equivalent to 35.5 nM ± 11 nM of oestradiol (data
200 not shown).

201

202 *Larval mortality (Fig. 1)*

203 Mortality was daily recorded to evaluate the toxicity of EE2 exposure. Log rank test results
204 indicated that there was no significant difference in the survival curves among groups ($p =$
205 0.06). Two-way ANOVA analyses demonstrated that the time ($p < 0.0001$) significantly
206 impacted daily mortality while the treatment ($p = 0.4$) had no effect. Regarding the curve
207 shapes, a peak in larval mortality is observed at 4 dph for the DMSO, 0.5 nM and 50 nM EE2
208 exposed groups.

209

210 *Effects of EE2 on target gene expression*

211 To assess the effect of EE2 exposure on the ontogenesis of the gonadotropic system, we
212 quantified the mRNA changes of different target genes at 2, 4, 6 and 8 dph.
213 For each gene and at each sampling, as no difference was observed between the control groups
214 (water and DMSO), the data were pooled into a single control group.
215

216 *Effect on cyp19a1b transcript level (Fig.2)*

217 *Cyp19a1b* mRNA was detected in each group and at each sampling time. A stimulatory effect
218 of EE2 on *cyp19a1b* expression was observed at all time points. At 2 and 4 dph, a 1.3-fold (P
219 = 0.026) and 1.87-fold (P = 0.039) increase was observed in 50 nM EE2 group as compared to
220 control. At 6 dph, significant increase in *cyp19a1b* transcript levels was detected in both 0.5
221 nM EE2 (6.62-fold increase, P = 0.003) and 50 nM EE2 (8.21-fold increase, P = 0.004) groups
222 respective to control. At 8 dph, a smaller significant effect was observed in both EE2 exposed
223 groups with a 3.1-fold increase as compared to control group (0.5 nM EE2, P = 0.006; 50 nM
224 EE2, P = 0.008).
225

226 *Effect on kiss1 and kiss2 transcript levels (Fig.3)*

227 Both *kiss1* and *kiss2* mRNAs were detected at each larval stage investigated. For *kiss1* gene
228 (Fig. 3A), a significant decrease in mRNA levels was observed in both EE2 exposed groups at
229 2 dph (0.5 nM EE2: 4.5-fold decrease, P = 0.003 and 50 nM EE2: 2.1-fold decrease, P = 0.036)
230 as compared to control. At the other time point, i.e. 4, 6, and 8 dph, no significant changes were
231 detected relative to control conditions.
232 Concerning *kiss2* gene expression (Fig. 3B), despite some small modulations, no significant
233 variations were observed among the different experimental conditions at any larval stages.
234

235 *Effect on gnrh1, gnrh2 and gnrh3 transcript levels (Fig.4)*

236 Transcripts for *gnrh1*, *gnrh2* and *gnrh3* were detected at each larval stage and in each
237 experimental condition. Regarding *gnrh1*, significant decrease in mRNA levels were detected
238 at 2 dph in both 0.5 nM EE2 (10.3-fold decrease, P = 0.0002) and 50nM EE2 groups (8-fold
239 decrease, P = 0.0003) as compared to control. In contrast, in the other time points, no significant
240 variation was detected among groups (Fig. 4A).
241 For *gnrh2* mRNA levels, no significant modulations were observed (Fig. 4B).
242 Some variations of *gnrh3* mRNA levels were observed but were not significant except at 4 dph

243 where a significant decrease was observed in 0.5nM EE2 group as compared to control (1.80-
244 fold decrease, P = 0.014) (Fig. 4C).
245
246 *Effect on lhβ and fshβ transcript levels (Fig.5)*
247 Measurements of *lhβ* and *fshβ* mRNAs were performed at each sampling time. Levels were,
248 however, not detectable at the earliest sampling time, i.e. 2 and 4dph. At 6 dph, there were
249 detectable in all samples but close to the limit of detection (mean Ct= 30.6) and at 8dph, both
250 *lhβ* and *fshβ* transcripts were detectable (mean Ct = 29.1). These results indicated an increase
251 in *lhβ* and *fshβ* expression during the larval development. When comparing *lhβ* and *fshβ* gene
252 expression at the different sampling time, no significant differences were detected between
253 control and EE2 exposed groups (Fig. 5A and 5B).
254

