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1 **Effects of 17 $\alpha$ -ethinylestradiol on the neuroendocrine gonadotropic system and behaviour**  
2 **of European sea bass larvae (*Dicentrarchus labrax*)**

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18

19

20 **Declarations**

21

22 *Ethics approval and consent to participate:* All procedures were performed in accordance  
23 with the French and European legislation concerning the protection of animals used in  
24 experimentation. Procedures undertaken were approved (#10263-2017061911009684v3)  
25 by the regional ethical committee

26

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28

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31 PENELOP) and the InterReg RedPol project (Réduction de la pollution des perturbateurs  
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33

34 *Authors' contributions:* S.A. and S.S. designed the study; S.A., S.O. A.P., and S.A. carried  
35 out the experiment and S.S and C.J.A. performed data analyses. S.S, MP.H.L, C.J.A, C.M  
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43

44 *Data availability statement:* Raw data were generated at SEBIO. Derived data supporting the  
45 findings of this study are available from the corresponding author [A.S.] on request.

46

47

48 **Abstract**

49  
50

51 The widespread use of 17 $\alpha$ -ethinylestradiol (EE2), and other estrogenic endocrine disruptors,  
52 results in continuous release of estrogenic molecules into aquatic environments. Some studies  
53 documented that xenoestrogens can interfere with the neuroendocrine system of aquatic organ-  
54 isms and cause various adverse effects. In the present study, we exposed sea bass larvae to EE2  
55 (0.5 and 50 nM) during 8 days and investigated the expression of brain aromatase (*cyp19a1b*),  
56 gonadotropin-releasing hormones (*gnrh1*, *gnrh2*, *gnrh3*), kisspeptins (*kiss1*, *kiss2*) and estrogen  
57 receptors (*esr1*, *esr2a*, *esr2b*, *gpera*, *gperb*). Growth and larval behaviours (*i.e.* locomotor activ-  
58 ity and anxiety-like behaviours) were also investigated after 8 days of EE2 exposure and after a  
59 depuration period of 20 days. Exposure to 0.5 nM EE2 induced a significant increase in *cyp19a1b*  
60 expression. Concerning larvae exposed to 50 nM EE2, standard length measured at the end of  
61 the exposure phase was significantly lower if compared to control fish; however, this effect was  
62 no longer observed after the depuration phase. Moreover, the upregulation of *gnrh2*, *kiss1* and  
63 *cyp19a1b* expression was observed along with an increase in larval activity and anxiety-like be-  
64 haviours. Such behavioural effects were still detected at the end of the depuration phase. If main-  
65 tained over long term, these effects may impact normal development and fitness of exposed fish.

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67  
68  
69

**Keywords:** teleost, neuroendocrine disruption, anxiety-like behaviours, locomotor activity, gonadotropic axis

## 70 Introduction

71 Xenoestrogens (XEs) are a group of endocrine disrupting chemicals (EDCs) that mimic the  
72 actions of endogenous estrogens (Aris *et al.*, 2014). Several naturals and synthetics chemicals  
73 belong to this group such as phytoestrogens, drugs, pesticides and industrial by-products and  
74 their presence in aquatic environments is ubiquitous (Aris *et al.*, 2014; Zhang *et al.*, 2016). 17 $\alpha$ -  
75 ethinylestradiol (EE2) is a synthetic estrogen found for instance in the contraceptive pill, and is  
76 commonly used as a model molecule of XEs in toxicological studies (Aris *et al.*, 2014). EE2  
77 environmental levels are highly uneven, ranging from undetectable levels to 24 ng/L in Jiaozhou  
78 Bay (China) (Zhou *et al.*, 2011), 34 ng/L in Venice lagoon (Italy) (Pojana *et al.*, 2007) and up to  
79 1250 ng/L in French surface water (INERIS Report, 2020).

80 Fishes are exposed to a mixture of EDCs in their natural environment and have become the most  
81 commonly used model organisms in ecotoxicological studies of EDCs (Celino-Brady *et al.*,  
82 2021), with a preference for freshwater over marine species (OECD, 2022). Owing to the central  
83 role of estrogens in the regulation of vertebrate reproductive functions, studies on XEs have  
84 mainly focused on gonadal/reproductive effects such as altered fecundity, vitellogenin  
85 production, male/female population ratio and sexual behaviours (Jobling *et al.*, 2003; Matozzo  
86 *et al.*, 2008; Söffker and Tyler, 2012). For instance, *in vivo* experiments showed that a two-month  
87 exposure to 10 ng/L of EE2 was sufficient to induce aromatase expression in medaka testes and  
88 to reduce growth and reproductive performance in three-spined stickleback. Similarly, induction  
89 in vitellogenin production was observed in male zebrafish after a 21-day exposure to 10 ng/L  
90 EE2 (reviewed in: Aris *et al.*, 2014). Comparable data were obtained in the wild where exposure  
91 to estrogenic EDCs was shown to induce widespread sexual disruption in freshwater fish from  
92 English rivers (Jobling and Tyler, 2003). Finally, a 7-year controlled experiment in a lake showed  
93 that exposure of minnows to 5-6 ng/L of EE2 led to population collapse due to reproductive  
94 failure (Kidd *et al.*, 2007).

95 While the noxious effects of XEs on reproduction are well-reported in juvenile/adult fish, a  
96 different approach should be considered for young life-stages (e.g. larvae) which are described  
97 as particularly sensitive to EDCs but do not yet have a functional reproductive system (Patino,  
98 1997; Diamanti-Kandarakis *et al.*, 2009). Indeed, estrogens are not only reproductive hormones,  
99 they are also involved in other major processes such as early development or behaviour, and XEs  
100 exposure during early-life stages may not only affect the functioning but also the ontogenesis of  
101 targeted systems. For instance, in zebrafish, XEs larval exposure modulated the expression and  
102 activity of the cerebral aromatase (aroB) (Hinfray *et al.*, 2006; Le Page *et al.*, 2011), an enzyme  
103 catalyzing the conversion of androgens into estrogens and involved in functions such as brain

104 sexualization and sexual behaviours (Diotel *et al.*, 2010). In addition, other studies demonstrated  
105 the sensitivity during the early life stages of key factors involved in the reproductive axis, namely  
106 the gonadotropin-releasing hormones (GnRH) and kisspeptins (Kiss) (Vosges *et al.*, 2012;  
107 Soloperto *et al.*, 2022a). In zebrafish embryos, exposure to XEs disrupted the ontogenesis of the  
108 forebrain GnRH system (Vosges *et al.*, 2012) and in sea bass eleutheroembryos, we recently  
109 showed that gene expression of *aroB*, *kiss1*, *gnrh1* and *gnrh3* were affected by EE2 exposure  
110 (Soloperto *et al.*, 2022a). Such effects may have long-term consequences on the fitness of an  
111 organism.

112 Regarding behaviour, several endocrinological studies conducted on mice have demonstrated the  
113 role of estrogens and their receptors in the regulation of non-reproductive behaviours by  
114 knockout/knockdown technologies. These behaviours include social motivation (Imwalle *et al.*,  
115 2002), aggression (Trainor *et al.*, 2006), feeding and social learning (Clipperton *et al.*, 2008) and  
116 anxiety-related behaviours (Lund *et al.*, 2005). In fish, relative knowledge remains scarce  
117 (Amenyogbe *et al.*, 2020), however, exposure to synthetic estrogens was shown to modulate a  
118 wide range of behavioural traits (Aris *et al.*, 2012; Celino-Brady *et al.*, 2021). For instance,  
119 exposure to EE2 (3-10 ng/L) induced an increase in anxiogenic behaviours (e.g. increased bottom  
120 dwelling or decreased boldness) in male guppies and zebrafish (Hallgren *et al.*, 2011; Porseryd  
121 *et al.*, 2017), effects that are persistent and even observed in unexposed zebrafish offspring  
122 (Volkova *et al.*, 2015). In addition, an increase in shoal cohesion and aggressiveness was  
123 described in zebrafish after long-term exposure to EE2 (3-10 ng/L) (Reyhalian *et al.*, 2011;  
124 Porseryd *et al.*, 2017). Finally, few hours exposure to EE2 (>15ng/L) altered male-to-male and  
125 male-to-female interactions in fighting fish (Dzieweczynski and Buckman, 2013; Forette *et al.*,  
126 2015). Interestingly, different authors have suggested (Söffker and Tyler, 2012; Melvin and  
127 Wilson, 2013) that behavioural biomarkers could be considered as sensitive as well-established  
128 biomarkers (e.g. vitellogenin production or developmental endpoints), as they both occur at  
129 similar concentrations. Nevertheless, most of the knowledge on EE2-mediated behavioural  
130 disruption has been obtained in model freshwater species, and it is important to determine  
131 whether these behavioural disruptions also occur in non-model organisms.

