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

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1 **Effects of 17 $\alpha$ -Ethinylestradiol (EE2) exposure during early life development on**  
2 **the gonadotropic axis ontogenesis of the European sea bass, *Dicentrarchus labrax*.**

3  
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18  
19  
20 **Highlights:**

- 21 • Gonadotropic axis components are expressed in sea bass larvae  
22 • 17 $\alpha$ -ethinylestradiol modulates the expression of gonadotropic axis components  
23 • 17 $\alpha$ -ethinylestradiol affects the expression of *cyp19a1b*, *kiss1*, *gnrh1* and *gnrh3*  
24 • Sea bass larvae are sensitive to xenoestrogen exposure

25

26 **Abstract**

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

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

43

44

45 **Keywords:** Neuroendocrine disruption, Xenoestrogens, Gonadotropin-Releasing hormones,  
46 Kisspeptins, Gonadotropins, Larva

## 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  
61 fish endocrine functions (Aris et al, 2014). The 17 $\alpha$ -ethinylestradiol (EE<sub>2</sub>) is a synthetic  
62 oestrogen used in oral contraceptives. Due to its ubiquitous occurrence, its chronic detection  
63 in aquatic environment (Aris et al, 2014; Du et al, 2020) and its high affinity to ERs (Denny et  
64 al, 2005), EE<sub>2</sub> is considered as a potent EDC. It has been used as a model molecule to  
65 evaluate the toxic potential of xenoestrogen in organisms (Aris et al, 2014; Martyniuk et al,  
66 2020). The reported effects range from changes in gene expression levels to complete sex  
67 reversion in laboratory fish models as well as in natural fish populations (for review: Aris *et*  
68 *al.*, 2014; Rutherford et al, 2020). Other commonly reported disorders are vitellogenin  
69 production in males, altered egg production in females or reduction of the fertilization rate  
70 (Matozzo *et al.*, 2008; Aris *et al.*, 2014; Volkova *et al.*, 2015). Finally, chronic exposure to an  
71 oestrogenic EDC leads to the 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  
74 the kisspeptins (Kiss) are the key factors that controlled the reproductive axis. At the pituitary  
75 level and under the control of the brain factors, the gonadotropin hormones (luteinizing  
76 hormone, LH and follicle stimulating hormone, FSH) are synthesized and released into the  
77 blood circulation. LH and FSH exert their actions on gonads controlling both steroidogenesis  
78 and gametogenesis. Gonadal steroids exert feedback on the HPG axis (Chedrese, 2009). In  
79 fish, most of the components of the HPG axis are expressed during early developmental

80 stages (Vissio et al, 2021).  
81 The oestrogenic EDCs might then act at the different levels of the HPG axis altering its  
82 normal functioning in juvenile and adult individuals (Aris et al, 2014; León-Olea et al, 2014).  
83 They may also affect the development/organization of the HPG axis when exposure occurs  
84 during early life stages (i.e embryos, larvae) (Dickerson et al, 2007 ; Gore et al, 2019) which  
85 are known to be highly sensitive to EDCs exposure (Hutchinson *et al.*, 1998). For example,  
86 fish exposure during the period of gonadal differentiation induced sex reversal in various  
87 species (zebrafish: Hill and Janz, 2003; roach: Lange et al, 2008; largemouth bass: Leet et al,  
88 2020). Or, in zebrafish, exposure of embryos to EE2 had various effects: it induced  
89 malformations such as oedema or abnormal body curvature (Santos et al., 2014); it impacted  
90 the expression of genes related to growth (Schiller et al., 2013) and disrupt the development  
91 of forebrain GnRH neurons (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  
97 (Conti *et al.*, 2015; Della Torre *et al.*, 2015; López *et al.*, 2015; Tornambè *et al.*, 2018;  
98 Mhadhbi *et al.*, 2020; Soloperto et al, 2021). Nevertheless, since 2010 a continuous decline of  
99 the north Atlantic fish stock is measured (ICES, 2018) conducting the European council to  
100 adopt fishing regulations (EU Regulation 2015/523; 2016/72; 2017/127; 2018/1308;  
101 2019/124). To explain this decline in fish stocks, several reasons are proposed, including  
102 fishing pressure and changing environmental conditions among which chemical pollution  
103 could play an important role. Indeed, during its life cycle and particularly during the larval  
104 and juvenile stages, the European sea bass lives in estuarine and coastal areas that are  
105 important nurseries areas (Kelley, 1988) and that are largely influenced by anthropogenic  
106 chemicals and estrogenic EDCs (Tappin and 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 17 $\alpha$ -ethinylestradiol and the expression of kisspeptins (*kiss1*, *kiss2*), gonadotropin-  
110 releasing hormones (*gnrh1*, *gnrh2*, *gnrh3*), gonadotropin subunits (*lh $\beta$*  and *fsh $\beta$* ) and brain  
111 type aromatase (*cyp19a1b*) were analyzed by quantitative real-time PCR.