255 Discussion

256
257 In vertebrates, the developmental period comprising the embryonic stages and/or the
258 larval/childhood period has been described to be particularly sensitive to environmental
259 stressors which can affect different physiological function and have long-lasting effects
260 (Lichtveld et al, 2018; Mughal et al, 2018; Besson et al, 2020; Eachus et al, 2021). In fish, most
261 of the components of the neuroendocrine gonadotropic axis are expressed in early life stages
262 (Vissio et al, 2021), modifications of their expression/production may disturb the
263 developmental process on which there are involved or may have delayed impact on adult life
264 (for review: Rosenfeld et al, 2017; Vissio et al, 2021). In this context, the present study
265 investigated the potential neuroendocrine effects of sea bass larvae exposure to a xenoestrogen,
266 the synthetic 17α-ethynilestradiol (EE2). Two concentrations of EE2 were tested: 0.5 nM (150
267 ng/L) representative of heavily polluted environments as in Venice lagoon where the total
268 estrogenic activity can reach 191 ng/L (Pojana *et al.*, 2007), and 50 nM of EE2 (15 µg/L) which
269 aimed at identifying mechanisms and targets of EE2 toxicity. Using the exposure protocol that
270 we previously developed (Soloperto et al, 2021) and quantitative real time PCR assays, we
271 demonstrated that EE2 interferes with the developing brain by modulating the expression of
272 some key genes of the neuroendocrine system.
273
274 The *cyp19a1b* gene coding for the brain aromatase (also named Aromatase B), due to its
275 remarkable sensitivity to oestrogens has been proposed as a biomarker of xenoestrogen

276 exposure both *in vivo* and *in vitro.* This ability to answer to oestrogens is given by the presence
277 of estrogen responsive element (ERE) in the *cyp19a1b* promoter (Kazeto *et al.*, 2001; Tong and
278 Chung, 2003; Piferrer and Blázquez, 2005), a structure that appears conserved in different
279 teleost species (zebrafish, catfish, fugu, goldfish, medaka and tilapia) (Diotel et al., 2010).
280 From this postulate, we analyzed *cyp19a1b* expression in order to assess the experimental
281 conditions of our experiment and the effects of EE2 in sea bass larvae. Our results demonstrated
282 that, as in other species, EE2 exposure triggered an up-regulation of the aromatase B expression
283 in sea bass larvae. Interestingly, at 2 and 4 dph the induction appears weaker than at 6 and 8
284 dph suggesting that sea bass early larval stages are less responsive to xenoestrogen exposure or
285 that long-time exposure enhance *cyp19a1b* response. As an enzyme implicated in the
286 neurosteroidogenesis, the aromatase B disruption may negatively influence the brain
287 homeostasis. Furthermore, in fish, this enzyme is expressed in radial glial cells, which are
288 neuronal progenitor cells, so disruption of these cells can affect several brain functions such as
289 neurotransmission, neurogenesis or neuronal organization (Coumailleau et al, 2015; Diotel et
290 al, 2018; Nasri et al, 2021).
291
292 *Xenoestrogen effects on kisspeptin system*
293 Regarding kiss genes, the present results show an early expression of both *kiss1* and *kiss2* genes
294 in sea bass larvae suggesting potential early roles during larval development, as demonstrated
295 in medaka embryos where knockdown of zygotic *kiss1* induced disruption in brain development
296 (Hodne et al, 2013). In addition, we observed that EE2 exposure of young larvae induced a
297 marked decrease in *kiss1* expression at 2 dph, an effect that was no longer observed in oldest
298 larvae. In contrast, EE2 exposure did not change *kiss2* expression levels during the followed
299 period. These effects appear in accordance with the results obtained by Escobar et al. (2013) in
300 juvenile sea bass showing by double *in situ* hybridization that *kiss1* neurons located in the
301 mediobasal hypothalamus express two types of nuclear estrogen receptor (ERα and ERβ2),
302 while no ER could be detected in *kiss2* neurons. Nevertheless, sensitivity to oestrogens of some
303 *kiss1* and *kiss2* neurons populations has been observed in adult sea bass after ovariectomy and
304 oestradiol replacement (Alvarado et al, 2016) suggesting that kiss genes could also be regulated
305 by oestrogens through indirect pathways. In teleosts, the situation concerning kiss neurons and
306 oestrogen responsiveness is varying according to species. In adult medaka, the situation appears
307 similar that the one we observed in sea bass with kiss1 neurons expressing ERα and sensitive
308 to oestrogens, while kiss2 neurons are not (Mitani et al, 2010). In juvenile zebrafish, an increase
309 of both *kiss1* and *kiss2* expression is observed after oestrogen treatments (Servili et al, 2011).

