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## Natural cortisol production is not linked to the sexual fate of European sea bass

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### Abstract :

In this study, we aimed to investigate the relationship between cortisol and the determination of sexual fate in the commercially important European sea bass (*Dicentrarchus labrax*). To test our hypothesis, we designed two temperature-based experiments (19 °C, 21 °C and 23 °C, experiment 1; 16 °C and 21 °C, experiment 2) to assess the effects of these thermal treatments on European sea bass sex determination and differentiation. In the fish from the first experiment, we evaluated whether blood cortisol levels and expression of stress key regulatory genes were different between differentiating (149 to 183 dph) males and females. In the second experiment, we assessed whether cortisol accumulated in scales over time during the labile period for sex determination as well as the neuroanatomical localisation of brain cells expressing brain aromatase (*cyp19a1b*) and corticotropin-releasing factor (*crf*) differed between males and females undergoing molecular sex differentiation (117 to 124 dph). None of the gathered results allowed to detect differences between males and females regarding cortisol production and regulatory mechanisms. Altogether, our data provide strong physiological, molecular and histochemical evidence, indicating that in vivo cortisol regulation has no major effects on the sex of European sea bass.

**Keywords :** Sex determination, Sex differentiation, Temperature, Cortisol, European sea bass

## 56 1. Introduction

57

58 The stress physiology of teleost fishes has been a long-standing object of research in the scientific community  
59 (Wendelaar Bonga 1997; Mommsen et al. 1999). In recent years, particular interest has been vested into the  
60 relationship between stress, reproduction and sexual development. The very well-described cross-talk between  
61 the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-interrenal (HPI) axes has further  
62 nourished the interest in the link between stress and sex (Rousseau et al. 2021). The HPI axis, analogous to the  
63 hypothalamic-pituitary-adrenal axis in mammals, is commonly known as the corticotropic or stress axis.  
64 Specifically, special attention has been given to cortisol, generally referred to as the dominant stress hormone in  
65 fishes (Sadoul and Geffroy 2019).

66

67 Sex determination in gonochoristic (fixed separate sexes) teleost fishes is generally categorised into two broad  
68 classes, those with a genotypic sex determination (GSD) in which sex is determined by inherited genetic elements;  
69 and those with an environmental sex determination (ESD) (Hattori et al. 2020). In ESD species, sexual fate is  
70 determined by environmental factors surrounding early development, most usually a temperature gradient (Bull  
71 1983). However, there are also some organisms which are affected by both strategies, and we refer to them as  
72 GSD + EE (environmental effects) species (Stelkens and Wedekind 2010; Sarre et al. 2011; Holleley et al. 2016).  
73 In most cases in which the phenotypic sex depends on environmental cues, this involves stressful factors (e.g.,  
74 high fish density, low pH, high temperature) triggering an increase in circulating cortisol (Devlin and Nagahama  
75 2002; Hattori et al. 2009; Stelkens and Wedekind 2010; Hayashi et al. 2010; Yamaguchi et al. 2010). Certainly,  
76 much of the little we know about the potential role of cortisol during sex determination and differentiation derives  
77 from studies investigating female-to-male sex reversal in these GSD + EE species, such as pejerrey (*Odontesthes*  
78 *bonariensis*), medaka (*Oryzias latipes*) or olive flounder (*Paralichthys olivaceus*) (Hattori et al. 2009; Hayashi et  
79 al. 2010; Yamaguchi et al. 2010). Such findings imply that cortisol may constitute a key element linking increased  
80 temperatures and masculinisation. Interestingly, conflicting results regarding the association between  
81 glucocorticoids and sex reversal have been found in reptilian systems (Geffroy and Douhard 2019), with  
82 experimental yolk corticosterone elevation shown to affect sex determination in some lizard species (Warner et  
83 al. 2009), but not in others (Uller et al. 2009; Castelli et al. 2021).

84

85 One of the most prominent examples of a GSD + EE species can be found in the European sea bass (*Dicentrarchus*  
86 *labrax*). This species has a polygenic sex determination system (Vandeputte et al. 2007; Geffroy et al., 2021a),  
87 and its temperature-induced masculinisation (TIM) has been described in detail in the literature (Piferrer et al.  
88 2005). In this species, the labile period for sex determination, which overlaps with the beginning of molecular sex  
89 differentiation, extends until the attainment of a size of around 8 cm of length at 180 – 200 dph (days post-  
90 hatching) (the exact size and age being dependant on the rearing temperature) (Piferrer et al. 2005).  
91 Thenceforward, histological sex differentiation proceeds and sex becomes fixed (Piferrer et al. 2005). However,  
92 sexual development of this captivating species is considered to include two thermolabile periods in which sexual  
93 fate may be affected by water temperature, biasing sex ratios (Vandeputte and Piferrer 2018; Vandeputte et al.  
94 2020). Fish kept at relatively high temperatures (> 20 °C) during their first months of life generally develop as  
95 males (Piferrer et al. 2005; Vandeputte and Piferrer 2018). Moreover, if kept for too long (more than 90 days after

96 fertilisation) under relatively a low temperature ( $< 16\text{ }^{\circ}\text{C}$ ), sea bass also mostly develop as males (Saillant et al.  
97 2002; Vandeputte et al. 2020). Here, we hypothesised that the temperature fish are exposed to would affect cortisol  
98 production (Alfonso et al. 2021; Bessa et al. 2021) which would, in turn, influence their phenotypic sex.

99

100 We previously found that cortisol was not involved in biasing sex ratios at the group level (Geffroy et al. 2021b)  
101 but a more complete evaluation at the individual level was lacking. The aim of the present work was to evaluate  
102 the effect of intrinsic cortisol regulation, expected to change in response to a thermal stress, on the sexual fate of  
103 European sea bass juveniles using fish from two different experimental set-ups involving a range of temperatures.  
104 Quantification of circulating cortisol at the time of molecular sex differentiation (Ribas et al. 2019) (Experiment  
105 1) and cortisol accumulated in scales over time during sex determination (Experiment 2) was used to evaluate the  
106 differences between fish from different sexes and temperature treatments. At the central level, the measurement  
107 of the expression of stress key regulatory genes in the hypothalamus was performed via qPCR (Experiment 1),  
108 and complemented by the neuroanatomical localisation of brain cells expressing brain aromatase (*cyp19a1b*) and  
109 corticotropin-releasing factor (*crf*) (Experiment 2).

110

## 111 **2. Materials and methods**

112

### 113 *2.1. Source of fish and experimental designs*

114 For Experiment 1, the fish population used originated from a complete factorial mating by artificial fertilisation  
115 between ten male and eight female European sea bass from a wild west Mediterranean Sea strain (Grima et al.  
116 2010). Eggs were then evenly distributed in 12 tanks of 500 L each, four replicate tanks per thermal treatment.  
117 Egg incubation, temperature monitoring and larvae rearing was performed as described in Goikoetxea et al.  
118 (2021). The temperature-increase protocol began at 85 dph and  $16\text{ }^{\circ}\text{C}$ , with a gradual increase of  $2\text{ }^{\circ}\text{C}$  per day  
119 until reaching the desired temperature for each treatment group:  $19\text{ }^{\circ}\text{C}$  (87 dph),  $21\text{ }^{\circ}\text{C}$  (88 dph) and  $23\text{ }^{\circ}\text{C}$  (89  
120 dph) (Fig. 1A). Experiment 1 targeted the late temperature-sensitivity window, whereby colder temperatures  
121 induce a higher proportion of males. Each thermal treatment was maintained until sampling when fish reached a  
122 body length of approximately 7.8 cm and 5.4 g, at 183 dph for those kept at  $19\text{ }^{\circ}\text{C}$  ( $n = 19$ ), 163 dph for those kept  
123 at  $21\text{ }^{\circ}\text{C}$  ( $n = 14$ ) and 149 dph for those kept at  $23\text{ }^{\circ}\text{C}$  ( $n = 18$ ), respectively, marking the end of the experiment.