132  
133 The European sea bass (*Dicentrarchus labrax*) is a marine teleost, native to European and North  
134 Africa coastlines (Bagni, 2020). This species has high cultural and economic value (Sánchez  
135 Vázquez and Muñoz-Cueto, 2014) and it appears as a promising test species representative of  
136 European coastal and pelagic environments (Conti *et al.*, 2015; Soloperto *et al.*, 2022 b).  
137 However, only few toxicological studies have focused on sea bass early life stages, even though

138 early development is particularly sensitive to chemical exposure (Hutchinson *et al.*, 1998).  
139 Indeed, throughout this period, the larvae undergo major morphological changes and the  
140 ontogenesis of several physiological systems is still ongoing (Barnabe *et al.*, 1976; Schilling,  
141 2002).

142  
143 In this study, we investigated the effects of EE2 on sea bass larvae after exposure to sublethal  
144 concentrations. For that, two concentrations of EE2 were chosen: 50 nM (15 µg/L), which aimed  
145 at identifying mechanisms and targets of EE2 toxicity, and 0.5 nM (150 ng/L) that can be  
146 considered as representative of severely polluted environments. The aim was to evaluate  
147 responses at different biological levels from molecular changes to behavioural effects. Endpoints  
148 analysed include standard length, expression of genes involved in the hypothalamus-pituitary-  
149 gonads (HPG) axis and fitness-related behaviours.

150

## 151 **Material and methods**

### 152 *Test species*

153 European sea bass larvae ( $20 \pm 2$  days post hatching) were purchased from a local hatchery (Les  
154 Poissons du Soleil, Balaruc les Bains, France) and transferred to the laboratory. For  
155 acclimatization to laboratory conditions, larvae were distributed into 10 L aquaria filled with  
156 filtered and aerated natural seawater (35 psu) at a density of 10 fishes per litre. Temperature was  
157 maintained at  $17.0 \pm 0.3$  °C and the photoperiod fixed at 12h light / 12h dark. These parameters  
158 were the same during both acclimatization and experiments. Larvae were fed twice a day with  
159 fish powder (néo supra S, Le Gouessant aquaculture, France) supplemented with alive artemia  
160 nauplii (A0). All procedures were performed in accordance with the French and European  
161 legislation on the protection of animals used in experimentation. Procedures undertaken were  
162 approved (#10263-2017061911009684v3) by the regional ethical committee (CENOMEXA;  
163 agreement number 54).

164

### 165 *Experimental design*

166 Two independent experiments were carried out in October 2019 (experiment 1) and March 2021  
167 (experiment 2) to study the effects of 17 $\alpha$ -ethinylestradiol (EE2, purity 99 %, Sigma–Aldrich,  
168 France) on sea bass. Larvae were exposed from 30 to 38 ( $\pm 2$ ) dph.

169 After preparation of a stock solution of EE2 ( $10^{-3}$  M) in ethanol, two test solutions (1 nM and  
170 100 nM) were made by serial dilutions in filtered natural seawater (35 psu). In experiment 1,  
171 larvae were dispatched in four experimental groups: 0.5 nM EE2 (150 ng/L), 50 nM EE2 (15

172  $\mu\text{g/L}$ ), ethanol (0,0005% v/v) or seawater only. As no difference was found between the two  
173 control groups in experiment 1, and to conform to the 3Rs framework (Replacement-Reduction-  
174 Refinement), only one control group was used in experiment 2: the ethanol group. To facilitate  
175 the reading, data from experiment 2 are presented below while data from experiment 1 are  
176 supplied as supplementary and supportive data (supplementary I).

177 The experimental protocol used for both experiments was divided into two phases: Phase I  
178 corresponding to an 8-day exposure period and Phase II corresponding to a 20-day depuration  
179 period (Fig. 1). On day 1 (1<sup>st</sup> day Phase I), larvae were exposed in duplicate aquaria (100 larvae  
180 per aquaria) to the different test solutions under semi-static conditions. As the half-life of EE2 is  
181 approximately  $33\pm 13$  h (Nagpal and Meays, 2009), half of the medium of each aquarium was  
182 renewed every 48h with the corresponding test solution, in accordance with the protocol followed  
183 by Vosges *et al.*, (2012). On day 8 (1<sup>st</sup> day Phase II), larvae were transferred into natural seawater  
184 (devoid of EE2) for 20 days (phase II). Half of the seawater was renewed every 48h.

185 During the experiment, aquaria were daily observed to monitor larval mortality.

186 Samples for qPCR analysis were collected at day 4 and day 8. Larvae (n = eight pools of two  
187 larvae per group) were euthanized using a solution of 250 mg/L of tricaine methanesulfonate  
188 (MS-222, Sigma–Aldrich, France), quickly frozen into liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until  
189 analysis. For behavioural analyses, naive larvae (n=12 per group) were randomly sampled and  
190 recorded at day 8 (end of Phase I) and day 28 (end of Phase II). After completion of behavioural  
191 testing, larvae were euthanized in MS-222 and pictures were taken using Leica MZ 10F  
192 microscope coupled with a Leica DFC425 C camera for standard length (SL) measurement.  
193 Standard length (n=10) was measured with ImageJ (1.8.0\_172). Growth rate calculated for the  
194 phase II of the experiment using the following formula: [(average SL day 28 – average SL day  
195 8)/20 (days)].

196

### 197 *Gene expression*

198 Analyses were performed on the heads of the larvae. Heads were dissected with a sterile razor  
199 blade between the operculum and the pectoral fins and homogenised in a Soft Tissue  
200 Homogenizer CK14 (Bertin, Montigny-le-Bretonneux, France). Total RNA was extracted in  
201 Trizol Reagent (Invitrogen, Illkirch, France) according to the manufacturer's instructions.  
202 Following extraction, samples were treated with TURBO DNA-free kit (Invitrogen) and the first  
203 strand of cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using Superscript Reverse Transcriptase  
204 III and random hexamers (Invitrogen).



205 Selected primers (Table 1) were ordered from Invitrogen (Thermo Fisher Scientific, US). qPCR  
206 analyses were performed using a Rotor Gene Q device (Qiagen, France). The qPCR mix  
207 consisted of 5µl of QuantiTect SYBR Green Master Mix (Qiagen), 1.5µl of diluted cDNA  
208 template (1:10) and forward/reverse primers (500 nM each) in a final volume of 10µl. The qPCR  
209 reactions were performed under the following conditions: 15 minutes at 95 °C activation, 15  
210 seconds at 94°C denaturation, 25 seconds at 57-60°C depending on the target gene (Table 1)  
211 annealing, and 25 s at 72°C elongation, with the last 3 steps repeated 35 times.

212 EFA, L13 and RPS14 were tested and validated as reference genes by comparing their stability  
213 using Bestkeeper software (© 2004 M. W. Pfaffl). Their geometric mean was used as reference.  
214 For each gene, a serial dilution (1:5) of cDNA prepared from a pool of cDNA from all conditions  
215 tested was analysed to determine the efficiency of the qPCR reaction. For each PCR run, samples  
216 were analysed in duplicate, and a non-template control was added to confirm the absence of  
217 contamination. A calibrator sample (pool of three randomly selected samples per group) was  
218 added in each run to adjust for assay to assay variations. The relative expression was calculated  
219 using the following formula:

$$220 \text{ relative expression} = Et^{Ct \text{ target (calibrator)} - Ct \text{ target (sample)}} \times Er^{Ct \text{ reference (sample)} - Ct \text{ reference (calibrator)}}$$

222 ***Et*** = efficiency of target gene; ***Er*** = efficiency of reference gene; ***Ct*** = cycle threshold

223 Results are presented as fold-change relative to the control group (i.e. relative expression target  
224 gene in group Y / relative expression target gene in control group).