## 112 **Material and Methods**

113

### 114 *Animals*

115 Fertilized eggs of sea bass ( $n \approx 5000$ ) were obtained from a local hatchery (Ecluserie 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  
118  $15^{\circ}\text{C}$  and a salinity of 33 psu and maintained in the dark. All procedures were in accordance  
119 with the French and European legislation concerning the protection of animals used for  
120 experimentation 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  $17\alpha$ -ethynilestradiol (EE2) (98% purity) and solvent control dimethyl sulfoxide (DMSO)  
126 (99% 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 (Mettmert BE500, France) at  $15^{\circ}\text{C}$  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  
133 (100 mM) was prepared in DMSO and serially diluted in filtered seawater to obtain the  
134 experimental solutions of 1 nM and 100 nM. The exposure started at hatching, half of each  
135 well content was replaced by 1 ml of the corresponding experimental solutions to reach the  
136 nominal concentration of 0.5 nM and 50 nM of EE2 (respectively  $0.15\mu\text{g/L}$  and  $15\mu\text{g/L}$ ), and  
137 for control groups to solvent (DMSO 0.0005% v/v) or to sea water only (14 plates,  $n=336$   
138 individuals /group). Plates were kept in an incubator at  $15^{\circ}\text{C}$  in the dark. Test solutions were  
139 renewed every 48 h to allow water oxygenation and avoid EE2 degradation. Exposure lasted 8  
140 days and the experiment was repeated twice. During the experiment, larvae were not fed as  
141 mouth opening 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-Aldrich, 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 -  
149 20°C until analysis by yeast oestradiol sensitive (YES) assay (Toxem, Le Havre, France) after  
150 a solid-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  
159 nm 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  
168 forward/reverse primers (500 nM each) in a final volume of 10µl. The following conditions  
169 were chosen: denaturation at 95 °C for 15min, followed by 45 cycles of 15s at 94°C, 25s at a  
170 temperature ranging from 57 to 60°C according to the target gene, 25s at 72°C. The first  
171 derivative of the melting curve (from 55°C to 95°C) was systematically plotted at the end of  
172 assay to control the amplification of a single product.

173 For each target gene, to determine the efficiency of the qPCR reaction, serial dilutions of  
174 cDNA prepared from a pool of samples were assayed in duplicate. In each run, samples were  
175 analyzed in duplicate and a non-template control was added to confirm the absence of  
176 contamination. Different reference genes were tested (*efl*, *L13* and *fau*) and their stability  
177 confirmed by Bestkeeper software (© 2004 M. W. Pfaffl). The relative expression levels were

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

180

### 181 *Statistical analysis*

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

192

## 193 **Results**

194

### 195 *Measurement of estrogenic activity in the exposure medium*

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

202

### 203 *Larval mortality (Fig. 1)*

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

210



211 *Effects of EE2 on target gene expression*

212 To assess the effect of EE2 exposure on the ontogenesis of the gonadotropic system, we  
213 quantified the mRNA changes of different target genes at 2, 4, 6 and 8 dph.

214 For each gene and at each sampling, as no difference was observed between the control  
215 groups (water and DMSO), the data were pooled into a single control group.

216

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

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

226

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

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

233 Concerning *kiss2* gene expression (Fig. 3B), despite some small modulations, no significant  
234 variations were observed among the different experimental conditions at any larval stages.

235

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

237 Transcripts for *gnrh1*, *gnrh2* and *gnrh3* were detected at each larval stage and in each  
238 experimental condition. Regarding *gnrh1*, significant decrease in mRNA levels were detected  
239 at 2 dph in both 0.5 nM EE2 (10.3-fold decrease,  $P$  = 0.0002) and 50nM EE2 groups (8-fold  
240 decrease,  $P$  = 0.0003) as compared to control. In contrast, in the other time points, no  
241 significant variation was detected among groups (Fig. 4A).