124

125 In Experiment 2, the fish population resulted from a complete factorial mating design with eight males and one  
126 female from a West Mediterranean Sea strain of European sea bass, performed by artificial fertilisation (March  
127 22<sup>nd</sup>, 2017). Eggs were then evenly distributed in six tanks of 500 L each, and temperature was gradually increased  
128 from  $14\text{ }^{\circ}\text{C}$  to  $16\text{ }^{\circ}\text{C}$  in the first 24h. Fish density after hatching was 50 larvae per litre. Then, larvae were  
129 maintained at  $16\text{ }^{\circ}\text{C}$  (in triplicates) or exposed to  $21\text{ }^{\circ}\text{C}$  (in triplicates) as described in Geffroy et al. (2021a) and  
130 Goikoetxea et al. (2021). For the  $21\text{ }^{\circ}\text{C}$ -treatment, temperature was increased from  $14\text{ }^{\circ}\text{C}$  to  $21\text{ }^{\circ}\text{C}$  during the first  
131 8 dph (Fig. 1B). Experiment 2 targeted the early temperature sensitivity window, whereby colder temperatures  
132 induce a higher proportion of females. For Experiment 2, each thermal treatment was maintained until sampling  
133 when fish in each group reached a body length of approximately 7.2 cm and 4.5g, at 127 dph ( $16\text{ }^{\circ}\text{C}$ ) and 117 dph  
134 ( $21\text{ }^{\circ}\text{C}$ ), respectively, marking the end of the experiment. For both experiments, fish were fed *Artemia nauplii* for  
135 40 days starting at 10 dph, then weaned onto a commercial sea bass diet (Pro Start and Pro Wean, BioMar). Fish

136 rearing was performed at the Ifremer Plateforme Expérimentale d'Aquaculture (Palavas-les-Flots, France),  
137 accredited to use and breed laboratory animals (n° C341926).

138

### 139 2.2. Sexing of fish

140 For Experiment 1, qPCR expression analysis of classical sex-pathway genes *cyp19a1a* (gonadal aromatase) and  
141 *gsdf* (gonadal soma derived factor) was used to assign the phenotypic sex to each individual (see Section 2.6. for  
142 details).

143

144 Regarding the fish included in Experiment 2, individuals had already been sexed as part of a previous experiment.  
145 In that case, sexing was done based on the difference in reads between *cyp19a1a* and *gsdf* within individuals,  
146 obtained via RNA-Seq, all data freely and openly available at <https://sextant.ifremer.fr/> (Geffroy 2018).

147

### 148 2.3. Plasma cortisol assessment

149 At the end of Experiment 1, blood plasma collected individually using a 1 mL-EDTA-treated syringe from the  
150 caudal vein of European sea bass exposed to 19 °C, 21 °C or 23 °C was diluted 10-fold, whenever feasible, and  
151 the level of cortisol was assessed using a Cortisol ELISA kit (Neogen Lexington, KY, USA). The lower limit of  
152 detection of the kit was 0.04 ng/mL. Samples were assayed in duplicate and intra- and inter-assay coefficients of  
153 variation were < 10%. The cross-reactivity of the antibody with other steroids is as follows: prednisolone 47.5%,  
154 cortisone 15.7%, 11-deoxycortisol 15.0%, prednisone 7.83%, corticosterone 4.81%, 6 $\beta$ -hydroxycortisol 1.37%,  
155 17-hydroxyprogesterone 1.36%. Steroids with cross-reactivity less than 1% are not presented. Plasma cortisol  
156 levels were normalised using the total protein level. Plasma protein level was estimated using a Protein  
157 Quantification Kit-Rapid (Sigma-Aldrich, St. Louis, MO, USA), as recommended by the manufacturer. Briefly,  
158 samples (diluted 100-fold) and standard (BSA standard stock solution) were added three times in each well and  
159 completed with a solution of Coomassie Brilliant Blue G. After one minute of incubation, the absorbance was  
160 measured at 630 nm with a microplate reader (Synergy HT, BioTek Instrument, VT, USA). Cortisol levels in  
161 plasma were expressed in micrograms per milligrams of proteins.

162

### 163 2.4. Scale cortisol assessment

164 Ontogenetic scales preparation, homogenisation and subsequent cortisol quantification with an Ultra-Performance  
165 Liquid Chromatography - Tandem Mass Spectrometer (UPLC-MS/MS) (XevoTQS, Waters, Milford, USA) were  
166 performed as previously described in Goikoetxea et al. (2021).

167

### 168 2.5. Extraction and reverse transcription of RNA from gonadal and hypothalamic tissues

169 Whole gonads and hypothalami from each fish (n=51) from Experiment 1 were homogenised using a ball mill  
170 (Retsch Mixer Mill MM 400, Haan, Germany) at 30 rpm for 30 s. Total RNA was extracted using 500  $\mu$ L (gonad)  
171 or 400  $\mu$ L (hypothalamus) of QIAzol<sup>®</sup> lysis reagent (Beverly, MA, USA) following manufacturer's instructions.  
172 Total RNA was measured using a NanoDrop<sup>®</sup> ND-1000 V3300 spectrophotometer (Nanodrop Technology Inc.,  
173 Wilmington, DE, USA). Each RNA sample was then diluted in DNase/RNase-free water for a final standard  
174 concentration of 100 ng (gonad) or 0.5  $\mu$ g (hypothalamus) of RNA. cDNA synthesis was performed using the

175 qScript™ cDNA SuperMix (Quantabio, QIAGEN, Beverly, MA, USA) following manufacturer's instructions.  
176 cDNA was then diluted 8-fold in DNase/RNase-free water prior to quantitative real-time PCR (qPCR).

177

## 178 2.6. qPCR gene expression analyses

179 European sea bass-specific primer sequences were obtained from the literature (Pavlidis et al. 2011; Navarro-  
180 Martín et al. 2011; Martins et al. 2015; Sadoul et al. 2018; Alfonso et al. 2019; Vandeputte et al. 2020) (Table 1).  
181 Ribosomal protein L13 (*l13*), eukaryotic translation elongation factor 1 alpha (*eef1a*) and beta-actin (*β-actin*) were  
182 used as reference genes. Our target genes in the hypothalamus included: *gr1* (glucocorticoid receptor 1), *gr2*  
183 (glucocorticoid receptor 2), *mr* (mineralocorticoid receptor), and *crf*. RefFinder (<https://www.heartcure.com.au>)  
184 (Xie et al. 2012) and BestKeeper (<https://www.gene-quantification.de>) (Pfaffl et al. 2004) approaches were used  
185 to determine the stability of gene expression of *l13*, *eef1a* and *β-actin* and their suitability as reference genes for  
186 the normalisation of qPCR results, and it was further validated that neither treatment nor sex had an effect on their  
187 expression profiles. Data were normalised based on the geometric mean of all three housekeeping genes. An  
188 Echo® 525 liquid handling system (Labcyte Inc., San Jose, CA, USA) was used to dispense 0.75 µL of  
189 SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK), 0.03 to 0.09 µL of each primer (forward and reverse  
190 primers between 0.2 and 0.6 µM final concentration), sufficient volume of ultra-pure water and 0.5 µL of diluted  
191 cDNA into a 384-well reaction plate. Each sample was run in duplicate. qPCR conditions were as follows:  
192 denaturation at 95 °C for 2 minutes, 45 cycles of amplification (95 °C, 15 s), hybridisation (60 °C, 5 s) and  
193 elongation (72 °C, 10 s), and a final step at 40 °C for 30 s. A melting curve program was performed to control the  
194 amplification specificity. Ultra-pure water was used as a no template control.

195

## 196 2.7. Histological processing of brain tissue and in situ hybridisation (ISH)

197 European sea bass juveniles from two temperature treatments (16 °C and 21 °C, n= 2-4 per experimental group  
198 and sex, Experiment 2) were euthanised (benzocaine 150 mg/L) at 127 (16 °C) and 117 dph (21 °C), respectively.  
199 The brain was quickly collected and fixed overnight (O/N) in 4% paraformaldehyde (PFA) at 4 °C. Tissues were  
200 dehydrated and embedded in paraffin before being transversally sectioned in series at 10 µm and mounted on  
201 SuperFrost® Ultra Plus Menzel Gläser adhesive slides (Thermo Fisher Scientific, Waltham, MA, USA). Slides  
202 were stored at 4 °C until processed for ISH. Riboprobes synthesis and ISH for *cyp19a1b* and *crf* genes were  
203 performed as described previously (Escobar et al. 2016) with few modifications.