310 Similar observations have been made in female juvenile rare minnows, while in juvenile male
311 both *kiss1* and *kiss2* expression decrease (Yang et al, 2016). In adult dabry's sturgeon and
312 juvenile orange-spotted grouper, *kiss2* but not *kiss1* expression are affected by oestrogens (Guo
313 et al, 2017; Yue et al, 2019). Finally, the study of kiss genes promoters in goldfish have
314 permitted to identify partial motif of oestrogen response element (ERE) in both genes (Wang
315 et al, 2013).
316 Altogether, the data collected in fish regarding oestrogen sensitivity of kiss genes suggest that
317 xenoestrogen exposure could disrupt the ontogeny and normal functioning of the kisspeptin
318 system. Nevertheless, it should be kept in mind that responses to oestrogen may vary according
319 to the developmental stage of the individual. Our results demonstrated that xenoestrogens are
320 able to transiently modulate kiss1 expression in young sea bass larvae. Further studies are
321 necessary to characterize the mode of action by, for example, locating the oestrogen receptors
322 at this stage of development, and to shed light on the biological effects induced by these
323 disruptions.

324 *Xenoestrogen effects on GnRH system*
325 In sea bass, three GnRH isoforms have been characterized and their neuroanatomical
326 distribution described (González-Martínez et al, 2002a, 2004). In the present study using qPCR
327 assays, the diverse GnRH isoforms were detected in sea bass larvae since 2dph, contrasting
328 with the results observed in a previous study using *in situ* hybridization where *gnrh2* expressing
329 cells were firstly detected at 4dph, *gnrh3* at 7dph and *gnrh1* at 30dph (González-Martínez et al,
330 2002b). This discrepancy may result from differences in the sensitivity of the techniques used,
331 but it should also be noted that in our study, the analyses were done on RNA extractions of
332 pooled whole larvae. In any case, the expression of GnRH isoforms in sea bass larvae suggests
333 roles for these genes during early stages of development, as demonstrated in zebrafish embryos
334 where knockdown of *gnrh2* or *gnrh3* affect brain and eye development (Wu et al, 2006).
335 We investigated the effects of EE2 on the diverse GnRH isoforms and we observed inhibitory
336 effects of EE2 exposure on *gnrh1* and *gnrh3* gene expression. In both cases, the effect was
337 transient (observed at one stage and no longer at later stages) suggesting that compensatory
338 mechanisms might occur at later larval stages. Regarding *gnrh2,* EE2 exposure did not induce
339 any variation of the gene expression.
340 It is classically admitted that sex steroids exert positive and negative feedbacks on the
341 gonadotropic axis (Zohar et al, 2010), with different effects on GnRH system depending on the
342 gene, the physiological stage and the species. In adult sea bass, a negative effect of oestrogens

343 has been demonstrated on *gnrh1* expression after ovariectomy and oestradiol (E2) replacement
344 (Alvarado et al, 2016). In contrast, in eels E2 stimulated gnrh1 while it had no effect on gnrh2
345 production (Montero et al, 1995). In immature tilapia, which possess three gnrh isoforms, E2
346 treatments induced an increase of gnrh1 neurons and have no effect on gnrh2 and gnrh3 neurons
347 (Parhar et al, 2000); and in prepubertal red seabream, E2 implants did not modify *gnrh1*
348 expression (Okuzawa et al, 2002). Because of this sensitivity of gnrh system, some studies have
349 raised the question of its potential disturbance by xenoestrogens in addition to other adverse
350 effects (for review: León-Olea et al, 2014).
351 During early developmental stages, even if several studies have investigated the impact of EE2
352 exposure (Lange et al, 2008; Vosges et al, 2010; 2012; Bhandari et al, 2015; Porseryd et al,
353 2018; Leet et al, 2020; Nasri et al, 2021), only limited ones performed in zebrafish have focused
354 on the oestrogens/xenoestrogens influence on GnRH. Vosges *et al.* (2010, 2012) observed that
355 larval exposure to EE2 induced, through an ER signaling pathways, an increase in gnrh3
356 forebrain neurons. Similar results have been obtained using molecules with oestrogeno-mimetic
357 activities as nonylphenol in zebrafish larvae (Vosges et al, 2012) and bisphenol A and
358 Bisphenol S in zebrafish embryos (Qiu et al, 2016).
359 Although the functions of the different gnrh in the developing brain are still not well known,
360 they are expressed, their silencing induced developmental alterations and they are sensitive to
361 disruptive action of xenoestrogens (for review: Vissio et al, 2021). Further studies should be
362 conducted with animals followed over a long period to decipher the biological effects induced
363 by these disruptions.
364
365 *Xenoestrogen effects on gonadotropin subunits LHβ and FSHβ*
366 In vertebrates, gonadotropins (LH and FSH) are heterodimeric hormones composed of a
367 common α subunit and a hormone-specific β subunit. In the present study, our analyses of *lhβ*
368 and *fshβ* subunits showed their low levels of expression in young sea bass larvae as they were
369 only detected from 6 dph near the detection limits. These observations of a precocious
370 expression of gonadotropin subunits during the embryonic and larval development are in
371 accordance with results obtained in other species (for review: Weltzien et al, 2014).
372 Several studies have demonstrated the direct and indirect control exerts by sex steroids on
373 gonadotropin expression, production and secretion (Aroua et al, 2007; Mazón et al, 2015;
374 Fontaine et al, 2020). In sea bass, Mateos et al (2002) measured the expression of gonadotropin
375 subunits in E2 treated animals and observed an increase in *lhβ* and a decrease in *fshβ* mRNA
376 levels. In addition, double label *in situ* hybridization demonstrated that both FSH and LH cells