225

### 226 *Behaviour analyses*

227 Behavioural analyses were performed on larvae at day 8 and day 28. Larvae were individually  
228 placed into a circular arena (glass beaker, diameter= 6 cm, height = 3 cm) filled with natural  
229 seawater (water volume = 50 ml). Arenas (four recorded at a time) were placed in a controlled  
230 light chamber with infrared back light and a video camera (Sony® FDR-AX53) placed 50 cm  
231 above. After 5 min of acclimatisation, larvae were video recorded for 10 min.

232 Video recordings were analysed using a video tracking software (EthoVision XT 10, Noldus®).  
233 Each arena was virtually divided into a central (½ well diameter) and a peripheral zone.  
234 Behavioural endpoints included: zone preference, distance travelled, time spent swimming, total  
235 number of rotations, maximal speed and acceleration. The total time spent swimming and the  
236 total distance travelled are common indicators of the locomotor activity level (Zhang et al.,  
237 2013). On the other side, an increase in (i) the time spent in the peripheral zone, (ii) the distance

238 travelled in the peripheral zone and/or (iii) the number of rotations performed are common  
239 indicators of anxiety-like behaviours.

240

#### 241 *Statistics*

242 Statistical analyses were done on GraphPad Prism (8.0.2) and R (4.1.1).

243 Effects of time (i.e. number of exposure days) and group (i.e. experimental group) on daily  
244 mortality were examined using generalized linear models fitted with a binomial distribution  
245 (GLM: *glm* function). Model selection for the GLM analyses was based on Akaike information  
246 criterion (Supplementary II). Simultaneous Tests for General Linear Hypotheses using Tukey  
247 Multiple Comparisons of Means were run as post-hoc tests.

248 For growth, gene expression and behaviour analyses, results were analysed using one-way  
249 ANOVA for data meeting the assumptions of normality (Shapiro-Wilk test) and equal variances  
250 (Brown-Forsythe test). Tukey's multiple comparisons test was used as post-hoc. For data that did  
251 not meet the equal variances assumption, Welch ANOVA tests were used with Games-Howell's  
252 multiple comparisons test as post-hoc. Data that did not meet the normality assumption were  
253 analysed with Kruskal-Wallis test, using Dunn's test as post-hoc.

254 Regarding behaviour, preliminary analyses were run to investigate the effects of different  
255 experimental variables (i.e. recording order, time of day) on behavioural endpoints. Pearson  
256 correlations showed that none of these experimental variables had a significant impact on the  
257 endpoints analysed (Supplementary III).

258

## 259 **Results**

260

### 261 *Survival rate*

262 During the experiment, the number of dead larvae was reported every day, and for all groups the  
263 survival curves were similar (Fig.2).

264 *Phase I:* At day 8, survival corresponded to 97% in the control group and 98% for both EE2  
265 groups. EE2 treatment had no significant impact on daily mortality ( $p = 0.87$ ) and no significant  
266 interaction was found.

267 *Phase II:* At day 28, survival reached 92 % in the control group and 93 % in the 0.5 nM EE2  
268 group, and 84% in the 50 nM EE2 group. Nevertheless, no significant difference in survival was  
269 detected among the three groups. The variable that best explained survival rate was time ( $p <$   
270  $0.001$ ), followed by treatment ( $p = 0.09$ ), with no significant interaction between these factors.

271

272 *Growth*

273 Standard length (SL) was measured on day 8 and day 28 (controls: 1.13 cm and 1.23 cm; 0.5 nM  
274 EE2: 1.15 cm and 1.23 cm; 50 nM EE2: 1.06 cm and 1.20 cm), and a significant difference was  
275 observed between the control and the 50 nM EE2 group ( $p = 0.044$ ) on day 8 (Fig.3).

276 Daily growth rates calculated during phase II (controls: 0.047 mm/day; 0.5 nM EE2: 0.041  
277 mm/day; 50 nM EE2: 0.071 mm/day) indicated faster growth in the 50 nM EE2 group (x 1.51  
278 compared to controls), suggesting the occurrence of compensatory growth.

279  
280 *Gene expression*

281 To evaluate the potential neuroendocrine effects of EE2 at a brain level, the expression of several  
282 genes was measured at day 4 and at day 8.

283 *Brain aromatase*: At both sampling times a significant upregulation of *cyp19a1b* was observed  
284 in EE2 groups compared to controls (Fig. 4A). After 4 days, mRNA levels for *cyp19a1b* showed  
285 a 3.0-fold increase in the 0.5 nM EE2 group ( $p = 0.0014$ ) and a 2.9-fold increase ( $p = 0.0035$ ) in  
286 the 50 nM EE2 group. This upregulation was confirmed at day 8 (0.5 nM EE2: 3.7-fold increase,  
287  $p = 0.0072$ ; 50 nM EE2: 7.1-fold increase,  $p = 0.0009$ ).

288 *Kisspeptins*: The gene expression of *kiss1* and *kiss2* was analysed. Whatever the sampling time,  
289 no difference was detected in *kiss1* expression between the control and the 0.5 nM EE2 groups.  
290 In contrast, a significant upregulation in *kiss1* expression was observed between the control and  
291 the 50 nM EE2 groups at day 8 (1.8-fold increase,  $p = 0.04$ ) (Fig. 4B).

292 No difference was observed in *kiss2* expression levels among groups (Fig. 4C).

293 *Gonadotropin-releasing hormones*: The expression of the three genes *gnrh1*, *gnrh2* and *gnrh3*  
294 was analysed. For both *gnrh1* and *gnrh3* mRNA levels, no statistical differences were observed  
295 between the groups at either time point (Fig. 4D and 4F).

296 For *gnrh2* mRNA levels, while there was no difference between the control and 0.5 nM EE2  
297 groups, a significant increase was observed between the control and 50 nM EE2 groups at day 4  
298 (2.3x;  $p = 0.03$ ) and day 8 (3.9x;  $p = 0.04$ ) (Fig. 4E).

299 *Estrogen receptors*: mRNA levels for the three nuclear (*esr1*, *esr2a* and *esr2b*) and the two  
300 membrane (*gpera* and *gperb*) estrogen receptors were analysed. Similar profiles were observed  
301 for *esr1* and *esr2a* mRNA levels, with a decreasing trend in both EE2-exposed groups at day 4  
302 (*esr1*: 0.5 nM - 0.77x, NS; 50 nM - 0.46x, NS; *esr2a*: 0.5 nM - 0.76x, NS; 50 nM - 0.38x, NS)  
303 and at day 8 (*esr1*: 0.5 nM - 0.57x, NS; 50 nM - 0.62x, NS; *esr2a*: 0.5 nM - 0.83x, NS; 50 nM -  
304 0.65x, NS) although no significant differences were observed. Regarding *esr2b*, no variation was  
305 observed between groups.

306 For membrane receptors, no significant difference was detected, although a small decrease in  
307 mRNA levels could be observed in EE2 groups for *gpera* at day 4 (0.5 nM: 0.42x, *NS*; 50 nM:  
308 0.33x, *NS*) and *gperb* at day 8 (0.5 nM: 0.67x, *NS*; 50 nM: 0.58x, *NS*).

309

### 310 *Behavioural analyses*

311 To assess the effects of XEs on behaviour, larvae were tested at day 8 (at the end of Phase I) and  
312 at day 28 (at the end of Phase II). Behavioural indicators of locomotor activity level and anxiety-  
313 like behaviours were analysed. Detailed results are presented for each Phase in Supplementary  
314 data IV.