204

205 For *cyp19a1b* and *crf* riboprobes synthesis, DNA fragments, obtained by PCR with the primers shown in Table  
206 2, were cloned into pCR™II-TOPO® (Invitrogen, Waltham, MA, USA). Plasmids were linearised with BamIII  
207 and NotI restriction enzymes. Digoxigenin-labelled sense and antisense RNA probes were synthesised by *in vitro*  
208 transcription using DIG RNA labelling mix and T7 or SP6 polymerases (Roche Applied Science, Indianapolis,  
209 IN, USA) following manufacturer's instructions. Slides were dewaxed and dehydrated by decreasing the  
210 concentration of ethanol before being washed twice in 0.1 M phosphate-buffered saline solution (PBS). After a  
211 20-minute post-fixation in 4% PFA and a further wash in PBS, sections were incubated in proteinase K (2 µg/mL)  
212 for 5 minutes in PBS at 37 °C. Slides were equilibrated in saline-sodium citrate solution (SSC 2X) before O/N  
213 hybridisation at 60 °C in humidified chambers with 4 µg/mL of one (*crf* or *cyp19a1b*) antisense or sense probe.  
214 Sections were then washed twice in 2X SSC at 60 °C, incubated with 2X SSC/50% formamide and finally washed

215 in 0.1X SSC. Immunodetection was processed after washing in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 (buffer  
216 1) and by incubation of slides for 30 minutes in buffer 1 with 0.5% blocking reagent and 0.2% Triton X-100. This  
217 was followed by incubation with anti-digoxigenin alkaline phosphatase-conjugated sheep Fab fragment antibodies  
218 (Roche Diagnostic, Indianapolis, IN, USA) at a dilution of 1/2000 O/N. Lastly, sections were incubated with  
219 HNPP/FastRed (Roche Diagnostic, Indianapolis, IN, USA) at room temperature for 4 (*crf* probes) to 12 hours  
220 (*cyp19a1b* probes). Photomicrographs were taken with an epifluorescent Olympus BX51 microscope equipped  
221 with camera Olympus DP71. Images were processed with the Olympus Analysis Cell software and plates  
222 assembled using Adobe Photoshop Element 2020.

223

## 224 2.8. Statistical analyses

225 For the gonadal qPCR analysis, a Fisher's test was used to evaluate any sex bias at the different temperatures (19,  
226 21 and 23 °C) with the molecular sex of the individuals analysed. For the ontogenetic scale cortisol, the ELISA  
227 for plasma cortisol and the hypothalamic gene expression qPCR analyses, a two factor (Temperature + Sex)  
228 ANOVA test was performed. A Principal Component Analysis (PCA) was used to visually discriminate males  
229 from females, based on gene expression levels (or RNAseq corrected reads) using the 'factoextra' package  
230 (Kassambara and Mundt 2020). All analyses were conducted in R (v. 1.4.1103) (Core Team 2020).

231

## 232 3. Results

233

### 234 3.1 Fish sexing

235 Based on qPCR expression levels of ovarian development gene *cyp19a1a* and testicular differentiation gene *gsdf*,  
236 the phenotypic sex was assigned to each individual from Experiment 1. We discarded 6 individuals that presented  
237 intermediate values (and were thus considered intersex, Fig. 2A) and otherwise found 30 males and 14 females in  
238 a total number of n = 44 individuals (Fig. 2A). Nevertheless, we tested for a potential sex bias at the three different  
239 temperatures with the molecular sexing of these individuals. None of the comparisons were significant (19 vs 21  
240 °C : p-value = 1; 19 vs 23 °C : p-value = 0.7; 21 vs 23 °C : p-value = 1).

241

242 For Experiment 2, following transcriptomic analysis, we detected on average 115x more *cyp19a1a* transcripts in  
243 gonads of future females and 4.5x more *gsdf* transcripts in gonads of future males, leaving no doubts about their  
244 phenotypic sex. We identified 10 males and 12 females based on the PCA (Fig. 2B). Detailed data on sex ratios  
245 for each thermal treatment from Experiment 2 can be found in our previously published work Geffroy et al.  
246 (2021a).

247

### 248 3.2 Plasma and scale cortisol

249 Cortisol concentration measured in the plasma of European sea bass (Experiment 1) was not significantly different  
250 between the three temperatures (19, 21 and 23 °C, p-value = 0.49) (Fig. 3). For each condition, mean ( $\pm$  SD  
251 (standard deviation)) values calculated were  $50.5 \pm 81.2$  SD,  $250.4 \pm 391.3$  SD and  $103.8 \pm 95.4$  SD  $\mu\text{g}/\text{mg}$  of  
252 proteins, respectively. Moreover, cortisol concentration in plasma did not differ between males and females in  
253 any treatment (p-value = 0.54) (Fig. 3). Regarding cortisol content in scales (Experiment 2), we did not observe

254 significant differences between phenotypic males (n=10) and females (n = 12) (p-value = 0.13), but there was a  
255 significant effect of temperature (p-value = 0.04) (Fig. 4).

256

### 257 3.3 Hypothalamic expression of genes involved in the glucocorticoid pathway

258 No significant differences between males and females were observed for any of the three thermal treatments  
259 evaluated via qPCR (19 °C, 21 °C and 23 °C, Experiment 1) for *gr1*, *gr2*, *mr*, or *crf* (Fig. 5). When differences in  
260 expression for each target gene were evaluated between treatments, statistically significant differences were found  
261 between the 19 °C and the 23 °C-fish for *gr2* (p-value < 0.05, Fig. 5B), and between the 21 °C fish and both other  
262 thermal treatments for *crf* (19 °C vs 21 °C, p-value < 0.001; 21 °C vs 23 °C, p-value < 0.05) but not for *gr1* or *mr*  
263 (Fig. 5D). No significant differences were found when analysing the effect of the interaction between sex and  
264 treatment.

265

### 266 3.4 Neuroanatomical localisation of cells expressing *cyp19a1b* and *crf*

267 No evident sexual dimorphism was observed regarding the expression pattern of *cyp19a1b* or *crf* cells. The  
268 location of expression sites of *crf* and *cyp19a1b* genes in the brain of European sea bass juveniles (180 dph) did  
269 not show any obvious variation associated with rearing temperature. Cells containing *cyp19a1b* were small and  
270 round shaped. They were consistently located, from the anterior region of the telencephalon until the posterior  
271 hypothalamus, along the boundary of the third ventricle. The neurons expressing *cyp19a1b* were seen in the medial  
272 dorsal telencephalic area (Dm, Figs. 6B-C) and in the dorsal (Vd) and ventral (Vv) part of the ventral  
273 telencephalon, respectively (Figs. 6B, 7A). Many scattered tiny positive cells were observed in the preoptic area  
274 (preoptic area, POA; nucleus preoptic parvocellularis, NPO and nucleus preopticus magnocellularis, PM) (Figs.  
275 6B-E and 7A-B.). Few cells containing *cyp19a1b* expressing cells were observed in the habenular and posterior  
276 commissures (Figs. 6E-F). Within the thalamus positive cells were evident in the posterior tubercle and the  
277 paraventricular organ (TPp, PVO; Figs. 6F-G and 7C, E). In more posterior regions *cyp19a1b* positive cells were  
278 observed in the synencephalon at the level of the periventricular pretectum (PPv) and the longitudinal medial  
279 fascicle (MLF, Figs. 6F-G, 7D).. Small *cyp19a1b* expressing cells were observed in the mesencephalic optic  
280 tectum and longitudinal and semicircular torus (OT, TLo and TS; Figs. 6G-H). In the posterior hypothalamus, the  
281 nucleus of the lateral tubercle (NLT) and the boundaries of the lateral recess (NRL) contained *cyp19a1b*  
282 expressing cells (Figs. 6G-H and 7E-F).

283

284 Expression sites of *crf* gene were made up of small groups of round or oval shaped cells bigger than *cyp19a1b*  
285 containing cells. The most anterior *crf* expression sites were located at the level of habenula (Fig. 6E) and the  
286 preoptic area (anteroventral part of the parvocellular preoptic nucleus, NPOav; gigantocellular part of the  
287 magnocellular preoptic nucleus, PMgc; NAPv, anterior periventricular nucleus; Figs. 6D-E and 8A-B). In a more  
288 posterior region of the hypothalamus *crf* positive cells were observed in the nucleus of the lateral tubercle (NLT)  
289 and the lateral recess (NRL) (Figs. 6F-H and 8E-G). Within the synencephalon, the longitudinal medial fascicle  
290 and the nucleus pretectalis periventricularis hosted few *crf* positive cells (MLF, Figs. 6F-G, 8H; PPv, Figs. 6G  
291 and 8H). In the posterior tubercle of the thalamus, *crf* containing cells appeared in the glomerular and  
292 preglomerular nuclei (Nga and NPGm; Figs. 6F-G and 8B), in the periventricular nucleus of the posterior tubercle  
293 (TPp, Figs. 6F and 8F) and in the paraventricular organ (nPVO, Figs. 6F). Scattered *crf* cells were observed in the



294 nucleus gustatorius tertius (NGT, Figs. 6G and 8E). The central pretectal nuclei also contained few oval *crf* cells  
295 (NPC, Figs. 6F and 8C-E). Tiny *crf* positive cells were observed into the mesencephalic optic tectum (OT),  
296 longitudinal torus (TL<sub>o</sub>) as well as in the ventral (TS<sub>v</sub>) and lateral (TS<sub>l</sub>) subdivisions of the semicircular torus (OT,  
297 TL<sub>o</sub>, TS<sub>l</sub>; Figs. 6G-H).