242 For *gnrh2* mRNA levels, no significant modulations were observed (Fig. 4B).

243 Some variations of *gnrh3* mRNA levels were observed but were not significant except at 4

244 dph where a significant decrease was observed in 0.5nM EE2 group as compared to control  
245 (1.80-fold decrease,  $P = 0.014$ ) (Fig. 4C).

246

247 *Effect on lh $\beta$  and fsh $\beta$  transcript levels (Fig.5)*

248 Measurements of *lh $\beta$*  and *fsh $\beta$*  mRNAs were performed at each sampling time. Levels were,  
249 however, not detectable at the earliest sampling time, i.e. 2 and 4dph. At 6 dph, there were  
250 detectable in all samples but close to the limit of detection (mean Ct= 30.6) and at 8dph, both  
251 *lh $\beta$*  and *fsh $\beta$*  transcripts were detectable (mean Ct = 29.1). These results indicated an increase  
252 in *lh $\beta$*  and *fsh $\beta$*  expression during the larval development. When comparing *lh $\beta$*  and *fsh $\beta$*  gene  
253 expression at the different sampling time, no significant differences were detected between  
254 control and EE2 exposed groups (Fig. 5A and 5B).

255

## 256 **Discussion**

257

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

274

275 The *cyp19a1b* gene coding for the brain aromatase (also named Aromatase B), due to its  
276 remarkable sensitivity to oestrogens has been proposed as a biomarker of xenoestrogen

277 exposure both *in vivo* and *in vitro*. This ability to answer to oestrogens is given by the  
278 presence of estrogen responsive element (ERE) in the *cyp19a1b* promoter (Kazeto *et al.*,  
279 2001; Tong and Chung, 2003; Piferrer and Blázquez, 2005), a structure that appears  
280 conserved in different teleost species (zebrafish, catfish, fugu, goldfish, medaka and tilapia)  
281 (Diotel *et al.*, 2010).

282 From this postulate, we analyzed *cyp19a1b* expression in order to assess the experimental  
283 conditions of our experiment and the effects of EE2 in sea bass larvae. Our results  
284 demonstrated that, as in other species, EE2 exposure triggered an up-regulation of the  
285 aromatase B expression in sea bass larvae. Interestingly, at 2 and 4 dph the induction appears  
286 weaker than at 6 and 8 dph suggesting that sea bass early larval stages are less responsive to  
287 xenoestrogen exposure or that long-time exposure enhance *cyp19a1b* response. As an enzyme  
288 implicated in the neurosteroidogenesis, the aromatase B disruption may negatively influence  
289 the brain homeostasis. Furthermore, in fish, this enzyme is expressed in radial glial cells,  
290 which are neuronal progenitor cells, so disruption of these cells can affect several brain  
291 functions such as neurotransmission, neurogenesis or neuronal organization (Coumailleau *et al.*  
292 *et al.*, 2015; Diotel *et al.*, 2018; Nasri *et al.*, 2021).

293

#### 294 *Xenoestrogen effects on kisspeptin system*

295 Regarding kiss genes, the present results show an early expression of both *kiss1* and *kiss2*  
296 genes in sea bass larvae suggesting potential early roles during larval development, as  
297 demonstrated in medaka embryos where knockdown of zygotic *kiss1* induced disruption in  
298 brain development (Hodne *et al.*, 2013). In addition, we observed that EE2 exposure of young  
299 larvae induced a marked decrease in *kiss1* expression at 2 dph, an effect that was no longer  
300 observed in oldest larvae. In contrast, EE2 exposure did not change *kiss2* expression levels  
301 during the followed period. These effects appear in accordance with the results obtained by  
302 Escobar *et al.* (2013) in juvenile sea bass showing by double *in situ* hybridization that *kiss1*  
303 neurons located in the mediobasal hypothalamus express two types of nuclear estrogen  
304 receptor (ER $\alpha$  and ER $\beta$ 2), while no ER could be detected in *kiss2* neurons. Nevertheless,  
305 sensitivity to oestrogens of some *kiss1* and *kiss2* neurons populations has been observed in  
306 adult sea bass after ovariectomy and oestradiol replacement (Alvarado *et al.*, 2016) suggesting  
307 that kiss genes could also be regulated by oestrogens through indirect pathways. In teleosts,  
308 the situation concerning kiss neurons and oestrogen responsiveness is varying according to  
309 species. In adult medaka, the situation appears similar that the one we observed in sea bass  
310 with *kiss1* neurons expressing ER $\alpha$  and sensitive to oestrogens, while *kiss2* neurons are not

