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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 ℃, 21 ℃ and 23 ℃, experiment 1; 16 ℃ and 21 ℃, 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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Source of fish and experimental designs

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

In Experiment 2, the fish population resulted from a complete factorial mating design with eight males and one female from a West Mediterranean Sea strain of European sea bass, performed by artificial fertilisation (March 22nd, 2017). Eggs were then evenly distributed in six tanks of 500 L each, and temperature was gradually increased from14 °C to 16°C in the first 24h. Fish density after hatching was 50 larvae per litre. Then, larvae were maintained at 16 °C (in triplicates) or exposed to 21 °C (in triplicates) as described in Geffroy et al. (2021a) and Goikoetxea et al. (2021). For the 21 °C-treatment, temperature was increased from 14 °C to 21 °C during the first 8 dph (Fig. 1B). Experiment 2 targeted the early temperature sensitivity window, whereby colder temperatures induce a higher proportion of females. For Experiment 2, each thermal treatment was maintained until sampling when fish in each group reached a body length of approximately 7.2 cm and 4.5g, at 127 dph (16 °C) and 117 dph (21 °C), respectively, marking the end of the experiment. For both experiments, fish were fed Artemia nauplii for 40 days starting at 10 dph, then weaned onto a commercial sea bass diet (Pro Start and Pro Wean, BioMar). Fish

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

2.2. Sexing of fish

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

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

2.3. Plasma cortisol assessment

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

2.4. Scale cortisol assessment

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

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

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

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

2.6. qPCR gene expression analyses

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

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

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

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

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

2.8. Statistical analyses

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

3. Results

3.1 Fish sexing

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

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

3.2 Plasma and scale cortisol

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

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

3.3 Hypothalamic expression of genes involved in the glucocorticoid pathway

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

3.4 Neuroanatomical localisation of cells expressing cyp19a1b and crf

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

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

nucleus gustatorius tertius (NGT, Figs. 6G and 8E). The central pretectal nuclei also contained few oval *crf* cells (NPC, Figs. 6F and 8C-E). Tiny *crf* positive cells were observed into the mesencephalic optic tectum (OT), longitudinal torus (TLo) as well as i the ventral(TSv) and lateral (TSl) subdivisions of the semicircular torus (OT, TLo, TSI; Figs. 6G-H).

4. Discussion

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

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

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

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

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

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

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

5. Conclusions

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

Declarations:

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Consent to participate:
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Consent for publication:
Not applicable.

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

Code availability:

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

Authors' contributions:

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 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 manuscript. All authors read and approved the final manuscript.

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Figure legends:

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

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

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

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

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

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

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

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

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

of the paraventricular organ (PVO) and the lateral tuberal nucleus (NLT). Tiny *cyp19a1b* positive cells run along the structure of the lateral recess (NRL) (F). Scale bar = 100 µm

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

List of abbreviations

BSA, bovine serum albumin; CCe, 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; NDLIl, 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; NPGl, nucleus preglomerulosus lateralis; NPGm, medial preglomerular nucleus; NPOav, anteroventral part of the parvocelullar 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 recces; NRLv, ventral part of the nucleus of the lateral recess; NRP, nucleus of the posterior recces; 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
cyp19a1a	DQ177458	cyp19a-F	AGACAGCAGCCCAGGAGTTG	101	1.97	Navarro-Martín et al. (2011)
		cyp19a-R	TGCAGTGAAGTTGATGTCCAGTT			
gsdf	DLAgn_00083310	gsdf2-F	TCCATCATCCCACACCAACG	168	1.99	Vandeputte et al. (2020)
		gsdf2-R	ATGTTGCCATGTTCACAGCC			
gr1	AY549305.1	gr1-F	GAGATTTGGCAAGACCTTGACC	401	1.915	Pavlidis et al. (2011)
		gr1-R	ACCACACCAGGCGTACTGA			
gr2	AY619996	gr2-F	GACGCAGACCTCCACTACATTC	403	1.683	Pavlidis et al. (2011)
		gr2-R	GCCGTTCATACTCTCAACCAC			
mr	JF824641.1	mr-F	GTTCCACAAAGAGCCCCAAG	197	1.938	Sadoul et al. (2018)
		mr-R	AGGAGGACTGGTGGTTGATG			
crf	JF274994.1	crf-F	GCAACGGGGACTCTAACTCT	217	1.956	Alfonso et al. (2019)
		crf-R	GTCAGGTCCAGGGATATCGG			
eef1a	AJ866727.1	eef1a-F	AGATGGGCTTGTTCAAGGGA	167	1.965	Sadoul et al. (2018)
		eef1a-R	TACAGTTCCAATACCGCCGA			
l13	DLAgn_00023060	l13-F	TCTGGAGGACTGTCAGGGGCATGC	148	2.023	Sadoul et al. (2018)
		l13-R	AGACGCACAATCTTGAGAGCAG			
β-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
crf	JF274994.1	sbHIS_CRF_F	ACCGTGATTCTGCTAGTTGC	475	This study
		sbHIS_CRF_R	CGAAGAGCTCCATCATTCTT		
cyp19a1b	AY138522.1	sbHIScyp19b_F	TGAGGTTTCATCCTGTGGTT	913	This study
		sbHIScyp19b_R	ATCCCAGTGTGTGCTGAAAT		