298

#### 299 4. Discussion

300

301 Analysis of circulating cortisol in the plasma of fish exposed to different temperature treatments demonstrated  
302 that no clear correspondence exists between cortisol concentrations and sex in the European sea bass. The same  
303 lack of association was observed during the evaluation of cortisol content accumulated in the scales over time of  
304 a second experiment fish. The latter were part of a previous study (Goikoetxea et al. 2021) in which we  
305 demonstrated the link between temperature and the induction of cortisol production in the European sea bass. In  
306 Goikoetxea et al. (2021), significant differences between thermal treatments (16 °C vs 21 °C) were reported  
307 regarding cortisol content in scales in the same individuals employed in the present study, in which we observed  
308 10x more cortisol in the scales of fish reared at 21 °C compared to the 16 °C group ( $21 \pm 6.3 \mu\text{g/g}$  vs  $2.1 \pm 0.3$   
309  $\mu\text{g/g}$ , respectively; Student's *t*-test, *p*-value < 0.01). These data suggested that fish exhibited increased cortisol  
310 production at a higher temperature. In that work, we also observed that all genes involved in pathways related to  
311 stress evaluated (e.g., *gr*, *mr*, *crf*, *hsp*, etc.) were overexpressed at 21 °C compared to 16 °C. Nevertheless,  
312 contrasting results have been reported in other species such as the emerald rockcod (*Trematomus bernacchii*), in  
313 which a correlation between a temperature increase and changes in basal cortisol levels was not observed (Hudson  
314 et al. 2008), suggesting that this relationship may be, to some extent, species-specific. Overall, our results suggest  
315 that males and females of this species undergo a similar glucocorticoid regulation when exposed to high  
316 temperatures, though significantly more males are produced (75% at 21°C vs 46% at 16°C). This is further  
317 reinforced by a most recent study by the authors in which the genotype by environment interaction in the European  
318 sea bass was described (Geffroy et al. 2021a) and where more males were produced at high temperature (75% at  
319 21 °C vs 46% at 16 °C). In that study, involving in-depth RNA-sequencing, we found no evidence that Gene  
320 Ontologies of stress were differentially regulated between future males and future females based on their estimated  
321 genetic sex tendency at the 'all fins' stage (between 50 and 80 dph) (Geffroy et al., 2021a). This previous work  
322 rather supports the idea that energetic and epigenetic pathways, and not the stress axis, may be pivotal in the  
323 determination of sexual fate (Geffroy et al. 2021a).

324

325 Although blood cortisol is routinely and reliably used as a biomarker of stress (Mommsen et al. 1999), it has been  
326 shown that during chronic stress, circulating cortisol levels are likely to return to their basal concentrations after  
327 reaching their maximum levels if the application of the stressor is prolonged in time (Vijayan and Leatherland  
328 1990; Mommsen et al. 1999). Because the thermal treatments implemented during Experiment 1 had a relatively  
329 long duration, varying from 149 (23 °C) to 183 days (19 °C), it could well be that the blood cortisol levels  
330 measured are not representative of the real direct effect of the temperatures applied, having dropped after reaching  
331 their maximum levels, and that the effect on sex is masked due to the treatment duration. The length of the  
332 treatment period may also have impacted our statistical power to detect significant differences between treatments,  
333 as circulating cortisol levels would have been expected to rise upon a prolonged temperature increase, as reported

334 in other species (Madaro et al. 2018; Samaras et al. 2018; Kim et al. 2019). We did not observe such pattern in  
335 our data, in which mean cortisol levels were 2.4-fold higher in the fish exposed to 21 °C compared to those at 23  
336 °C. In the future, this issue could be overcome by the use of alternative stress biomarkers, for example, scale  
337 cortisol content (Aerts et al. 2015; Laberge et al. 2019; Samaras et al. 2021), as we did for the second experiment.  
338 Measurement of cortisol concentrations in ontogenetic scales has been successfully employed previously as a  
339 precise proxy of chronic thermal stress (Goikoetxea et al. 2021). Therefore, even though measurement of  
340 circulating cortisol could be considered a limitation for our first experiment, data from this experiment are  
341 coherent with results emerging from our second experiment, in both cases reinforcing the hypothesis that there is  
342 no link between cortisol production and sex determination and/or differentiation in the European sea bass.

343

344 In addition to cortisol, we deemed important to study the regulators of the HPI axis, such as *gr1*, *gr2*, *mr*, and *crf*,  
345 in the hypothalamus, to confirm the relationship between stress and sex ratios. Like cortisol, no significant  
346 differences in expression were observed between males and females for any of the four genes measured,  
347 supporting the data obtained from the hormonal and histochemical analyses. The genes evaluated in this study  
348 were carefully chosen due to their well-studied role in the mediation of the stress response in fishes. When  
349 analysing the differences between thermal treatments, a pattern of expression upregulation as temperature  
350 increased was observed for *gr1* and *gr2*, although statistically significant differences between treatments were  
351 only observed for the latter (i.e., 19 °C vs 23 °C). This increase in expression across thermal treatments was  
352 expected, given the well-described link between cortisol and increased temperatures in other species, such as the  
353 olive flounder or the Atlantic salmon (*Salmo salar*) (Madaro et al. 2018; Kim et al. 2019). Moreover, our data  
354 correlates well with studies on rainbow trout (*Oncorhynchus mykiss*) involving the investigation of *gr1* mRNA  
355 expression during long-term cortisol exposure (Rosewicz et al. 1988; Yudit and Cidlowski 2002; Vijayan et al.  
356 2003). Contrary to these results, *gr1* was found to be downregulated in a different experiment involving European  
357 sea bass larvae maintained at 21 °C compared to those maintained at 16 °C (Goikoetxea et al. 2021). In that case,  
358 however, authors concluded that such differences were due to the younger age of the larvae analysed (i.e., flexion  
359 stage), as older and bigger larvae are predicted to produce a higher number of glucocorticoid receptors than their  
360 younger counterparts (Goikoetxea et al. 2021).

361

362 Unexpectedly, mean *mr* mRNA levels were observed to be virtually equal in the 21 °C-treatment fish compared  
363 to those maintained at 23 °C. Furthermore, for *crf*, mean values in the 21 °C-group were 1.54-fold higher than in  
364 the fish reared at 23 °C, a statistically significant difference. Higher *mr* expression as temperature increased was  
365 predicted and correlates well with the data observed for *gr1* and *gr2*. Indeed, it has been argued that cortisol  
366 affinity to *mr* could be even higher than that to the *grs* (Prunet et al. 2006). In the case of *crf*, our results were  
367 expected based on the lack of differential expression in circulating cortisol levels between males and females from  
368 the same experiment. While we might have expected the expression of this gene to peak in the fish reared at 23 °C  
369 when more males are induced, as previously observed in medaka (Castañeda Cortés et al. 2019), our gene  
370 expression data matches very well the steroid measurement of cortisol, where plasma cortisol concentration was  
371 observed to reach the highest recorded values also in the 21 °C-group, despite differences between treatments not  
372 being significant. Interestingly, no differences were observed in the expression of *crf* between males and females,  
373 as was previously observed in medaka (Castañeda Cortés et al. 2019), where both sexes respond equally to

374 environmental stress. Somehow, intriguingly, we detected two groups of individuals based on the expression level  
375 of *gr2* and *mr* that were markedly observable at 23 °C. Since all sexes were confounded in these two groups, one  
376 might wonder which intrinsic individual characteristics would drive this pattern. In fact, it could well be related  
377 to the personality of each individual, since both genes were shown to present higher expression levels in the brain  
378 of shy compared to bold individuals (Alfonso et al. 2019).