315

#### 316 *Phase I:*

317 Regarding indexes of locomotor activity, we observed an increasing trend in the *time spent*  
318 *swimming* in EE2 exposed groups. While the difference was not significant for the 0.5 nM EE2  
319 group (2x, *NS*), a significant increase was observed for the 50 nM EE2 group as compared to  
320 control (2.6x,  $p = 0.016$ ) (Fig. 5A). A similar profile was observed for the *total distance travelled*  
321 with a non-significant increase in the 0.5 nM EE2 group (2.1x, *NS*) and a significantly longer  
322 distance swam for larvae in the 50 nM EE2 group (2.5x;  $p = 0.03$ ) as compared to control (Fig  
323 5B). In contrast, the *maximal speed* (cm/s) and the *maximal acceleration* (cm/s<sup>2</sup>) were not  
324 affected by exposure to EE2 (data not shown).

325 In addition, three different parameters were measured as indexes of anxiety-like behaviours: *time*  
326 *spent in the periphery*, *distance travelled in the periphery* and *number of rotations*.

327 In general, whatever the group, the larvae preferred to stay in the peripheral zone rather than in  
328 the central zone of the arena (average time spent in the periphery = 90 %) (Fig. 6A). No difference  
329 in *time spent in the periphery* was observed between the control and 0.5 nM EE2 groups, while  
330 the 50 nM EE2 group spent significantly more time (1.2x,  $p=0.004$ ) in the periphery of the arena  
331 than the control.

332 Similar results were observed for the parameters *distance travelled in the periphery* (Fig 6B) and  
333 *number of rotations* (Fig 6C). No significant differences were observed between the control and  
334 0.5 nM EE2 groups. In contrast, larvae in the 50 nM EE2 group swam a significantly longer  
335 distance (2.8x;  $p = 0.044$ ) and had a significantly higher number of rotations (3.3x,  $p = 0.033$ )  
336 than larvae in the control group.

337

#### 338 *Phase II:*

339 At day 28, indexes of locomotor activity were affected by the exposure to EE2. For both  
340 parameters, namely *time spent swimming* and *total distance travelled*, similar trends were  
341 observed. While non-significant increases were observed in 0.5 nM EE2 group (*time spent*  
342 *swimming*: 1.7x, NS; *total distance travelled*: 1.69x, NS), the increases became significant in the  
343 50 nM EE2 group as compared to control (*time spent swimming*: 2.2x,  $p = 0.041$ , Fig 5A; *total*  
344 *distance travelled*: 2.38x,  $p = 0.010$ , Fig. 5B). As observed in Phase I, neither *maximal speed* nor  
345 *maximal acceleration* differed between groups (data not shown).

346 Regarding anxiety-like behaviours, in all groups, larvae preferred the peripheral zone over the  
347 central zone of the arena (average time spent in the periphery = 91 %) (Fig. 6A). Comparison of  
348 the *time spent in the periphery* showed no significant difference between groups.

349 For the *distance travelled in the periphery*, an increasing trend was observed at the end of the  
350 depuration phase (Fig. 6B). This increase was significant for the 50 nM EE2 group (2.5x,  
351  $p=0.007$ ) but not for the 0.5 nM EE2 group (1.7x, NS) as compared to control.

352 Regarding the *number of rotations*, it was higher in the 0.5 nM EE2 group (2.4x, NS) and  
353 significantly higher in the 50 nM EE2 group (3.7x,  $p = 0.002$ ) as compared to control (Fig. 6C).

354

## 355 **Discussion**

356 Exposure to endocrine disruptors during early development can have adverse effects on exposed  
357 organisms (Diamanti-Kandarakis *et al.*, 2009). In order to assess the effects of EE2 on European  
358 sea bass larvae, different biological and behavioural parameters were measured during and after  
359 an exposure period (Phase I) and following a depuration period (Phase II).

360

### 361 *Effects of EE2 exposure on larval survival and growth*

362 In the present study, sea bass larvae were exposed to 0.5 nM EE2 (150 ng/L) or 50 nM EE2 (15  
363  $\mu\text{g/L}$ ) and survival was followed throughout the experiment. Good survival rate was recorded in  
364 each group and no difference was detected in daily mortality among groups confirming that  
365 larvae were exposed to sublethal concentrations of EE2. The impact of EE2 on fish survival has  
366 been investigated in different studies. While no effect was observed in fathead minnow after  
367 short- or long-term exposure to 10 or 64 ng/L of EE2 (Länge *et al.*, 2001; Van Aerle *et al.*, 2002),  
368 adverse effects were noted in juvenile sea bass exposed via the trophic route to 12.5mg/kg food  
369 (Gorshkov *et al.* 2004) and in adult male minnow exposed during a year to 3ng/L of EE2  
370 (Schwindt *et al.*, 2014). These results suggest that exposure to EE2 induces varying effects on  
371 individual survival depending on the species, the dose, the timing and the duration of exposure.

372 In fish, growth is rather “plastic” and depends on environmental and endogenous factors  
373 (Weatherley, 1990). In our study, the results showed that at the end of the exposure period, larvae  
374 exposed to 50 nM EE2 had a lower standard length than controls (consistent in both experiments,  
375 Supplementary Data I-a), a difference that was no longer observed after the depuration period.  
376 This indicated that compensatory growth occurred after the stressor EE2 was removed.  
377 Both stimulatory and inhibitory effects of EE2 were reported on fish growth. For instance, in  
378 juvenile yellow catfish, exposure to EE2 induced an increase in growth rate (Chen et al., 2017)  
379 while in subadult fathead minnow, it induced a decrease in standard length (Länge et al., 2001).  
380 Regarding compensatory growth, it was observed in zebrafish (Xu et al., 2008 and mangrove  
381 rivulus (*Kryptolebias marmoratus*) (Voisin et al. 2016) following exposure to EE2 and a recovery  
382 period. In contrast, Gorshkov et al. (2004) did not find this in juvenile sea bass. The timing (age  
383 of exposure), the intensity and duration of the stress period appear to be important factors leading  
384 to the occurrence of compensatory growth (Hector and Nakagawa 2012).

385

#### 386 *Effects of EE2 exposure on the gonadotropic axis components*

387 In vertebrates, the establishment of the neuroendocrine system takes place during early life  
388 stages. An impairment or a disequilibrium during this period may impact the development and  
389 functioning of the neuroendocrine system in the young and adult stages (Diamanti-Kandarakis *et*  
390 *al.*, 2009; Vissio *et al.*, 2021). To assess the effects of EE2 on the establishment of some key  
391 components of the neuroendocrine gonadotropic axis in sea bass, gene expression of brain  
392 aromatase, kisspeptins, gnrhs and estrogen receptors was analysed.

393 Aromatase b, encoded by the *cyp19a1b* gene, is an enzyme involved in estrogen synthesis. This  
394 enzyme is expressed in radial glial cells (Le Page et al. 2011). An increase in *cyp19a1b*  
395 expression after exposure to XEs was reported in various teleost species (zebrafish: Le Page et  
396 al. 2006; medaka: Kuhl et al., 2005; rare minnow: Wang et al. 2010) and our results in sea bass  
397 larvae are in agreement with these findings. In fish, aromatase b and neuroestrogens have been  
398 described to play key roles in brain sexual differentiation, cell proliferative activity, sexual  
399 behaviours and might also be involved in sensory perception (Diotel et al., 2010 ; Shaw, 2018).  
400 Consequently, deregulation of *cyp19a1b* transcription by XEs can have an impact on all these  
401 functions/processes mentioned above. Further investigations are necessary to unravel the long-  
402 term consequences of such disruption of *cyp19a1b* during the development. The potential link  
403 between *cyp19a1b* upregulation and the alteration of non-reproductive behaviours is discussed  
404 below.

405

406 Kisspeptins are considered essential regulators of the HPG axis in vertebrates, responding  
407 directly to estrogenic stimuli. While this process is well documented in mammals, different  
408 situations have been observed in teleosts, with species possessing either one or two *kiss* genes  
409 (Pasquier *et al.*, 2014). In sea bass, two genes have been identified (Felip *et al.*, 2009) and in the  
410 present study, both *kiss1* and *kiss2* gene expression were analysed.