Figure 1

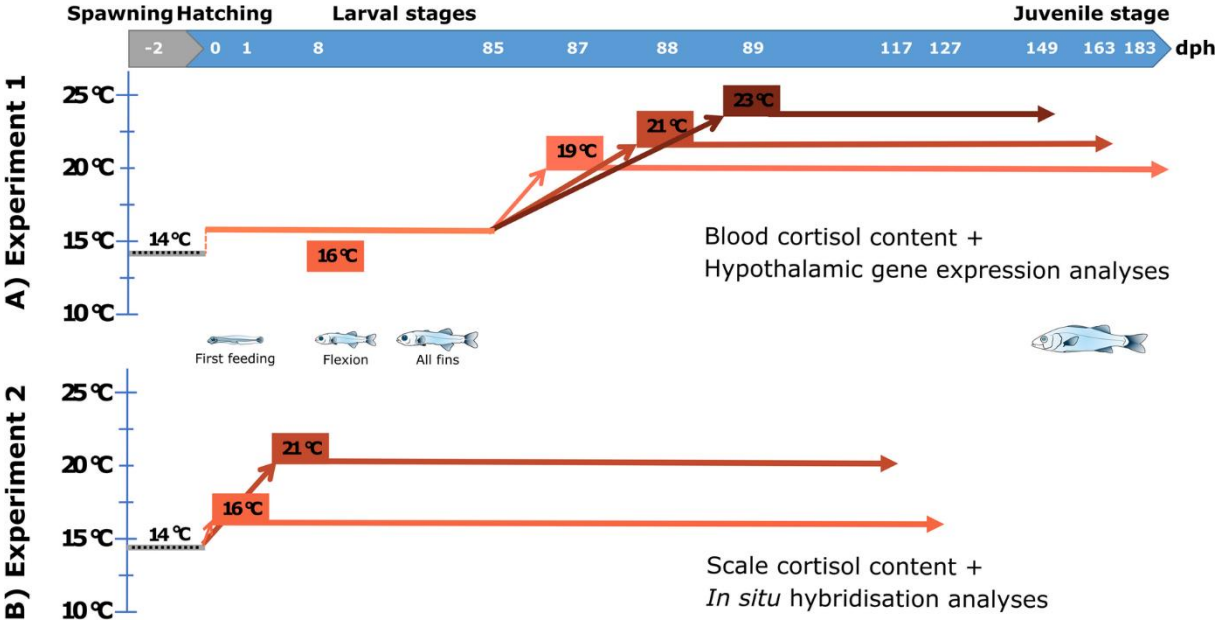


Figure 2

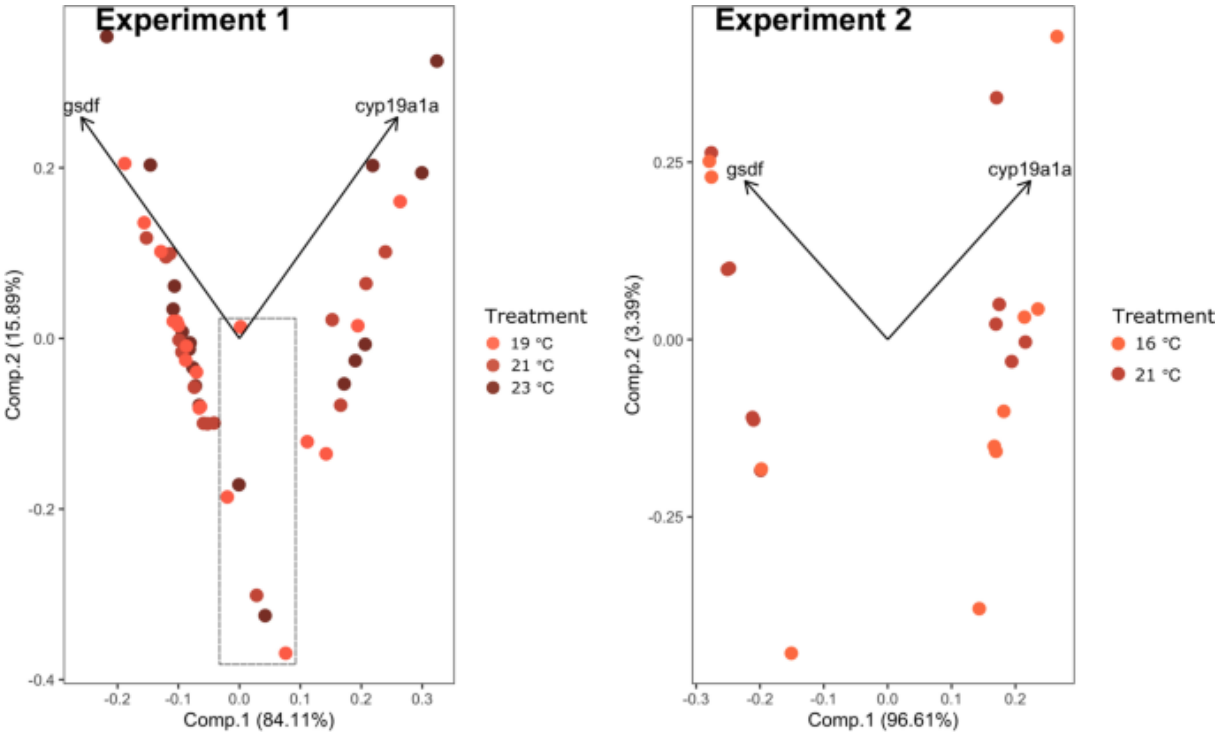


Figure 3