379

380 Considering the unchanged levels of cortisol in fish reared at different temperatures, we proceeded to analyse the  
381 distribution of two brain genes involved in sexual fate (Diotel et al. 2010; Castañeda Cortés et al. 2019). Gene  
382 *cyp19a1b* is the brain-specific paralogue of *cyp19a*, which resulted from a third whole-genome duplication unique  
383 to teleost fish (Holland and Ocampo Daza 2018). This duplicate gene is a critical element of sexual differentiation  
384 and sexual behaviour mechanisms at the level of the brain, and controls the local biosynthesis of oestrogens (Diotel  
385 et al. 2010; Thomas et al. 2019). In the present work, neural cells expressing *cyp19a1b* were found to be primarily  
386 located in the periventricular region of the brain, specifically in the olfactory bulb, the telencephalon and preoptic  
387 area, the posterior tubercle, the ventral hypothalamus, the lateral recess, the posterior recess, and the optic tectum.  
388 The neural localisation of *cyp19a1b* was not affected by the sex of the individuals evaluated or by the thermal  
389 treatment applied (16 °C vs 21 °C, Experiment 2). The distribution pattern of *cyp19a1b* observed in this study  
390 globally agrees with the *cyp19a1b* mapping by immunohistochemistry generated by Diotel and colleagues (2016)  
391 on the brain of zebrafish (*Danio rerio*), as well as of the African Catfish (*Clarias gariepinus*) (Timmers et al.  
392 1987). However, most studies on *cyp19a1b* to date have focused on the localisation and/or activity of this gene  
393 without taking into account that differences between males and females may exist. For this reason, in the future,  
394 comparative approaches between sexes may help elucidate the differential organisation, regulation and function  
395 of *cyp19a1b* during fish sex differentiation. Likewise, the neuroanatomical analysis of brain cells expressing *crf*  
396 revealed that their localisation did not vary based neither on sex nor on temperature. These cells were  
397 predominantly located in the ventral and dorsal telencephalon, preoptic area, ventral hypothalamus, pretectum,  
398 paraventricular organ, optic tectum and glomerular nuclei. This distribution was similar to reports in male adult  
399 zebrafish (Alderman and Bernier 2007). Again, although the localisation of *crf* in the fish brain has been evaluated  
400 for several species (Olivereau et al. 1984; Vallarino et al. 1989; Alderman and Bernier 2007), most studies fail to  
401 discuss potential differences between sexes. The differential localisation of *crf* between males and females was,  
402 however, investigated in the European eel (*Anguilla anguilla*), in which male silver and female yellow eels were  
403 observed to have a similar distribution of *crf* (Olivereau and Olivereau 1988). Due to the great importance of *crf*  
404 release following a stressful event, had the thermal-induced cortisol release had an effect on sex, we would have  
405 expected to see this reflected in the histochemical analysis. Overall, our findings are coherent with data from a  
406 recent study showing no bias in whole-body cortisol in individuals sampled during the labile period for sex  
407 determination, individuals which originated from groups in which an effect on sex ratios was observed (Geffroy  
408 et al. 2021b). In that work, Geffroy and colleagues (2021b) demonstrated that not only temperature but also other  
409 EE, such as density, can also affect sex ratios in the European sea bass. However, following measurement of  
410 cortisol release they reported, in agreement with our observations, that there was no link between cortisol  
411 production and sex bias at the group level, providing further support that cortisol does not mediate the  
412 determination of sexual fate in this dazzling species.

413

414 **5. Conclusions**

415

416 In this study, we demonstrated that cortisol does not have a major impact over sexual fate in European sea bass  
417 in early stages of development. The temperature treatments used during our experiments included known  
418 thermolabile periods of European sea bass sex determination. Nevertheless, an effect of cortisol release on the sex  
419 of each individual was not observed in any of the two experimental set-ups, nor with any of the approaches  
420 (hormonal, histochemical, molecular) employed. Ultimately, this suggests that the relevance attributed to cortisol  
421 in the redirection of sexual fate in gonochoristic fishes may not be a general mechanism in this group of  
422 vertebrates. Why the maximum levels of circulating cortisol and the highest hypothalamic expression of *mr* and  
423 *crf* did not occur in the fish undergoing the highest thermal treatment should be investigated in the future.  
424 Moreover, whenever possible, we encourage the use of scale cortisol as a biomarker of chronic thermal stress.  
425 Future comparative studies should shed light on this knowledge gap. Based on our work, we encourage the shift  
426 in the focus in the investigation of the pathways underlying sex determination and sex reversal to alternative  
427 proposed mechanisms (e.g., epigenetic reprogramming, energy dynamics, calcium redox regulation) (Todd et al.  
428 2019; Ortega-Recalde et al. 2020; Sakae et al. 2020; Castelli et al. 2020). Studying the determination of sexual  
429 gonadal fate as a continuous process in which different effectors can contribute together or with different  
430 strategies, depending on the species, may hold the key to the full understanding of these fascinating mechanisms.

431

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433

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441

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443 This project was approved by the Animal Care Committee # 36 COMETHEA under project authorisation numbers  
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445

446 Consent to participate:

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448

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451

452 Availability of data and material/ Data availability:

453 All data generated or analysed during this study are included in this published article.

454 Code availability:

455 The code used during analysis in the current study is available from the corresponding author on reasonable  
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457

458 Authors' contributions:

459 B.G., F.A., and M.V. designed research; A.G., A.S., C.H., O.M., S.H., F.C., J.A., E.B.B., and B.G. performed  
460 research; A.G., A.S., C.H., J.A., E.B.B., and B.G. analysed data; A.G., A.S., C.H., J.I.F., and B.G. wrote the  
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462

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654

655 **Figure legends:**

656

657 **Table 1** List of specific primers used for European sea bass hypothalamus gene expression: sequences, GenBank  
658 accession numbers and amplicon sizes

659

660 **Table 2** Specific primers used for RNA riboprobe synthesis: sequences, GenBank accession numbers and  
661 amplicon sizes

662

663 **Fig. 1** Experimental design for (A) Experiment 1 and (B) Experiment 2, assessing the effect of different  
664 temperatures (19, 21 and 23 °C, Experiment 1; 16 and 21 °C, Experiment 2) on the sex of European sea bass  
665 during its developmental process. Complementary information is available in the Materials and Methods section

666

667 **Fig. 2** Principal component analysis (PCA) showing clustering of sex in Experiment 1 and Experiment 2, based  
668 on the expression of *cyp19a1a* and *gsdf*. In both PCAs, the principal component 1 explains most of variation (>  
669 84%). Fish with a positive comp1 value are considered female, whereas those with a negative comp1 value are  
670 considered male. Individuals considered intersex are enclosed in a dashed rectangle

671

672 **Fig. 3** Cortisol content in plasma collected from European sea bass exposed to three temperatures (Experiment  
673 1). Plasma from 7, 13 and 11 fish was collected at 19, 21 and 23 °C, respectively, and cortisol levels were  
674 measured. Males are represented by squares and females by circles

675

676 **Fig. 4** Cortisol content (µg/mg) in ontogenetic scales of fishes from Experiment 2

677

678 **Fig. 5** Hypothalamic gene expression analysis of *gr1*, *gr2*, *mr* and *crf* from European sea bass individuals kept at  
679 19, 21 or 23°C. Values are shown as normalised relative to the geometric mean of reference genes *eef1a*, *l13* and  
680 *β-actin*. Letters denote a statistically significant difference between treatments. Males are represented by squares  
681 and females by circles

682

683 **Fig. 6** Panel A represents the lateral view of the sea bass brain. Lettered lines indicate the level of representative  
684 transverse sections shown in B-H taken from the *Dicentrarchus labrax* brain atlas (Cerdá-Reverter et al. 2001a,  
685 b, 2008). B-H represent schematic drawings of rostrocaudal transverse sections showing the location of cells  
686 expressing *cyp19a1b* (small grey dots on the right side) and *crf* (big black dots on the left side), respectively. Scale  
687 bars = 1 mm. See Abbreviation list for the nomenclature of brain nuclei

688

689 **Fig. 7** Neuroanatomical localisation of representative *cyp19a1b* expressing sites in European sea bass brain. Cells  
690 containing *cyp19a1b* are revealed by *in situ* hybridisation in the periventricular regions of the ventral  
691 telencephalon (Vv) (picture A) and the preoptic area (NPO, NPOpc, NPOav, PM) (pictures A-B). Pictures C-E  
692 show *cyp19a1b* containing cells in the central posterior thalamic nucleus (CP) and in the ventral region in the  
693 periventricular nucleus of the posterior tuberculum (TPp), the nucleus posterioris periventricularis (NPPv) and  
694 the anterior tuberal nucleus (NAT). In a more posterior area, *cyp19a1b* expressions sites include the boundaries