411 Firstly, we observed that both genes are expressed during larval development. In addition, we  
412 detected an upregulation of *kiss1* expression after EE2 exposure while *kiss2* expression was not  
413 modified (observed in experiment 1 and 2 Supplementary data I-b, with minor inconsistencies  
414 that can be associated to interindividual variance). This action of EE2 on *kiss* gene expression  
415 may be related to the presence of nuclear estrogen receptors (ER). Indeed, in the brain of adult  
416 sea bass, Escobar *et al.* (2013) demonstrated by double *in situ* hybridization that *kiss1*-expressing  
417 neurons located in the mediobasal hypothalamus co-express *esr1* and *esr2a*. In contrast, no co-  
418 expression with ER was observed in *kiss1* neurons of the habenula, nor in the *kiss2* neurons.

419 These results suggest a potential direct action of estrogens in *kiss1* regulation, in accordance with  
420 our findings. Moreover, upregulation of *kiss1* has already been reported in fish species such as  
421 medaka and zebrafish after exposure to estrogens (Mitani *et al.*, 2010; Servili *et al.*, 2011) or  
422 xenoestrogens (Faheem *et al.*, 2019; Thayil *et al.*, 2020), and we already observed it in sea bass  
423 eleutheroembryos (Soloperto *et al.*, 2022.a). Regarding *kiss2*-expressing cells, estrogens seem to  
424 not directly regulate *kiss2* expression in the adult sea bass as cells do not express ER (Escobar *et al.*,  
425 2013) and as no variation in *kiss2* expression was observed after gonadectomy and estradiol  
426 replacement (Carrillo *et al.*, 2016). Our current results in larvae, like those obtained previously  
427 in eleutheroembryos (Soloperto *et al.*, 2022.a), confirm that exposure to XEs does not affect *kiss2*  
428 expression in sea bass. Nevertheless, in some fish species as zebrafish or sturgeon, *kiss2*  
429 expression was stimulated after estradiol treatment (Servili *et al.*, 2011; Yue *et al.*, 2019).

430 Disruption of *kiss* expression can lead to different effects. In sea bass, some studies have  
431 suggested the involvement of the *kiss2/gnrh1* system in the control of gonadotropic activity  
432 (Espigares *et al.*, 2015), and of the *kiss1/gnrh2* system in the connection between the integration  
433 of environmental stimuli (*i.e.* photoperiod) and the onset of puberty (Espigares *et al.*, 2017).  
434 Studies carried out during early developmental stages led to somewhat different results, while  
435 normal development was observed in *kiss1* and *kiss2* knockout zebrafish (Tang *et al.*, 2015), in  
436 medaka, *kiss1* knockdown induced major disruption of embryonic and brain development  
437 (Hodne *et al.*, 2013). Therefore, XEs exposure can lead to different adverse effects depending on  
438 the species and the exposure windows. In the case of the European sea bass, further research is

439 needed to identify the developmental roles and potential adverse effects exerted by a disruption  
440 of *kiss* expression.

441 Regarding the GnRH system, teleost display two or three different GnRH isoforms. All of them  
442 are detectable from early life stages, suggesting a role in larval development (Muñoz-Cueto *et al.*,  
443 *et al.*, 2020). In sea bass, three *gnrh* genes have been identified and their ontogenesis described  
444 (Gonzales-Martinez *et al.*, 2002b). In the present study, the influence of EE2 exposure on the  
445 different isoforms was investigated, showing that the expression of *gnrh1* and *gnrh3* is not (or  
446 not consistently) impacted (Supplementary data I-b). For *gnrh2*, an up-regulation in larvae  
447 exposed to 50 nM EE2 was observed (experiment 1 and 2, Supplementary I-b). Interestingly, in  
448 our previous study on eleutheroembryos, the effects were different with variation in the  
449 expression of the *gnrh1* and *gnrh3* genes but not the *gnrh2* gene (Soloperto *et al.*, 2022.a). So  
450 far, only a few studies have investigated the effects of XEs on GnRH in fish (Le Page *et al.*,  
451 2011). In zebrafish, exposure to EE2 or nonylphenol has been shown to alter the number, the size  
452 and the migration pattern of GnRH3 neurons (Vosges *et al.*, 2012). Exposure to other known  
453 XEs as bisphenol A and bisphenol F have been shown to altered *gnrh3* and *gnrh2* expression in  
454 rare minnow (Qin *et al.*, 2013) and *Catla Catla* (Faheem *et al.*, 2019) respectively, and to impair  
455 GnRH3 neuronal development in medaka (Inagaki *et al.*, 2016) and zebrafish (Qiu *et al.*, 2019).  
456 Further in medaka, embryonic exposure to EE2 triggered an increase in *gnrh1* mRNA levels  
457 while no effects were reported on *gnrh2* (Thayil *et al.*, 2020).

458 The effects of XEs on GnRH neurons can be mediate through an ER pathway, as demonstrated  
459 in zebrafish (Vosges *et al.*, 2012). In adult sea bass, the brain distribution of ER has been  
460 described (Muriach *et al.*, 2008a; 2008b) showing that *esr1a* and *esr2b* expression was detected  
461 in the same cerebral area as *gnrh2*-expressing cells, and that *esr2a* expression was detected where  
462 *gnrh1*- and *gnrh3*-expressing neurons are located (Gonzales-Martinez *et al.*, 2002a; 2002b).  
463 Anyhow, there are contrasting results on the expression of ER in GnRH neurons in teleost, with  
464 co-expression found in some species as tilapia and medaka, but not in others as silverside or  
465 rainbow trout (Navas *et al.*, 1995; Le Page *et al.*, 2011; Ogawa and Parhar, 2020; Macedo-Garzón  
466 *et al.*, 2021). Furthermore, it is of interest to note that estrogen binding sites were detected in the  
467 promotor regions of the *gnrh* gene in zebrafish and sea bream, supporting the idea that estrogen  
468 can directly modulate GnRH expression (Torgersen *et al.*, 2002; Hu *et al.*, 2008).

469 The different functions exert by the GnRH isoforms have been recently summarized by Muñoz-  
470 Cueto *et al.* (2020). In fish having three GnRH isoforms, the preoptic area GnRH1 is defined as  
471 the hypophysiotropic form, the other forms are described as neuromodulators with GnRH2  
472 potentially regulating circadian activities, feeding and reproduction, and GnRH3 potentially



473 involved in sexual behaviours (Muñoz-Cueto *et al.*, 2020). Regarding possible developmental  
474 roles, a knockdown study on zebrafish showed that the lack of either GnRH2 or GnRH3 altered  
475 the regionalization of the brain and the formation of eye cup (Wu *et al.*, 2006). Therefore, all  
476 these functions could be affected by disruptions in the expression of the *gnrh* genes and future  
477 studies should investigate this point.

478 At last, the expression of the nuclear estrogen receptors (ER) and the membrane estrogen  
479 receptors (GPER) were studied. As in most teleost species, the European sea bass possess three  
480 ER (*esr1*, *esr2a*, *esr2b*) (Halm *et al.*, 2004) and two GPER (*gpera*, *gperb*) (Pinto *et al.*, 2018)  
481 genes. Reported effects of XEs on ER seem to largely vary among species, tissues, age-class and  
482 the sex (Zhang *et al.*, 2012; Nikoleris *et al.*, 2016). For instance, in the loach exposed to EE2, ER  
483 expression appeared to be more responsive in the liver and gonads compared to the brain (Zhang  
484 *et al.*, 2012). In sea bass larvae, our results showed that neither *er* nor *gper* were modulated by  
485 EE2 exposure, indicating that at this developmental stage, the estrogen receptors located in the  
486 head are not sensitive to estrogen.

487

#### 488 *Effects of EE2 exposure on larval behaviour*

489 Estrogen and the brain aromatase are involved in the control of sexual and non-sexual behaviours  
490 (Diotel *et al.*, 2010). In sea bass, no standardized or referenced behavioural tests exist for larvae  
491 and, to our knowledge, our study is the first to report the effects of EE2 on the behaviour of sea  
492 bass larvae. Our results demonstrated an EE2-mediated increase in locomotor activity and  
493 anxiety-like behaviours, well conserved after a 20 day depuration period.