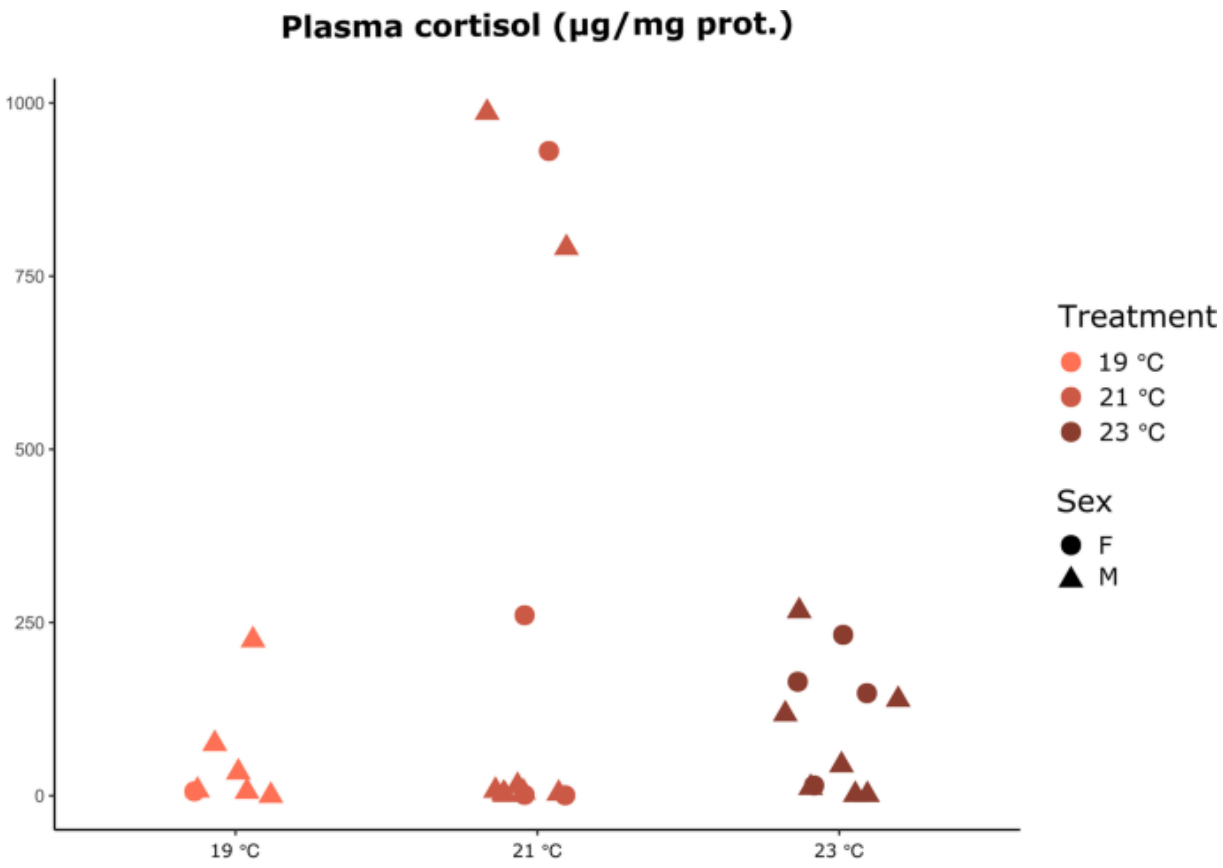

Plasma cortisol (µg/mg prot.)
1000
750
500
250
0
19 °C
21 °C
23 °C
Treatment
19 °C
21 °C
23 °C
Sex
F
M