311 (Mitani et al, 2010). In juvenile zebrafish, an increase of both *kiss1* and *kiss2* expression is  
312 observed after oestrogen treatments (Servili et al, 2011). Similar observations have been made  
313 in female juvenile rare minnows, while in juvenile male both *kiss1* and *kiss2* expression  
314 decrease (Yang et al, 2016). In adult dabry's sturgeon and juvenile orange-spotted grouper,  
315 *kiss2* but not *kiss1* expression are affected by oestrogens (Guo et al, 2017; Yue et al, 2019).  
316 Finally, the study of kiss genes promoters in goldfish have permitted to identify partial motif  
317 of oestrogen response element (ERE) in both genes (Wang et al, 2013).

318 Altogether, the data collected in fish regarding oestrogen sensitivity of kiss genes suggest that  
319 xenoestrogen exposure could disrupt the ontogeny and normal functioning of the kisspeptin  
320 system. Nevertheless, it should be kept in mind that responses to oestrogen may vary  
321 according to the developmental stage of the individual. Our results demonstrated that  
322 xenoestrogens are able to transiently modulate *kiss1* expression in young sea bass larvae.  
323 Further studies are necessary to characterize the mode of action by, for example, locating the  
324 oestrogen receptors at this stage of development, and to shed light on the biological effects  
325 induced by these disruptions.

#### 326 *Xenoestrogen effects on GnRH system*

327 In sea bass, three GnRH isoforms have been characterized and their neuroanatomical  
328 distribution described (González-Martínez et al, 2002a, 2004). In the present study using  
329 qPCR assays, the diverse GnRH isoforms were detected in sea bass larvae since 2dph,  
330 contrasting with the results observed in a previous study using *in situ* hybridization where  
331 *gnrh2* expressing cells were firstly detected at 4dph, *gnrh3* at 7dph and *gnrh1* at 30dph  
332 (González-Martínez et al, 2002b). This discrepancy may result from differences in the  
333 sensitivity of the techniques used, but it should also be noted that in our study, the analyses  
334 were done on RNA extractions of pooled whole larvae. In any case, the expression of GnRH  
335 isoforms in sea bass larvae suggests roles for these genes during early stages of development,  
336 as demonstrated in zebrafish embryos where knockdown of *gnrh2* or *gnrh3* affect brain and  
337 eye development (Wu et al, 2006).

338 We investigated the effects of EE2 on the diverse GnRH isoforms and we observed inhibitory  
339 effects of EE2 exposure on *gnrh1* and *gnrh3* gene expression. In both cases, the effect was  
340 transient (observed at one stage and no longer at later stages) suggesting that compensatory  
341 mechanisms might occur at later larval stages. Regarding *gnrh2*, EE2 exposure did not induce  
342 any variation of the gene expression.

343 It is classically admitted that sex steroids exert positive and negative feedbacks on the

344 gonadotropic axis (Zohar et al, 2010), with different effects on GnRH system depending on  
345 the gene, the physiological stage and the species. In adult sea bass, a negative effect of  
346 oestrogens has been demonstrated on *gnrh1* expression after ovariectomy and oestradiol (E2)  
347 replacement (Alvarado et al, 2016). In contrast, in eels E2 stimulated *gnrh1* while it had no  
348 effect on *gnrh2* production (Montero et al, 1995). In immature tilapia, which possess three  
349 *gnrh* isoforms, E2 treatments induced an increase of *gnrh1* neurons and have no effect on  
350 *gnrh2* and *gnrh3* neurons (Parhar et al, 2000); and in prepubertal red seabream, E2 implants  
351 did not modify *gnrh1* expression (Okuzawa et al, 2002). Because of this sensitivity of *gnrh*  
352 system, some studies have raised the question of its potential disturbance by xenoestrogens in  
353 addition to other adverse effects (for review: León-Olea et al, 2014).