695 of the paraventricular organ (PVO) and the lateral tuberal nucleus (NLT). Tiny *cyp19a1b* positive cells run along  
696 the structure of the lateral recess (NRL) (F). Scale bar = 100  $\mu\text{m}$

697

698 **Fig. 8** Photomicrographs showing representative *crf* expressing sites in the brain of European sea bass. The  
699 preoptic area (PMgc) and the anterior periventricular nucleus (NAPv) contain small populations of *crf* expressing  
700 cells (pictures A, C). Bigger *crf* containing cells are consistently observed in the glomerular (Nga), the central  
701 pretectal nuclei (NPC) and the lateral tuberal nuclei (NLT) (B-E). In a more periventricular region, the  
702 periventricular nucleus of the posterior tuberculum TPp reveals *crf* cells as shown in Fig. 4F. The most posterior  
703 regions of the nucleus of lateral recess (NRL), and in the dorsal region, the nucleus of the medial longitudinal  
704 fasciculus (MLF) and the ventral periventricular pretectal nucleus (PPv) constantly host *crf* populations. Scale bar  
705 = 100  $\mu\text{m}$

706

707

## List of abbreviations

BSA, bovine serum albumin; Cc, corpus of the cerebellum; CE, cerebellum; CM, corpus mammillare; CP, central posterior thalamic nucleus; Dc2, area dorsalis telencephali, pars centralis subdivision 2; Dld, area dorsalis telencephali, pars lateralis dorsal; Dlp, lateral posterior part of the dorsal telencephalic area; Dlv2, area dorsalis telencephali, pars lateralis ventral, subdivision 2; Dm2, Dm3, Dm4, subdivisions 2, 3 and 4 of the medial dorsal telencephalic area; Dph, days post hatching; DWZ, deep white zone of the optic tectum; E, entopeduncular nucleus; FR, fasciculus retroflexus; HCo, horizontal commissure; IL, inferior lobe of the hypothalamus; LFB, lateral forebrain bundle; LT, nucleus lateralis thalami; MaOT, marginal optic tract; NAPv, anterior periventricular nucleus; NAT, anterior tuberal nucleus; NC, nucleus corticalis; NDII, lateral part of the diffuse nucleus; NGa, nucleus glomerulosus, pars anterioris; NGT, tertiary gustatory nucleus; NHd, dorsal habenular nucleus; NHv, ventral habenular nucleus; NLT, lateral tuberal nucleus; NLTd, dorsal part of the lateral tuberal nucleus; NLTi, inferior part of the lateral tuberal nucleus; NLTm, medial part of the lateral tuberal nucleus; NLTv, ventral part of the lateral tuberal nucleus; nMLF, nucleus of the medial longitudinal fasciculus; NPC, central pretectal nucleus; NPGa, anterior preglomerular nucleus; NPGc, nucleus preglomerulosus commissuralis; NPGI, nucleus preglomerulosus lateralis; NPGm, medial preglomerular nucleus; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of paraventricular organ; NPPv, nucleus posterioris periventricularis; NPT, nucleus posterior tuberis; nPVO, nucleus of the paraventricular organ; NRL, nucleus of the lateral recess; NRLd, dorsal part of the nucleus of the lateral recess; NRLl, lateral part of the nucleus of the lateral recess; NRLv, ventral part of the nucleus of the lateral recess; NRP, nucleus of the posterior recess; NT, nucleus taenia; nTPI, nucleus of the tractus pretectoisthmicus; OB, olfactory bulbs; OC, optic chiasm; OpN, optic nerve; OT, optic tectum; P, pituitary; PCo, posterior commissure; pgd, nucleus periglomerulosus dorsalis; Pin, pineal gland; PMgc, gigantocellular part of the magnocellular preoptic nucleus; PMmc, nucleus preopticus magnocellularis, pars magnocellularis; PMpc, nucleus preopticus magnocellularis, pars parvocellularis; POA, preoptic area; PPd, dorsal periventricular pretectal nucleus; PPv, ventral periventricular pretectal nucleus; PSm, nucleus pretectalis superficialis, pars magnocellularis; PSp, parvocellular superficial pretectal nucleus; PVO, paraventricular organ; SV, saccus vasculosus; TEG, tegmentum; TEL, telencephalon; TLa, nucleus of the torus lateralis; TLo, torus longitudinalis; TPp, periventricular nucleus of the posterior tuberculum; TSl, torus semicircularis, pars lateralis; Tsv, torus semicircularis pars ventralis; VAO, ventral accessory optic nucleus; Vc, central nuclei of the ventral telencephalon; VCe, valvula of the cerebellum; VI, area ventralis telencephali, pars lateralis; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventral optic tract; Vp, area ventralis telencephali, pars postcommissuralis; Vv, ventral nuclei of the ventral telencephalon.

Table 1

From: [Natural cortisol production is not linked to the sexual fate of European sea bass](#)

| Gene            | GeneBank accession numbers | Primers  | Primer sequence 5' to 3' | Amplicon size (bp) | Efficiency | Bibliography                 |
|-----------------|----------------------------|----------|--------------------------|--------------------|------------|------------------------------|
| <i>cyp19a1a</i> | DQ177458                   | cyp19a-F | AGACAGCAGCCCAGGAGTTG     | 101                | 1.97       | Navarro-Martín et al. (2011) |
|                 |                            | cyp19a-R | TGCAGTGAAGTTGATGTCCAGTT  |                    |            |                              |
| <i>gsdf</i>     | DLAgn_00083310             | gsdf2-F  | TCCATCATCCCACACCAACG     | 168                | 1.99       | Vandeputte et al. (2020)     |
|                 |                            | gsdf2-R  | ATGTTGCCATGTTCACAGCC     |                    |            |                              |
| <i>gr1</i>      | AY549305.1                 | gr1-F    | GAGATTTGGCAAGACCTTGACC   | 401                | 1.915      | Pavlidis et al. (2011)       |
|                 |                            | gr1-R    | ACCACACCAGGCGTACTGA      |                    |            |                              |
| <i>gr2</i>      | AY619996                   | gr2-F    | GACGCAGACCTCCACTACATTC   | 403                | 1.683      | Pavlidis et al. (2011)       |
|                 |                            | gr2-R    | GCCGTTCATACTCTCAACCAC    |                    |            |                              |
| <i>mr</i>       | JF824641.1                 | mr-F     | GTTCCACAAAGAGCCCCAAG     | 197                | 1.938      | Sadoul et al. (2018)         |
|                 |                            | mr-R     | AGGAGGACTGGTGGTTGATG     |                    |            |                              |
| <i>crf</i>      | JF274994.1                 | crf-F    | GCAACGGGGACTCTAACTCT     | 217                | 1.956      | Alfonso et al. (2019)        |
|                 |                            | crf-R    | GTCAGGTCCAGGGATATCGG     |                    |            |                              |
| <i>eef1a</i>    | AJ866727.1                 | eef1a-F  | AGATGGGCTTGTCAAGGGA      | 167                | 1.965      | Sadoul et al. (2018)         |
|                 |                            | eef1a-R  | TACAGTTCCAATACCGCCGA     |                    |            |                              |
| <i>l13</i>      | DLAgn_00023060             | l13-F    | TCTGGAGGACTGTCAGGGGCATGC | 148                | 2.023      | Sadoul et al. (2018)         |
|                 |                            | l13-R    | AGACGCACAATCTTGAGAGCAG   |                    |            |                              |
| $\beta$ -actin  | AY148350.1                 | act1-F   | TGACCTCACAGACTACCT       | 176                | 1.795      | Martins et al. (2015)        |
|                 |                            | act1-R   | GCTCGTAACTCTTCTCCA       |                    |            |                              |

Table 2

From: [Natural cortisol production is not linked to the sexual fate of European sea bass](#)

| Gene            | GeneBank accession numbers | Primers       | Primer sequence 5' to 3' | Amplicon size (bp) | Bibliography |
|-----------------|----------------------------|---------------|--------------------------|--------------------|--------------|
| <i>crf</i>      | JF274994.1                 | sbHIS_CRF_F   | ACCGTGATTCTGCTAGTTGC     | 475                | This study   |
|                 |                            | sbHIS_CRF_R   | CGAAGAGCTCCATCATTCTT     |                    |              |
| <i>cyp19a1b</i> | AY138522.1                 | sbHIScyp19b_F | TGAGGTTTCATCCTGTGGTT     | 913                | This study   |
|                 |                            | sbHIScyp19b_R | ATCCCAGTGTGTGCTGAAAT     |                    |              |