494 Locomotor activity is a peculiar behavioural endpoint as it can be measured in several ways, such  
495 as time spent swimming, swimming burst or distance travelled. After introduction to a new  
496 environment or introduction of a novel object, a decrease in locomotion is usually observed for  
497 the first minutes (three to six), which gradually diminished as a consequence of habituation  
498 (Wong *et al.*, 2010). This behaviour is commonly interpreted as an indicator of acute stress. To  
499 overcome handling/novelty stress, acclimation to the novel environment or direct recording in  
500 experimental set-ups are two alternatives, although there is no consensus on the duration of  
501 acclimation (from 1 to 60 minutes) (Melvin *et al.*, 2017). In our study, larvae were acclimated  
502 for 5 minutes before recordings and in the two independent experiments, the larvae exposed to  
503 EE2 showed an increased in locomotor activity. This increase is observed at the end of the  
504 exposure period (significant in experiment 2 and strong trend in experiment 1) and persisted after  
505 the 20-day depuration phase (experiments 1 and 2) (Supplementary I-c). In the literature,  
506 estrogens or xenoestrogens effects on locomotor activity in fish have shown variable results. In

507 accordance with our results, Sali *et al.* (2012) reported hyperactivity in zebrafish larvae after  
508 embryonic exposure to estradiol or bisphenol A (BPA). These results were confirmed by Kinch  
509 *et al.* (2015) who, in addition, showed that hyperactivity was associated with an increase in  
510 hypothalamic neurogenesis. Other studies in zebrafish have reported a decrease in larval activity  
511 after embryonic exposure to EE2 (Martínez *et al.*, 2020) and in juvenile after a chronic exposure  
512 for 40 days (Sárria *et al.*, 2011). Similar observations have been made in medaka after chronic  
513 exposure of embryos and early larval stages to estradiol or BPA (Inagaki *et al.*, 2016). Thus,  
514 disruption of locomotion appears to vary according to life-stage, duration of exposure, levels of  
515 anxiety and behavioural test systems. Interestingly, Morgan *et al.* (2015) suggested that estrogen  
516 might increase locomotor activity in a safe environment while it might reduce it in an  
517 environment perceived as threatening.

518 Our results show an increase in locomotion after EE2 exposure which is here interpreted as an  
519 induced hyperactivity. Sustained hyper-activity could be detrimental for fish as it can affect  
520 predator avoidance, prey catch and transport, as well as social behaviours and overall energy  
521 balance (Sárria *et al.*, 2011).

522

523 The occurrence of anxiety-like behaviours was also investigated *via* an increase in thigmotaxis  
524 (“wall-hugging”: time spent and distance travelled in the periphery) and erratic movements  
525 (number of rotations). Thigmotaxis (Richendrfer *et al.*, 2012) and erratic movements (Egan *et al.*  
526 *et al.*, 2009) have been validated as proxies for anxiety in zebrafish using different psychoactive  
527 drug treatments. Based on these indications, we examined the behavioural responses of sea bass  
528 larvae. Whatever the conditions, the larvae preferred to stay in the peripheral zone of the arena.  
529 Nevertheless, a higher thigmotactic behaviour and an increase in the number of rotations were  
530 observed in larvae exposed to EE2 than in controls (day 8). In addition, these effects were  
531 conserved after 20 days of depuration (in both experiments), indicating that EE2 may have  
532 permanently impaired anxiety-like behaviour in the developing sea bass. Exposure to chemicals  
533 during early life stages has already been shown to permanently disrupt behaviour, with effects  
534 lasting after a depuration period or persisting into adulthood (Weis, 2014). This long-term effects  
535 indicate that a chemical can permanently modify brain areas associated with specific behaviours,  
536 as proposed by Volkova *et al.* (2012).

537 In the literature, EE2 exposure was repeatedly shown to increase fear, anxiogenic and depressive  
538 behaviours (e.g. *freezing*, bottom-dwelling and scototaxis) in several teleost species. Both long-  
539 and short-term exposure resulted in increased anxiety-like behaviours in larvae and adult  
540 zebrafish, increased bottom dwelling and latency to swim in the upper half of the tank in the

541 Novel Tank Test (Reyhalian *et al.*, 2011; Volkova *et al.*, 2015). Same effects were observed in  
542 guppy maternally exposed to 20 ng/L of EE2 and raised in clean sea water (Volkova *et al.*, 2012)  
543 and in female fighting fish after exposure to 10 ng/L of EE2 for only few hours (Dzieweczynski  
544 *et al.*, 2014). It is hard, however, to extrapolate the real ecological/individual consequences of  
545 such behavioural impairments in the environment as few data exist on such topic.

546 The above-mentioned behavioural changes detected in larvae exposed to EE2 could be related to  
547 the disruption of some brain genes. Among them, the previously mentioned *Cyp19a1b* gene has  
548 a recognized role in the regulation of sexual behaviours and also of non-reproductive behaviours  
549 such as depression, cognition and sociability (Diotel *et al.*, 2010; Shaw, 2018; Brocca and Garcia-  
550 Segura, 2019). In rodents, altered anxiety and fear responses have been observed after inhibition  
551 of aromatase b (Graham and Milad, 2014). Another candidate gene is *kiss1*, indeed, the  
552 administration of kisspeptins has been shown to have anti-depressant, anxiolytic and/or  
553 anxiogenic effects in vertebrates (Mills *et al.*, 2019). In zebrafish, kisspeptin1 injection induced  
554 a decrease in fear responses and anxiety-like behaviour (Nathan *et al.*, 2015). This is in contrast  
555 with our results, where an increase in *kiss1* expression is correlated to an increase in anxiety-like  
556 behaviours, but it suggests a potential role for *kiss1* gene in the regulation of anxiogenic states.  
557 Based on our observations, we can hypothesize that there is a link between effects on *cyp19a1b*  
558 and/or *kiss1* gene expression and the anxiety-like behaviours measured after EE2 exposure of sea  
559 bass larvae that should be investigated in future studies.

560 Overall, our results show that EE2 disrupts behaviour of sea bass larvae, potentially causing a  
561 long-term increase in locomotor activity and anxiogenic behaviour. As fish behaviour appears to  
562 be a sensitive target of EDCs, the standardisation of behavioural tests is of primary importance  
563 for non-model organisms. Furthermore, as the neuroendocrine control of behaviour is  
564 multifactorial and rather complex, future studies are needed to identify the specific cellular  
565 mechanisms linking chemical exposure to behavioural responses.

566

## 567 **Conclusion**

568 In this study sea bass larvae were exposed to EE2 to investigate potential neuroendocrine effects.  
569 Exposure to 0.5 nM of EE2, representative of environmentally relevant concentration of  
570 xenoestrogens, triggered a significant increase in *cyp19a1b*. Moreover, exposure to 50 nM of  
571 EE2 resulted in the upregulation of *gnrh2*, *kiss1* and *cyp19a1b*. A strong increase in larval  
572 locomotor activity and anxiety-like behaviours was also observed, with effects persisting after a  
573 20-day depuration phase. Alteration of gene expression levels and fitness-related behaviours

574 during early development might lead to deleterious consequences, and long-term effects cannot  
575 be excluded.