377 express ER transcripts in female adult sea bass (Muriach et al, 2008a; 2008b).
378 Nevertheless, in the present study, we observed that EE2 exposure did not affect the expression
379 of the two gonadotropin beta subunits in sea bass larvae. However, the situation differs in other
380 species as in fathead minnow where 7 days exposure to EE2 (2, 5 and 50ng/L) during early life
381 stages modify the gene expression of several genes including *lhβ* (Johns et al, 2009), or in
382 zebrafish where exposure of embryos for 5 days to bisphenol A or bisphenol S induced an
383 increase in *lhβ* and *fshβ* expression (Qiu et al, 2016).
384 Altogether, these results indicate that the pathways regulating gonadotropin beta expression
385 might be stage and species specific. In the case of sea bass, our results indicate that at these
386 larval stages, *lhβ* and *fshβ* subunits are not sensitive to oestrogenic molecules. Further studies
387 investigating the localization of gonadotropins and ER expressing site in larval stages might
388 help in the understanding of these responses.
389
390 **Conclusion**
391 Finally, our study demonstrated that sea bass larvae are sensitive to xenoestrogen exposure and
392 that the ontogenesis of the gonadotropic system has been influenced by EE2. These disruptions
393 occurring during the early larval stages could lead to deleterious effects in individuals such as
394 alterations in brain organization, behavior or sexual differentiation. In sea bass, where the first
395 signs of sexual differentiation are observed around 150 dph (Blázquez et al, 2008), a sex ratio
396 deviation in favor of females has been shown after larval exposure (48 dph to 88 dph) to EE2
397 through food (10mg/kg) (Blázquez et al, 1998). These effects could be the result of disturbances
398 affecting different levels of the reproductive axis (brain, pituitary and gonads) and need to be
399 further investigated.
400
401
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410

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716 # Tables

717

718 *Table 1: Selected primers for qPCR analysis*

719

Gene	Accession n°	Annealing T °C	Efficiency %	Forward sequence	Reverse sequence
Ef1a	AJ866727	60	100	CTAAGAACGGACAGACCCGC	TGACTCCAACGATGAGCTGC
L13	DQ836931	60	101	GGCTAAGGCCCGTCTCATTG	GCACGAACCTTGGTGTGGTA
fau	EF190887	57	103	CAAACTGAGAGCGACCGGAG	GCGTCCGATCTTCATCCCTG
cyp19a1b	AY138522	57	99	GGCTACACCCTCAACAGCAT	CCACCAAAACCCCTCTGTGT
kiss 1	FJ008914	57	101	CTCAAAGCTGGTCCTCCCTC	GGTCTTGTGTCACTTTCCGT
kiss 2	FJ008915	57	101	GGATTCCAGCCCGTGTTTCT	TAGGCACCTCCAGTTCTCGT
gnrh1	AF224279	60	102	GGTCCTATGGACTGAGTCCAGG	TGATTCCTCTGCACAACCTAA
gnrh2	AF224281	60	104	GTGTGAGGCAGGAGAATGCA	CTGGCTAAGGCATCCAGAATG
gnrh3	AF224280	57	99	TGTGGGAGAGCTAGAGGCAAC	GTTTGGGCACTCGCCTCTT
lhβ	AF543314	60	98	ACCAACATCAGCATCCAAGTG	TTCTCTGTTCAGGCCTCTCATAGT
fshβ	AF543314	60	98	TTGAGCTTCCTGACTGTCCA	GCAGGCTCTCGAAGGTACAG