Figure 1

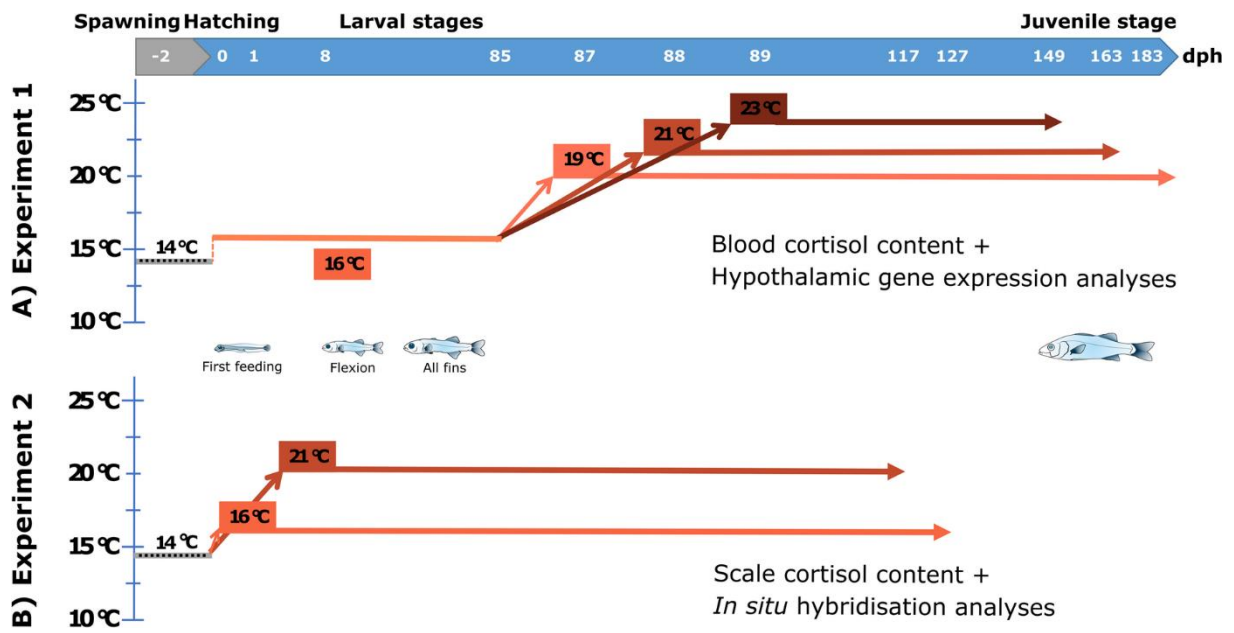


Figure 2

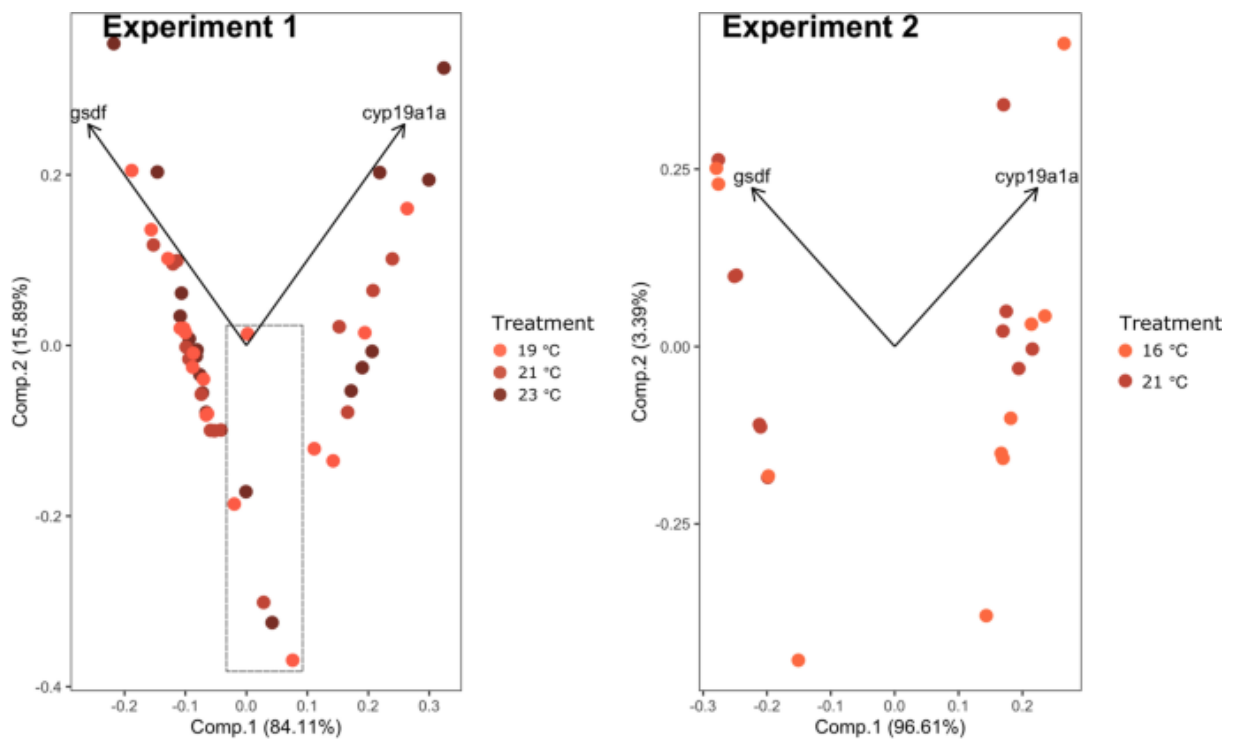






Figure 4