Figure 4

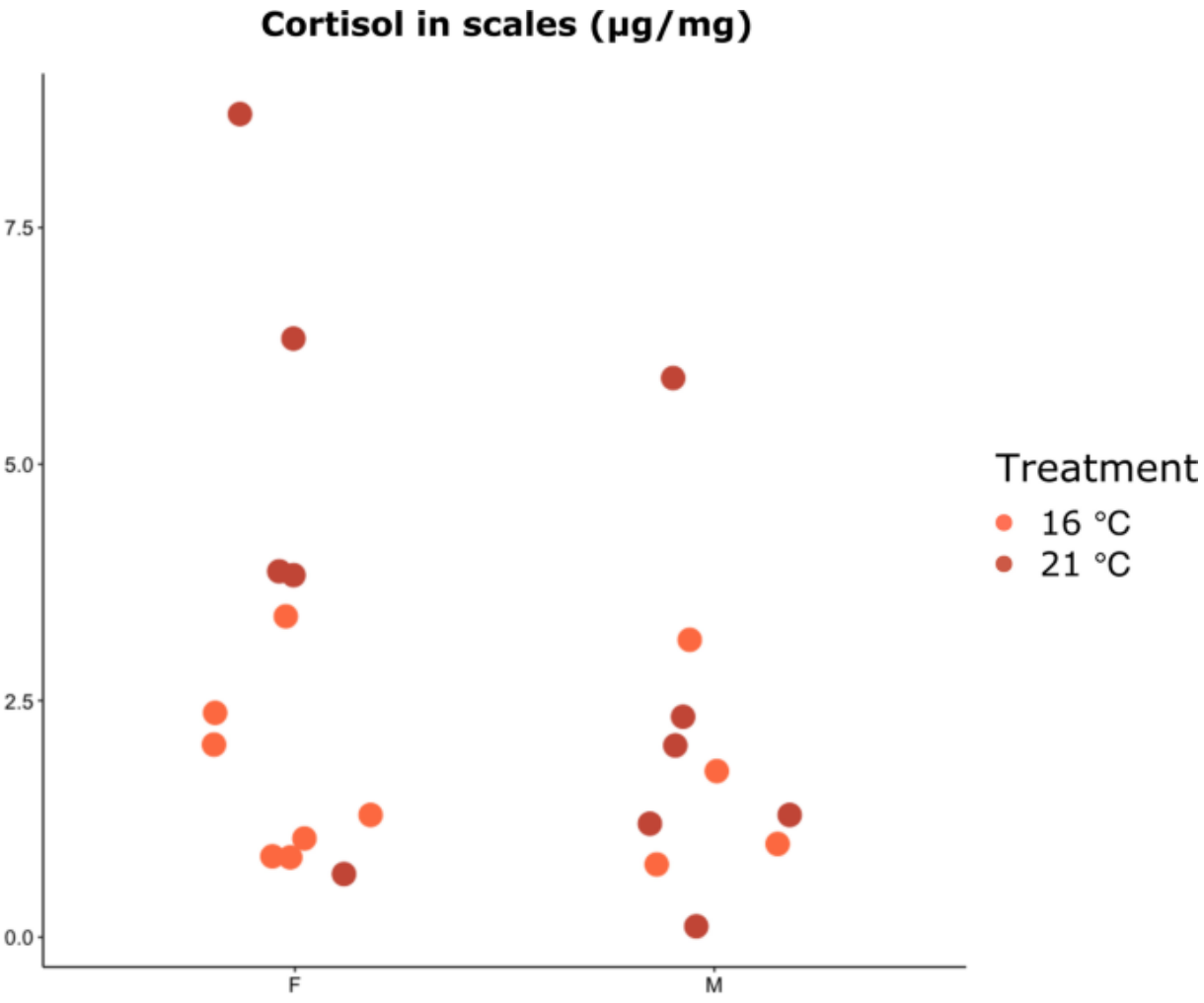


Figure 5

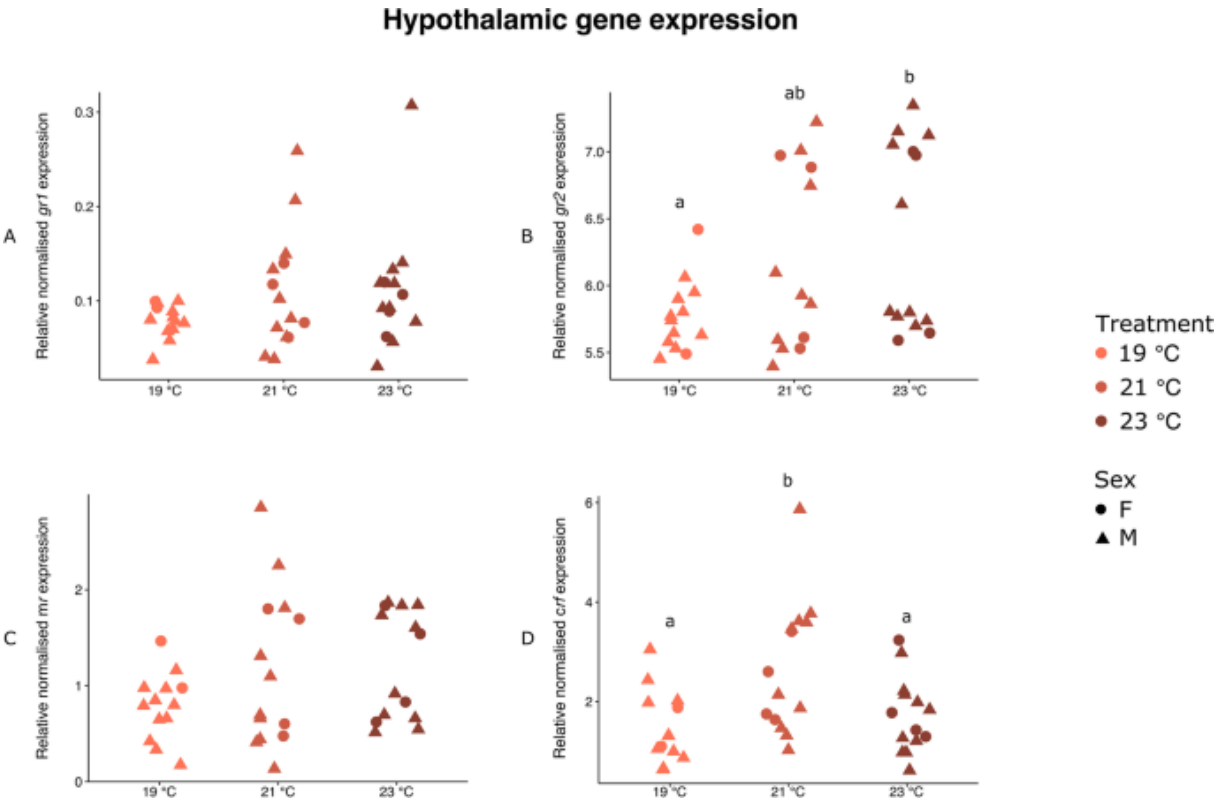
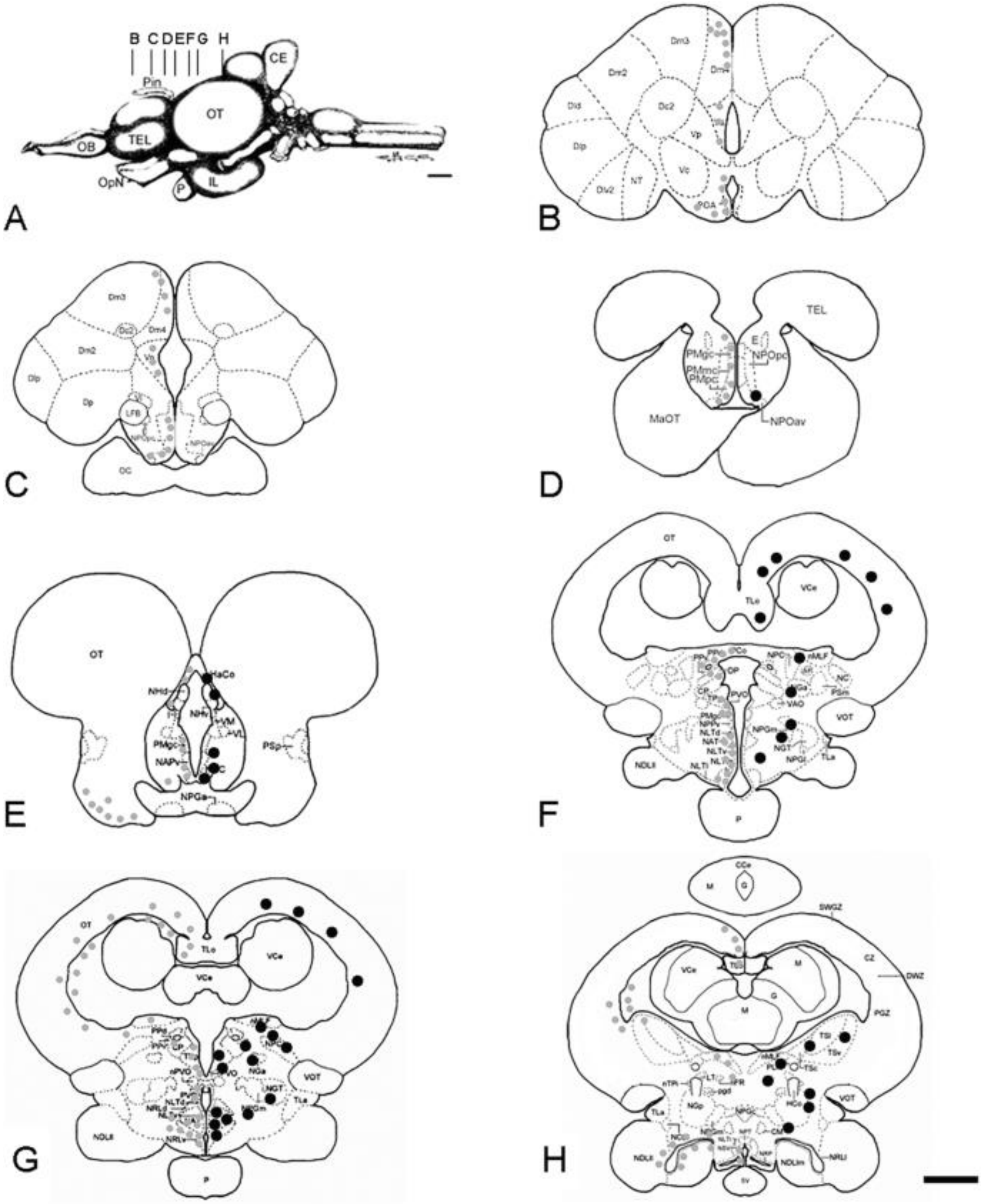

Hypothalamic gene expression
A
Relative normalised gr1 expression
0.3
0.2
0.1
19 °C
21 °C
23 °C
B
Relative normalised gr2 expression
7.0
6.5
6.0
5.5
a
ab
b
C
Relative normalised mr expression
2
1
0
D
Relative normalised crf expression
6
4
2
a
b
a
Treatment
19 °C
21 °C
23 °C
Sex
F
M