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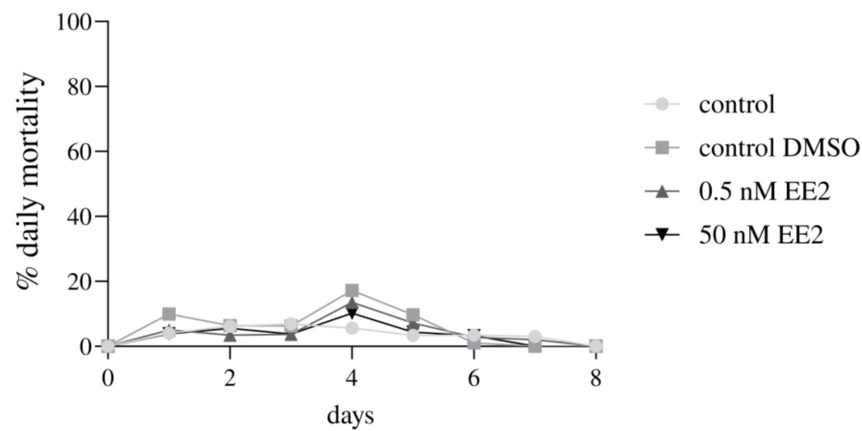
724 **Figure legends**

725



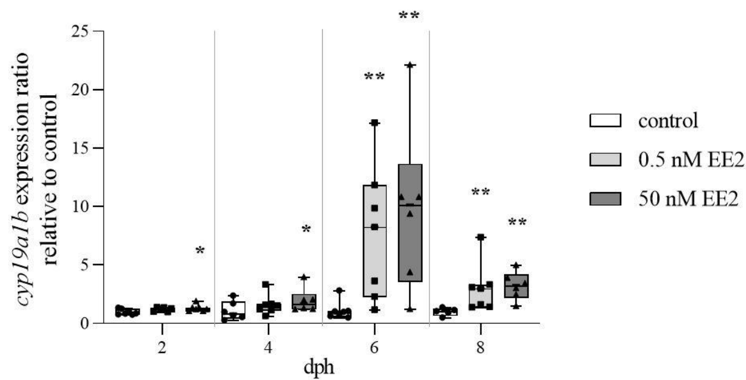
726

727 Figure 1

728 Daily mortality (%) of sea bass larvae during the experimental exposure to 17α-ethinylestradiol

729 (EE2).

730



731

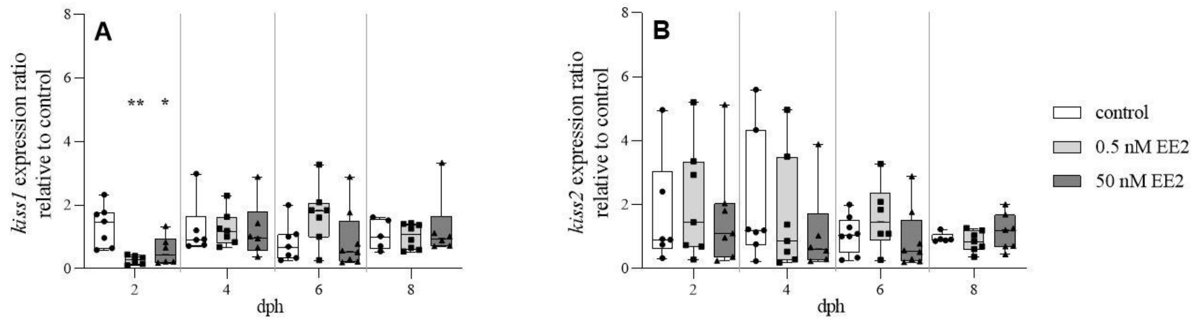
732 Figure 2

733 Relative gene expression of *cyp19a1b* in sea bass larvae during the experimental exposure to

734 17α-ethinylestradiol (EE2). Results are presented as group average (average Pflaff ratio ± SEM)