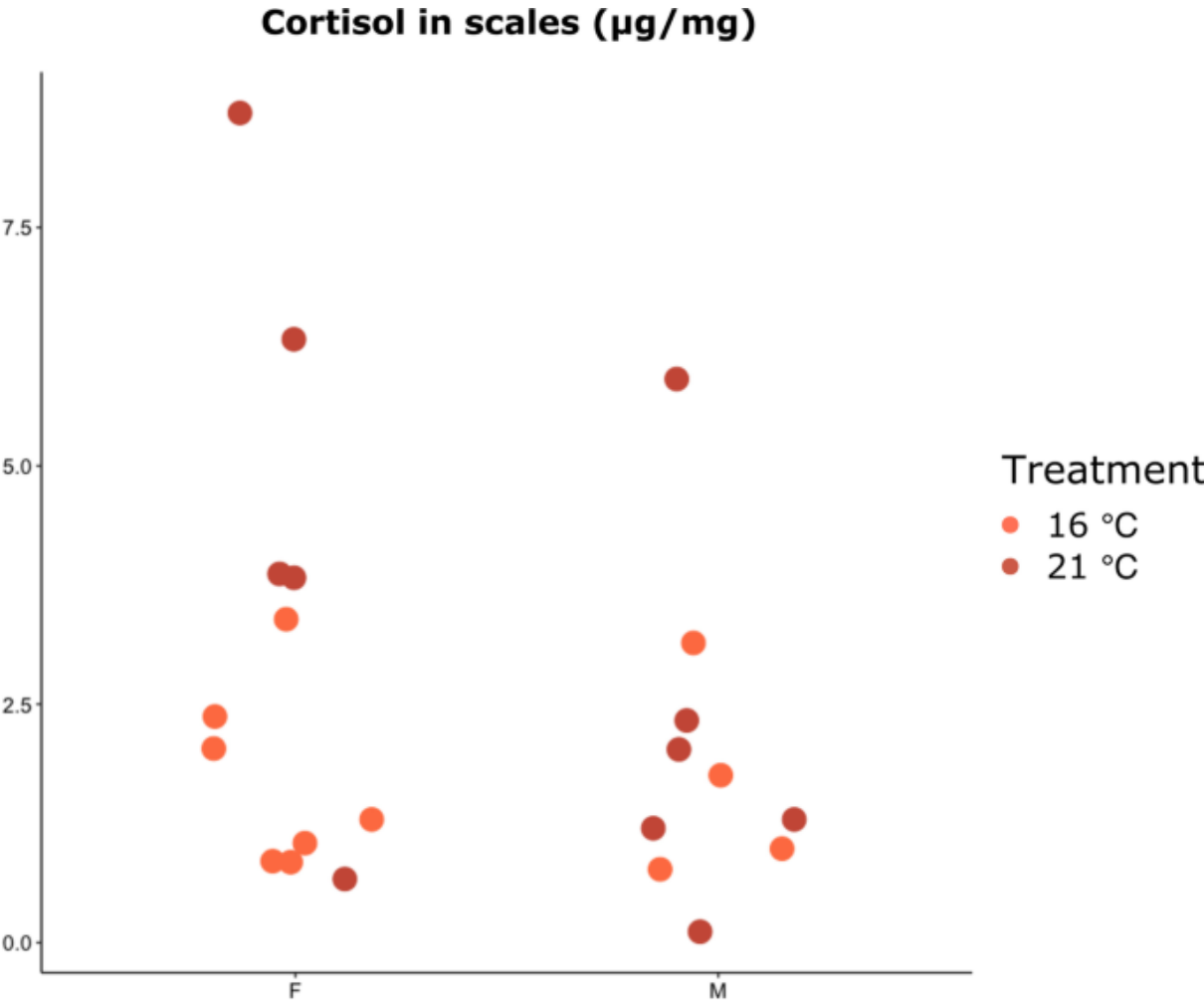


Figure 5

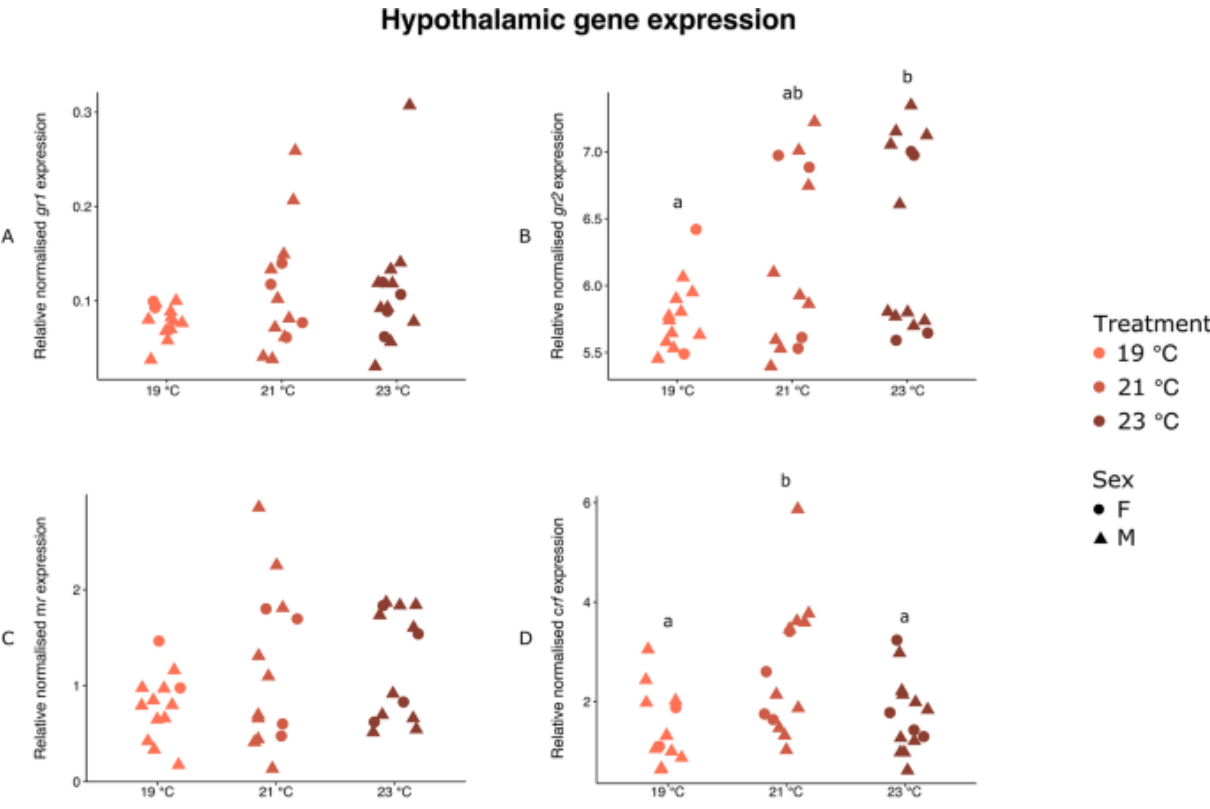




Figure 7

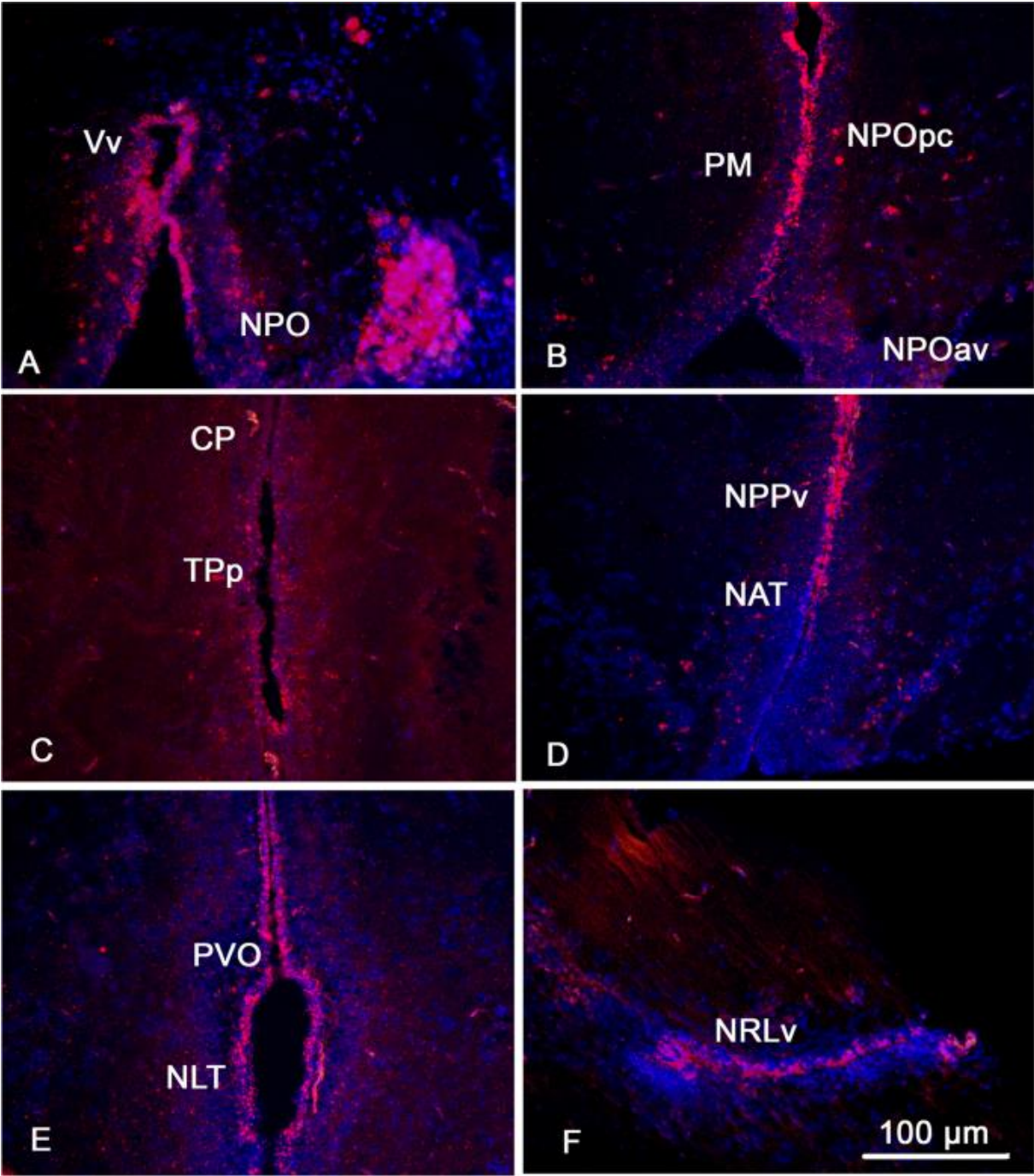


Figure 8