354 During early developmental stages, even if several studies have investigated the impact of  
355 EE2 exposure (Lange et al, 2008; Vosges et al, 2010; 2012; Bhandari et al, 2015; Porseryd et  
356 al, 2018; Leet et al, 2020; Nasri et al, 2021), only limited ones performed in zebrafish have  
357 focused on the oestrogens/xenoestrogens influence on GnRH. Vosges *et al.* (2010, 2012)  
358 observed that larval exposure to EE2 induced, through an ER signaling pathways, an increase  
359 in *gnrh3* forebrain neurons. Similar results have been obtained using molecules with  
360 oestrogeno-mimetic activities as nonylphenol in zebrafish larvae (Vosges et al, 2012) and  
361 bisphenol A and Bisphenol S in zebrafish embryos (Qiu et al, 2016).

362 Although the functions of the different *gnrh* in the developing brain are still not well known,  
363 they are expressed, their silencing induced developmental alterations and they are sensitive to  
364 disruptive action of xenoestrogens (for review: Vissio et al, 2021). Further studies should be  
365 conducted with animals followed over a long period to decipher the biological effects induced  
366 by these disruptions.

367

### 368 *Xenoestrogen effects on gonadotropin subunits LH $\beta$ and FSH $\beta$*

369 In vertebrates, gonadotropins (LH and FSH) are heterodimeric hormones composed of a  
370 common  $\alpha$  subunit and a hormone-specific  $\beta$  subunit. In the present study, our analyses of *lh $\beta$*   
371 and *fsh $\beta$*  subunits showed their low levels of expression in young sea bass larvae as they were  
372 only detected from 6 dph near the detection limits. These observations of a precocious  
373 expression of gonadotropin subunits during the embryonic and larval development are in  
374 accordance with results obtained in other species (for review: Weltzien et al, 2014).

375 Several studies have demonstrated the direct and indirect control exerts by sex steroids on  
376 gonadotropin expression, production and secretion (Aroua et al, 2007; Mazón et al, 2015;  
377 Fontaine et al, 2020). In sea bass, Mateos et al (2002) measured the expression of

378 gonadotropin subunits in E2 treated animals and observed an increase in *lhβ* and a decrease in  
379 *fshβ* mRNA levels. In addition, double label *in situ* hybridization demonstrated that both FSH  
380 and LH cells express ER transcripts in female adult sea bass (Muriach et al, 2008a; 2008b).  
381 Nevertheless, in the present study, we observed that EE2 exposure did not affect the  
382 expression of the two gonadotropin beta subunits in sea bass larvae. However, the situation  
383 differs in other species as in fathead minnow where 7 days exposure to EE2 (2, 5 and 50ng/L)  
384 during early life stages modify the gene expression of several genes including *lhβ* (Johns et al,  
385 2009), or in zebrafish where exposure of embryos for 5 days to bisphenol A or bisphenol S  
386 induced an increase in *lhβ* and *fshβ* expression (Qiu et al, 2016).  
387 Altogether, these results indicate that the pathways regulating gonadotropin beta expression  
388 might be stage and species specific. In the case of sea bass, our results indicate that at these  
389 larval stages, *lhβ* and *fshβ* subunits are not sensitive to oestrogenic molecules. Further studies  
390 investigating the localization of gonadotropins and ER expressing site in larval stages might  
391 help in the understanding of these responses.

392

### 393 **Conclusion**

394 Finally, our study demonstrated that sea bass larvae are sensitive to xenoestrogen exposure  
395 and that the ontogenesis of the gonadotropic system has been influenced by EE2. These  
396 disruptions occurring during the early larval stages could lead to deleterious effects in  
397 individuals such as alterations in brain organization, behavior or sexual differentiation. In sea  
398 bass, where the first signs of sexual differentiation are observed around 150 dph (Blázquez et  
399 al, 2008), a sex ratio deviation in favor of females has been shown after larval exposure (48  
400 dph to 88 dph) to EE2 through food (10mg/kg) (Blázquez et al, 1998). These effects could be  
401 the result of disturbances affecting different levels of the reproductive axis (brain, pituitary  
402 and gonads) and need to be further investigated.

403

404

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407

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723 **Tables**

724

725 *Table 1: Selected primers for qPCR analysis*

726

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

727

728

729

730

731 **Figure legends**

732

733 Figure 1

734 Daily mortality (%) of sea bass larvae during the experimental exposure to 17 $\alpha$ -  
735 ethinylestradiol (EE2).

