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1 **Effects of 17 α -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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33

34 *Authors' contributions:* S.A. and S.S. designed the study; S.A., S.O. A.P., and S.A. carried
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43

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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 α -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.

66

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

69

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 α -
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 ± 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 ± 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 α -ethinylestradiol (EE2, purity 99 %, Sigma–Aldrich,
168 France) on sea bass. Larvae were exposed from 30 to 38 (± 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 (1st 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 ± 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 (1st 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°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 μg 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/s²) 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*
425 *al.*, 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*
526 *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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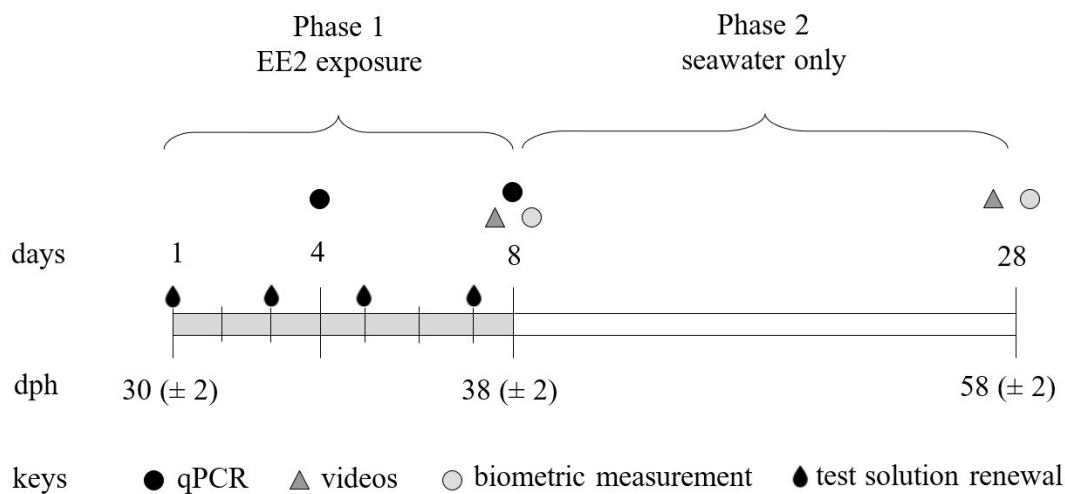
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953 **Tables**954 *Table 1: Selected primers for qPCR analysis*

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	CCACCAAACCCCTCTGTGT
<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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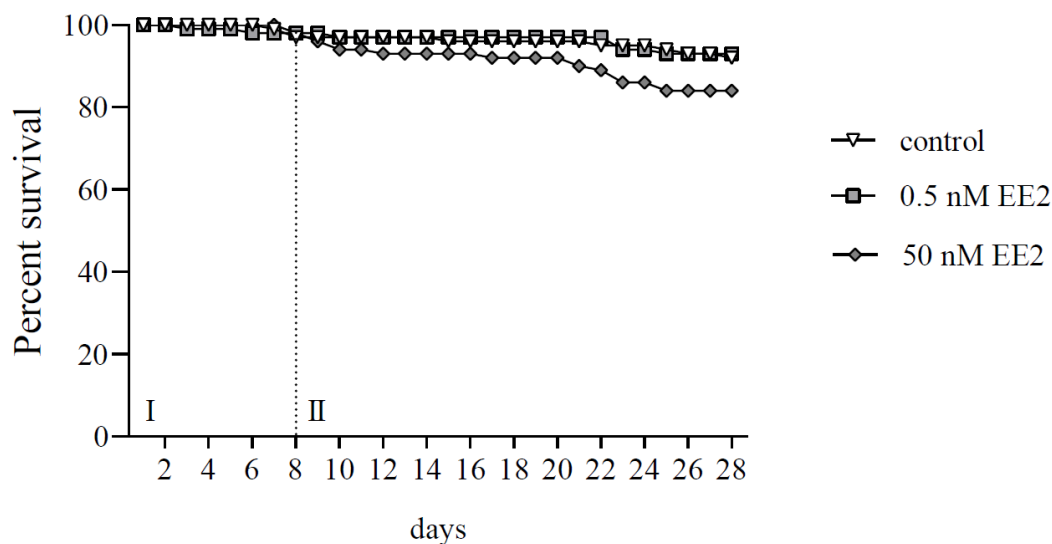