Figure 6



B C D E F G H
CE
Pin
OT
OB
TEL
OpN
P
IL
A
Dm3
Dm4
Dm2
Dc2
Dld
Dlp
Vp
Vc
NT
Dlv2
POA
B
Dm3
Dc2
Dm4
Dm2
Dlp
Dp
Vs
LFB
OC
C
TEL
E
PMgc
NPOpc
PMmci
PMpc
NPOav
MaOT
D
OT
NHd
HaCe
NHv
VM
VL
PMgc
NAPv
PSp
NPGa
E
OT
TLo
VCe
VOT
NDLII
P
F
OT
TLo
VCe
VCe
VOT
TLa
NDLII
P
G
CCe
M
G
SWGZ
CZ
DWZ
PGZ
VCe
M
G
M
VOT
TLa
NDLII
NDLIm
NRLl
SV
H

Figure 7

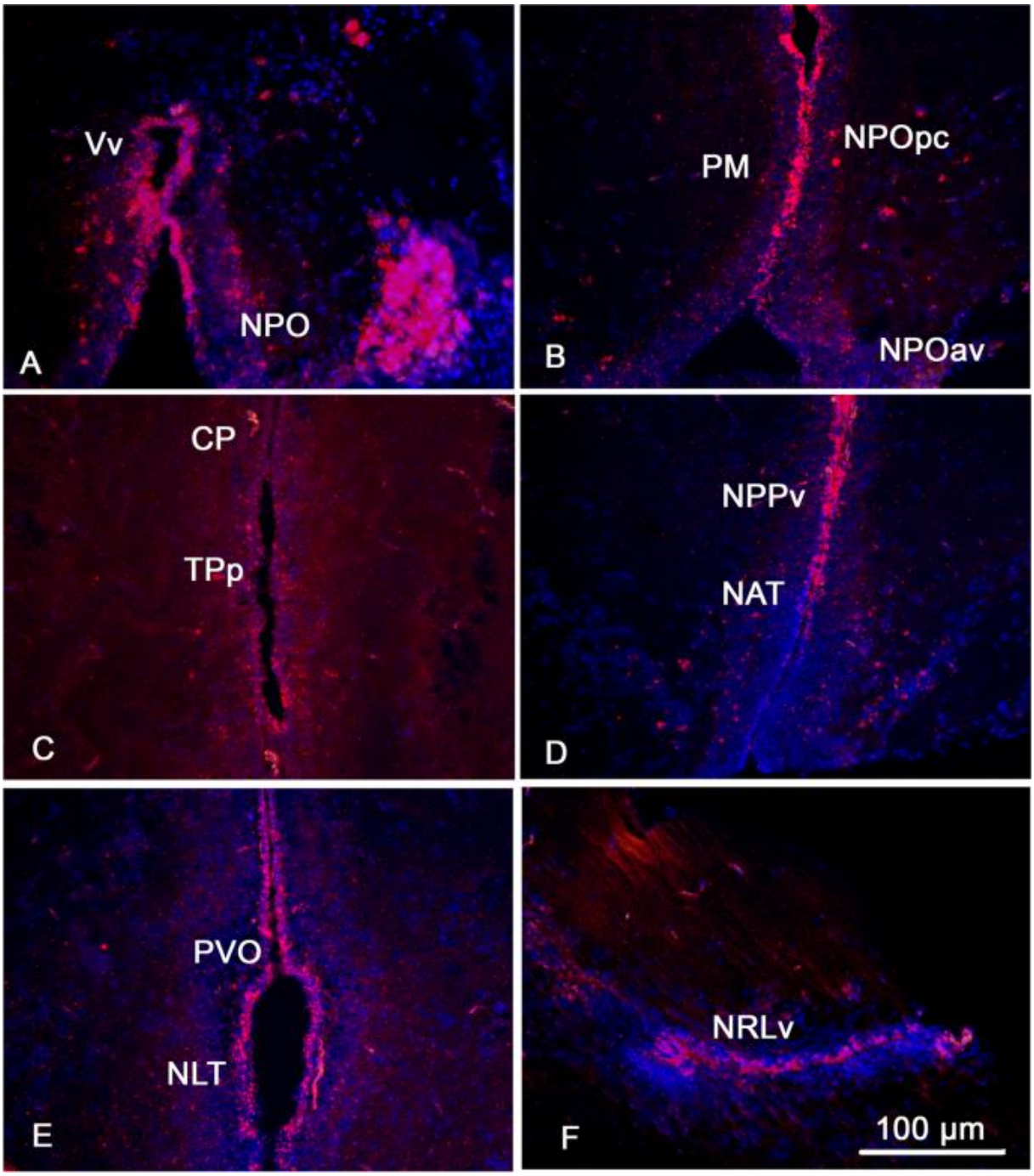
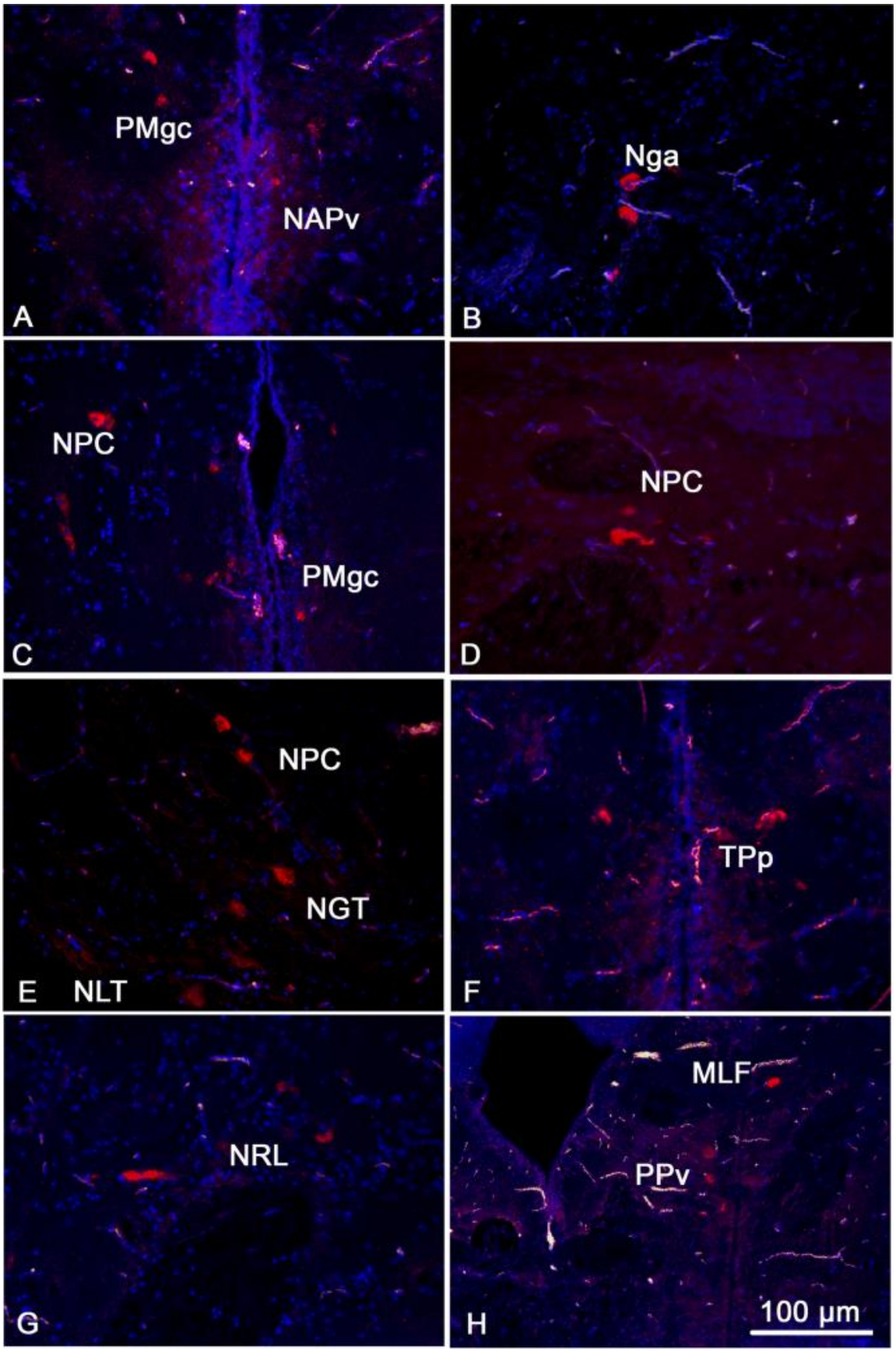

Vv
NPO
A
PM
NPOpc
B
NPOav
CP
TPp
C
NPPv
NAT
D
PVO
NLT
E
NRLv
F
100 µm

Figure 8


PMgc
NAPv
A
Nga
B
NPC
PMgc
C
NPC
D
NPC
NGT
E NLT
TPp
F
NRL
G
MLF
PPv
H
100 µm