736

737 Figure 2

738 Relative gene expression of *cyp19a1b* in sea bass larvae during the experimental exposure to  
739 17 $\alpha$ -ethinylestradiol (EE2). Results are presented as group average (average Pflaff ratio  $\pm$   
740 SEM) relative to control conditions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$

741

742 Figure 3

743 Relative gene expression of *kiss1* (A) and *kiss2* (B) in sea bass larvae during the experimental  
744 exposure to 17 $\alpha$ -ethinylestradiol (EE2). Results are presented as group average (average  
745 Pflaff ratio  $\pm$  SEM) relative to control conditions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$

746

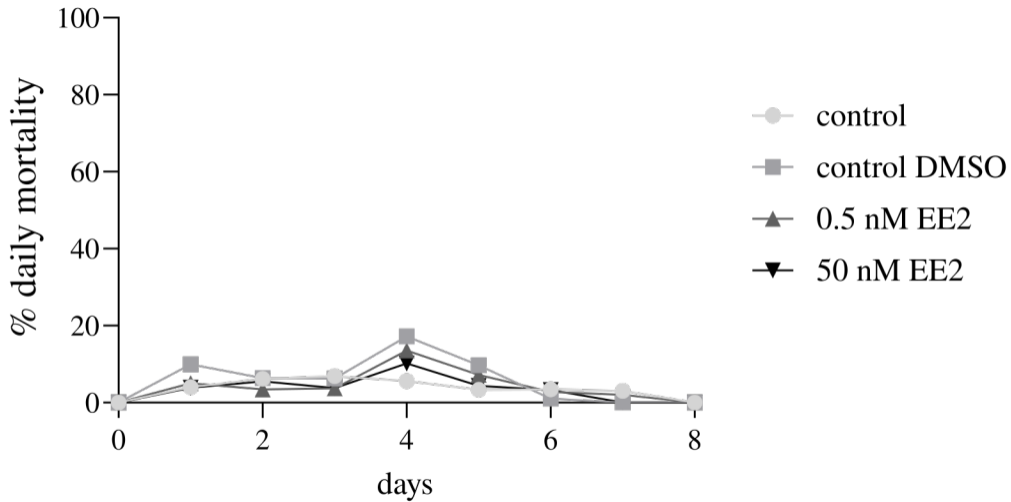
747 Figure 4

748 Relative gene expression of *gnrh1* (A) and *gnrh2* (B) and *gnrh3* (C) in sea bass larvae during  
749 the experimental exposure to 17 $\alpha$ -ethinylestradiol (EE2). Results are presented as group  
750 average (average Pflaff ratio  $\pm$  SEM) relative to control conditions. \*:  $p < 0.05$ ; \*\*\*:  $p < 0.001$