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959 Figure 1: Scheme of the two-phases experiment indicating specific measurements (symbols). Sea
 960 bass larvae were firstly exposed to EE2 during 8 days (phase I) and afterwards maintained into
 961 natural seawater during 20 days (phase II). At day 0, larvae (30 ± 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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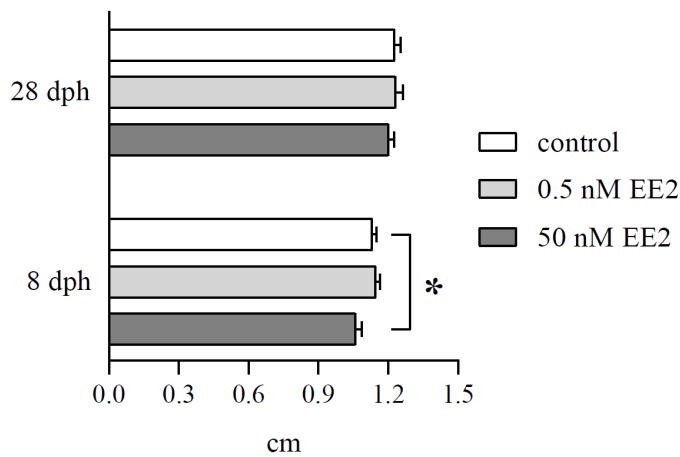
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969 Figure 2: Sea bass larvae survival during phase I (from Day 0 until Day 8, I) and phase II (from
 970 day 9 to day 28, II). Survival rate is presented as daily cumulative survival percentage for each
 971 treatment.

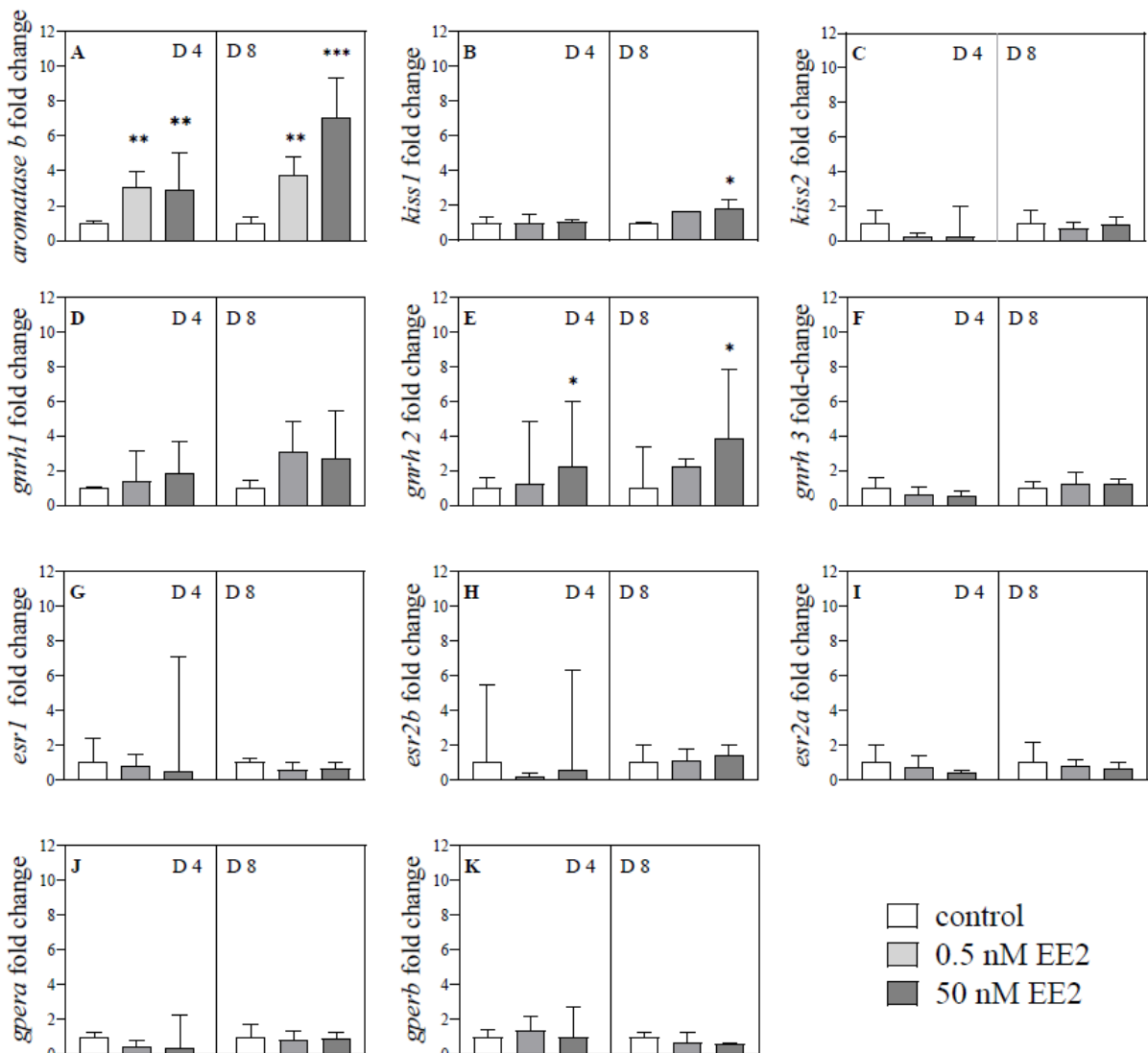
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973