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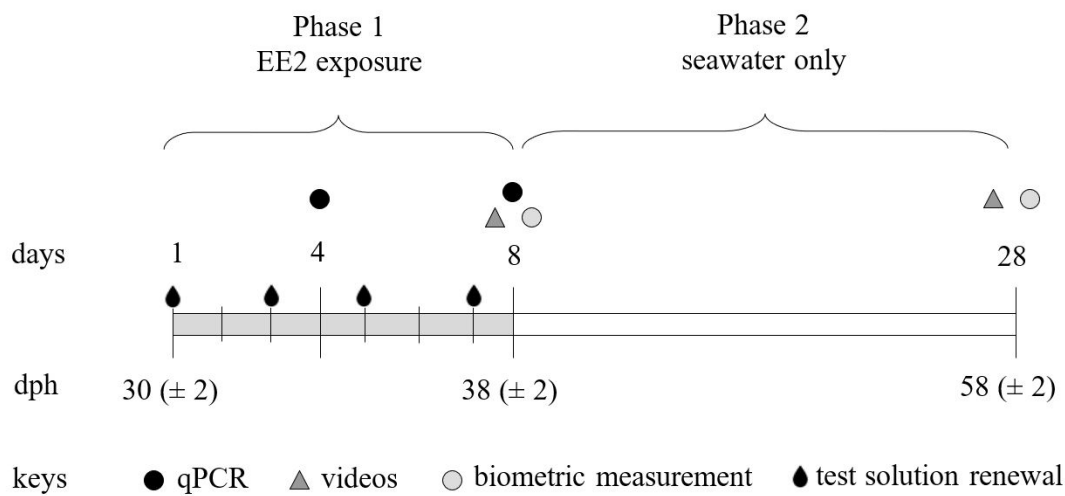
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953 **Tables**954 *Table 1: Selected primers for qPCR analysis*

Name	Acces. n°	Ann. T °C	Efficiency	Forward sequence	Reverse sequence
<i>ef1a</i>	AJ866727	60	2,00	CTAAGAACGGACAGACCCGC	TGACTCCAACGATGAGCTGC
<i>l13</i>	DQ836931	60	2,03	GGCTAAGGCCCGTCTCATTG	GCACGAACCTTGGTGTGGTA
<i>RPS14</i>	EF190887	57	1,99	CAAACCTGAGAGCGACCGGAG	GCGTCCGATCTTCATCCCTG
<i>cyp19a1b</i>	AY138522	57	2,02	GGCTACACCCTCAACAGCAT	CCACAAAACCCCTCTGTGT
<i>kiss 1</i>	FJ008914	57	2,03	CTCAAAGCTGGTCCTCCCTC	GGTCTTGTGTCACCTTCCGT
<i>kiss 2</i>	FJ008915	57	2,03	GGATTCCAGCCCGTGTCTTCT	TAGGCACCTCCAGTTCTCGT
<i>gnrh1</i>	AF224279	60	1,97	GGTCCTATGGACTGAGTCCAGG	TGATTCCTCTGCACAACCTAA
<i>gnrh 2</i>	AF224281	60	2,01	GTGTGAGGCAGGAGAATGCA	CTGGCTAAGGCATCCAGAATG
<i>gnrh 3</i>	AF224280	57	1,97	TGTGGGAGAGCTAGAGGCAAC	GTTTGGGCACTCGCTCTT
<i>esr1</i>	AJ505009	63	2,00	GGCAAACCTCATCTTGCACAG	ACTGCTGCTGAGCCGAACAC
<i>esr2b</i>	AJ489523	61	2,01	CTGTCCTCACAGAGTAGAACAC	GCAGGTGCACCTGGTCCAAG
<i>esr2a</i>	AJ489524	63	1,98	GTGGACTCCAGACTCGGGAC	ATCATGCTAGCCTCGGTGAAG
<i>gpera</i>	MF508726	63	2,00	GCCACCCTTCTCCCTTCACC	TTCGCCAATCAGAGAGTAGCAT
<i>gperb</i>	MF508727	59	2,02	ACAGCAGCGTCTTCTTCTTAACC	AGATGAGGACACCCAGATAAGGCAG

955 Access. n°: gene accession number ; Ann T °C : annealing temperature in °C

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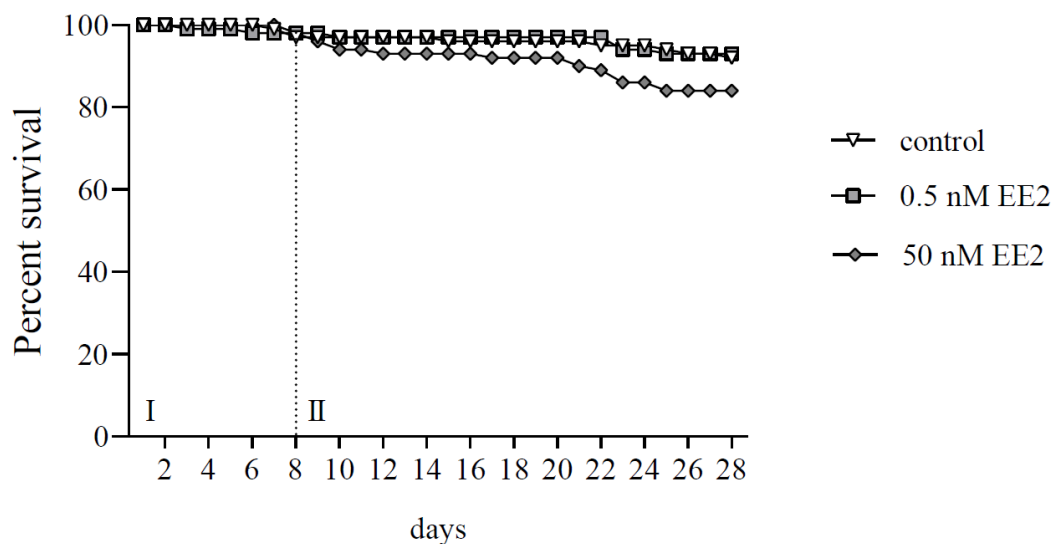


958

959 Figure 1: Scheme of the two-phases experiment indicating specific measurements (symbols). Sea  
 960 bass larvae were firstly exposed to EE2 during 8 days (phase I) and afterwards maintained into  
 961 natural seawater during 20 days (phase II). At day 0, larvae ( $30 \pm 2$  dph) were exposed under  
 962 semi-static conditions to 0.5 nM of EE2, 50 nM of EE2, or seawater only. Experimental medium  
 963 was renewed every 48 h (droplet) until day 7. qPCR samples were collected at day 4 and day 8  
 964 (phase I). Samples for standard length and video recordings for behavioural analysis were taken  
 965 at day 8 (phase I) and day 28 (phase II).

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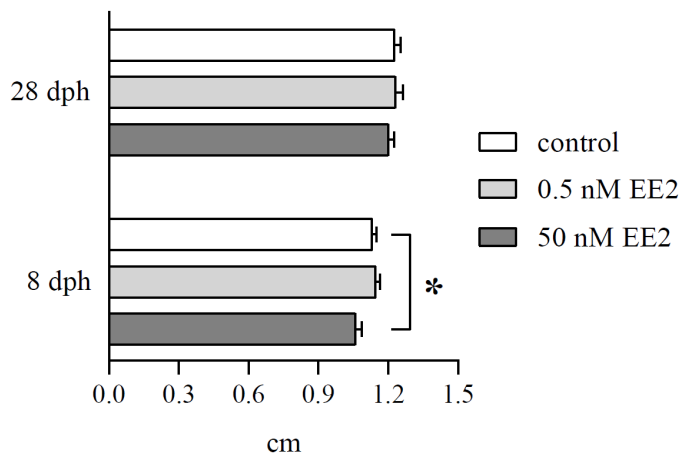
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968

969 Figure 2: Sea bass larvae survival during phase I (from Day 0 until Day 8, I) and phase II (from  
 970 day 9 to day 28, II). Survival rate is presented as daily cumulative survival percentage for each  
 971 treatment.