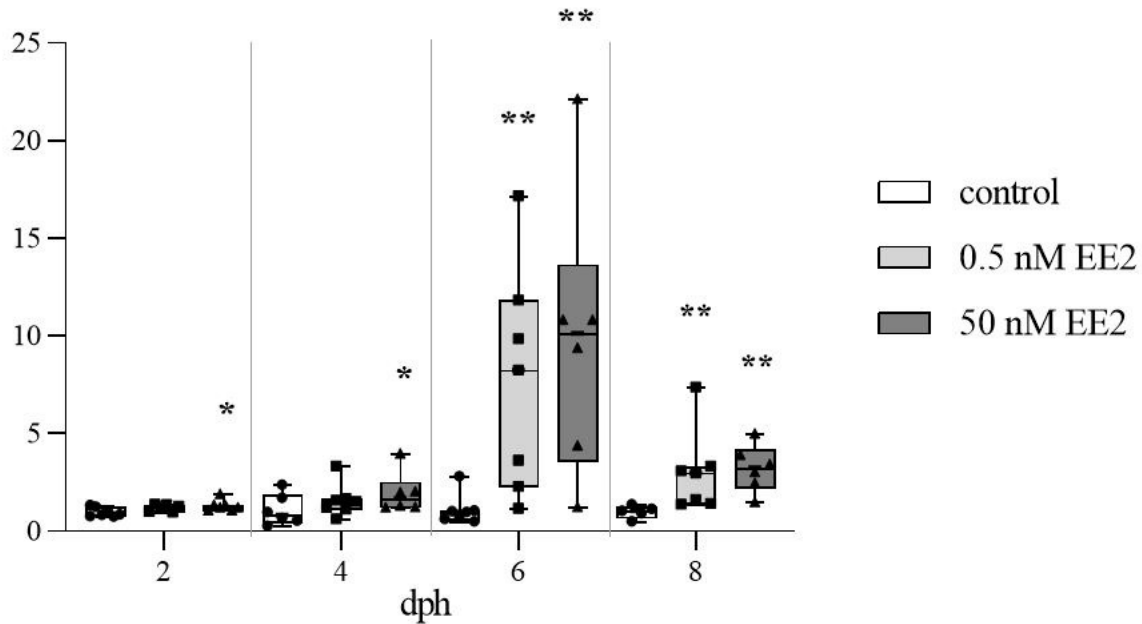
751

752 Figure 5

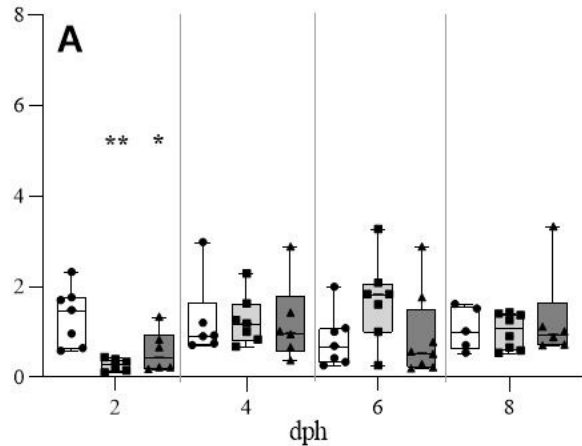
753 Relative gene expression of *lh $\beta$*  (A) and *fsh $\beta$*  (B) in sea bass larvae during the experimental  
754 exposure to 17 $\alpha$ -ethinylestradiol (EE2). Results are presented as group average (average  
755 Pflaff ratio  $\pm$  SEM) relative to control condition.



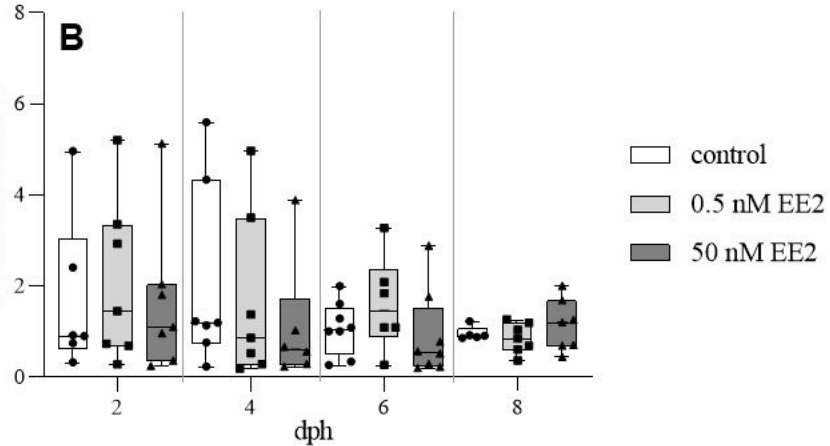
*cyp19a1b* expression ratio  
relative to control