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

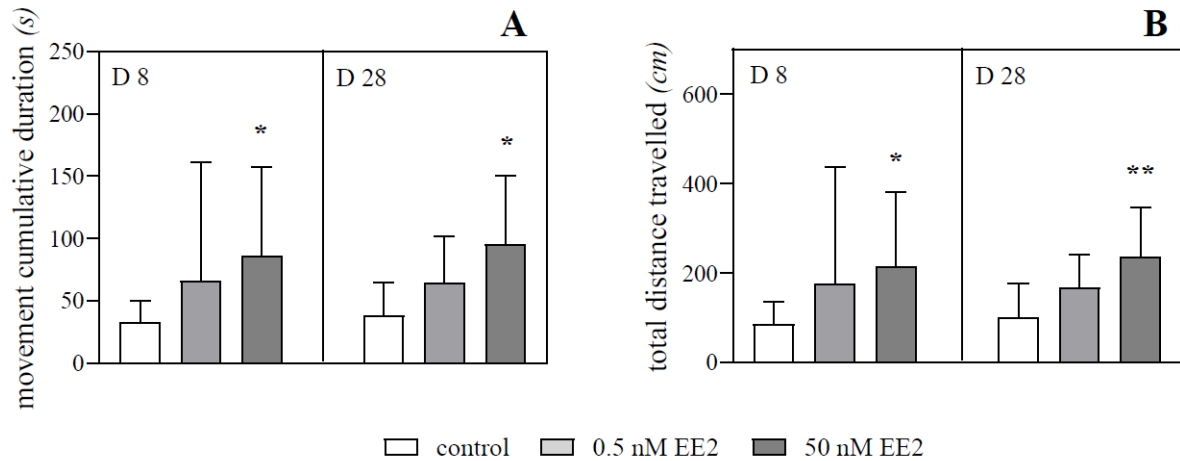
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979 Figure 4: Relative expression of genes measured in the head of sea bass larvae (n = 8 per group)
 980 exposed to 17 α -ethinylestradiol. Gene expression for aromatase b (A), kiss (B-C), gnrs (D-F),
 981 nuclear (G-I) and membrane (J-K) estrogen 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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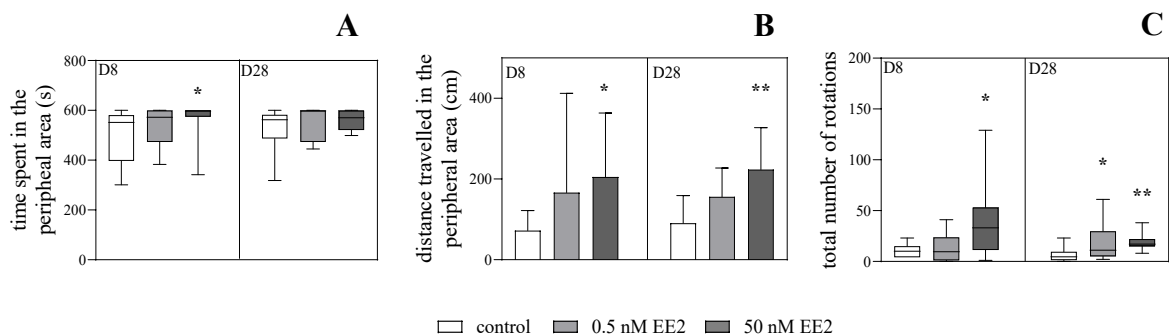


985

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