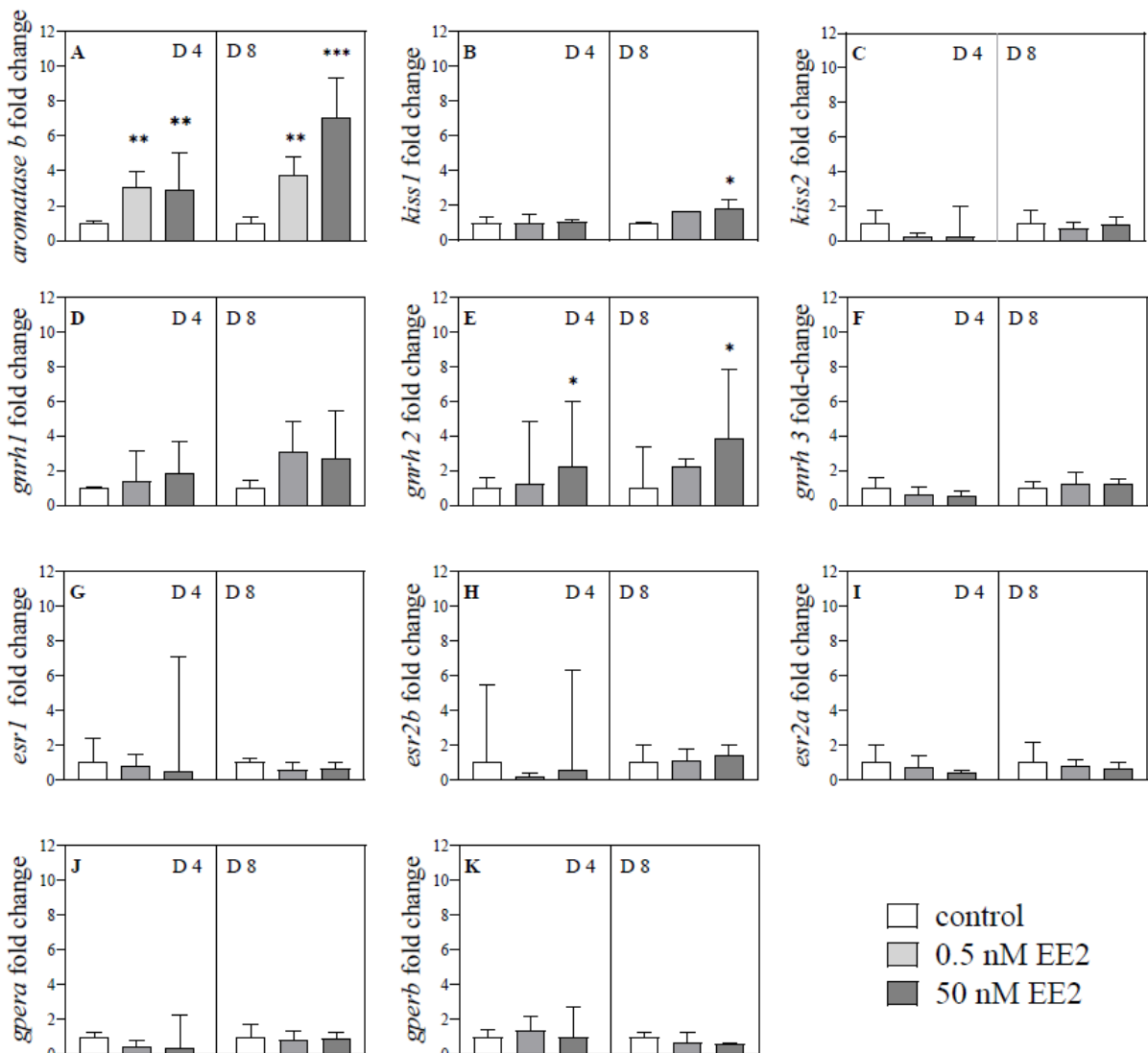
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974 Figure 3: Standard length measured in larvae (n = 10 per group) at the end of phase I (8<sup>th</sup> day)  
 975 and at the end of phase II (28<sup>th</sup> day) of the experiment. Values are presented as mean ± SEM. \*:   
 976 p < 0.05

977

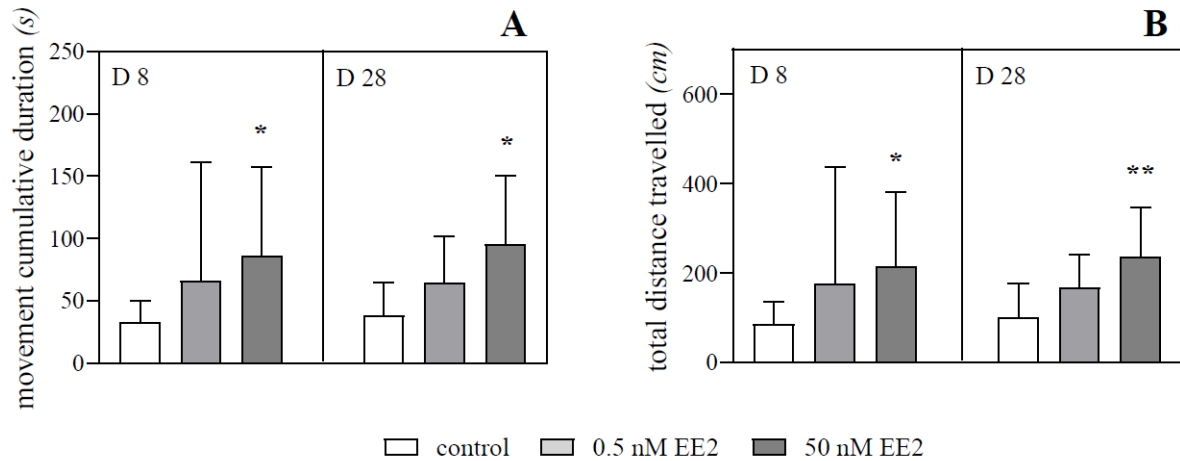


978



979 Figure 4: Relative expression of genes measured in the head of sea bass larvae (n = 8 per group)  
 980 exposed to 17 $\alpha$ -ethinylestradiol. Gene expression for aromatase b (A), kiss (B-C), gnrhs (D-F),  
 981 nuclear (G-I) and membrane (J-K) estrogen receptors are presented at day 4 (D 4) and at day 8  
 982 (D 8) of phase I. Results are presented as average fold change  $\pm$  SD relative to control conditions.  
 983 \*: p < 0.05; \*\*: p < 0.01; \*\*\*\*: p < 0.0001 if compared to control conditions.

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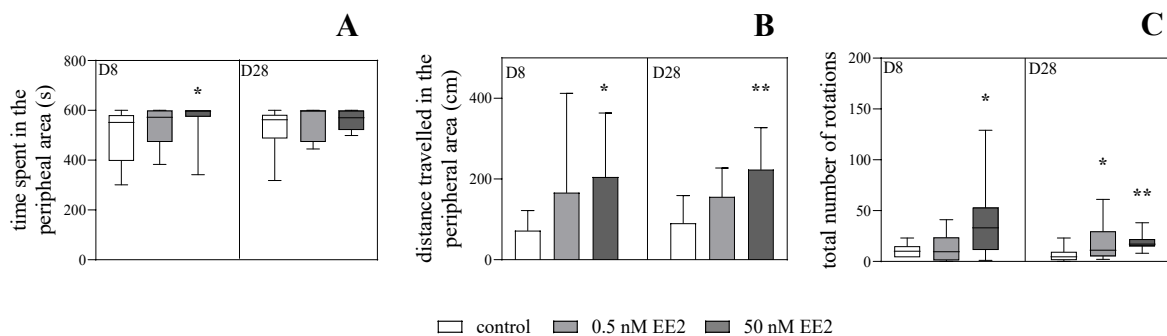


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986 Figure 5: Behavioural indicators of locomotor activity of sea bass larvae (n = 12 per group)  
 987 recorded at day 8 (D 8, end of phase I) and day 28 (D 28, end of phase II). Graphs show the time  
 988 spent swimming or movement cumulative duration (A, geometric mean + 95% CI) and the total  
 989 distance travelled (B, geometric mean + 95% CI). \*: p < 0.05; \*\*: p < 0.01 compared to control  
 990 conditions.

991

992



993

994 Figure 6: Behavioural indicators of anxiety-like behaviours in sea bass larvae (n = 12 per group)  
 995 recorded at day 8 (D 8, end of phase I) and day 28 (D 28, end of phase II). Graphs show  
 996 cumulative time spend in peripheral area of the arena (A, min-max boxplot), total distance  
 997 travelled in peripheral area of the arena (B, geometric mean + 95% CI) and the total number of  
 998 rotation (C, min-max boxplot). \*: p < 0.05; \*\*: p < 0.01 compared to control conditions.

999