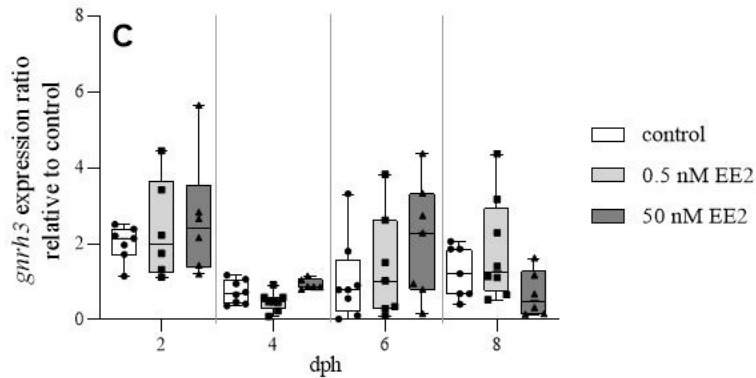
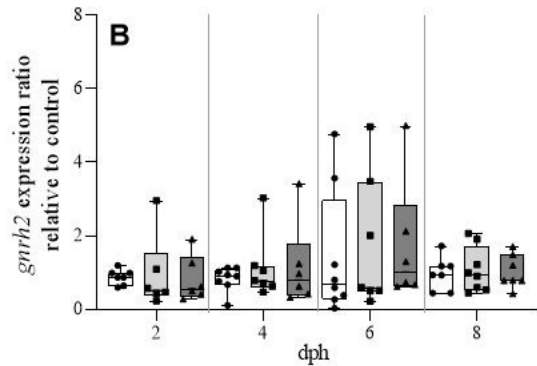
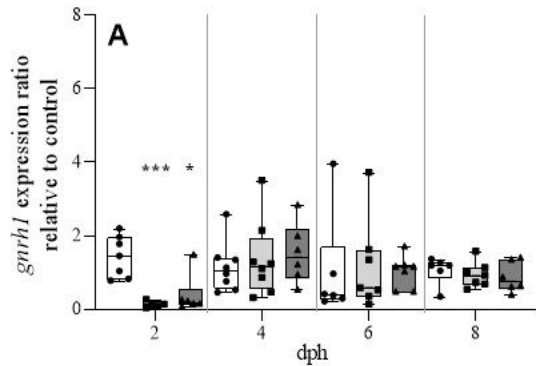


*kiss1* expression ratio  
relative to control

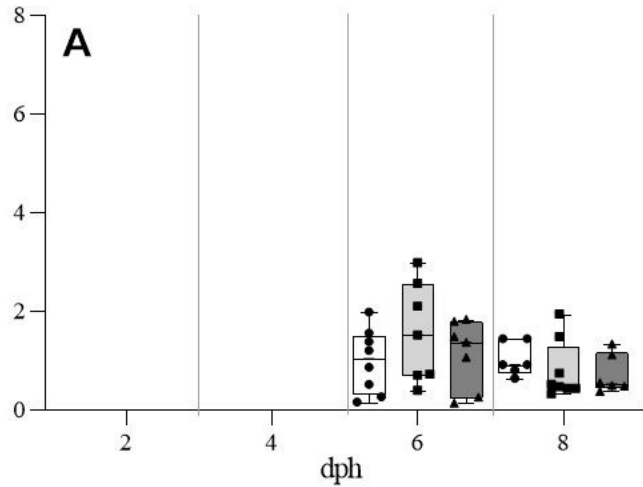


*kiss2* expression ratio  
relative to control





lh $\beta$  expression ratio  
relative to control



fsh $\beta$  expression ratio  
relative to control

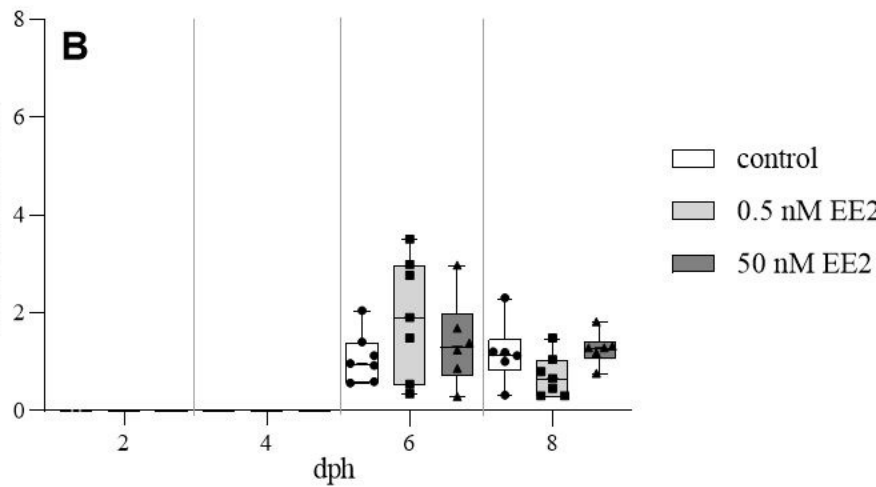






Figure 10.10: A 3D wireframe model of a mechanical assembly.



Figure 10.11: A 3D shaded model of a mechanical assembly.



Figure 10.12: A 3D wireframe model of a mechanical assembly.



Figure 10.13: A 3D shaded model of a mechanical assembly.