735 relative to control conditions. *: $p < 0.05$; **: $p < 0.01$

736



737

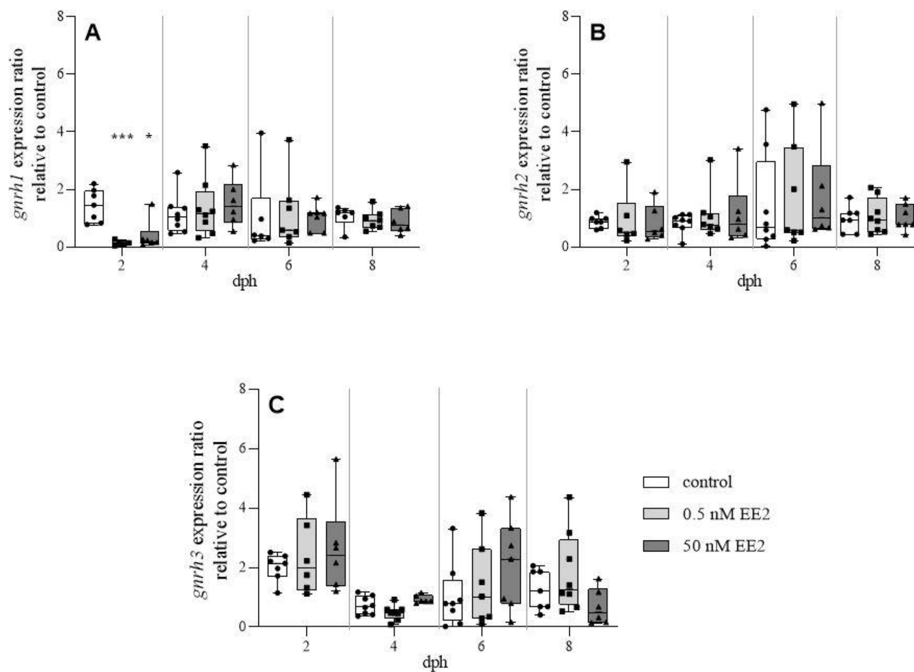
738 Figure 3

739 Relative gene expression of *kiss1* (A) and *kiss2* (B) in sea bass larvae during the experimental

740 exposure to 17α-ethinylestradiol (EE2). Results are presented as group average (average Pflaff

741 ratio ± SEM) relative to control conditions. *: $p < 0.05$; **: $p < 0.01$

742



743

744 Figure 4

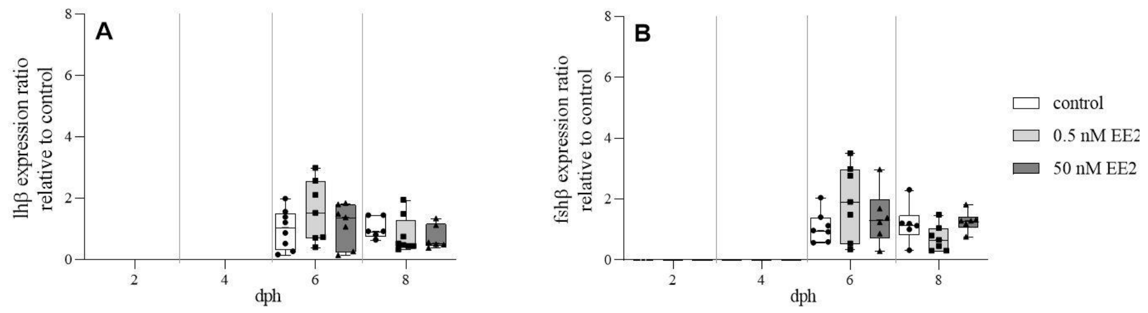
745 Relative gene expression of *gnrh1* (A) and *gnrh2* (B) and *gnrh3* (C) in sea bass larvae during

746 the experimental exposure to 17α-ethinylestradiol (EE2). Results are presented as group

747 average (average Pflaff ratio ± SEM) relative to control conditions. *: $p < 0.05$; ***: $p<0.001$

748

749



750 Figure 5

751 Relative gene expression of *lhβ* (A) and *fshβ* (B) in sea bass larvae during the experimental

752 exposure to 17α-ethinylestradiol (EE2). Results are presented as group average (average Pflaff

753 ratio ± SEM) relative to control condition.

754

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