ACTIONS OF SEX STEROIDS ON KISSPEPTIN EXPRESSION AND OTHER REPRODUCTION-RELATED GENES IN THE BRAIN OF THE TELEOST FISH EUROPEAN SEA BASS

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Summary Statement

Kisspeptins are attributed to be key factors in mediating gonadal steroid feedback in mammalian hypothalamus and in the teleost fish sea bass, suggesting that this property is conserved across vertebrates.

ABSTRACT

Kisspeptins are well known as mediators of the coordinated communication between the brain-pituitary axis and the gonads in many vertebrates. To test the hypothesis that gonadal steroids regulate *kiss1* and *kiss2* mRNA expression in European sea bass (a teleost fish), we examined the brains of gonad-intact (Control) and castrated animals, as well as castrated males (GDX) and females (OVX) that received testosterone (T) and estradiol (E_2) replacement, respectively, during recrudescence. In GDX males, low expression of *kiss1* mRNA is observed by *in situ* hybridization in the caudal hypothalamus (CH) and the mediobasal hypothalamus (MBH), although hypothalamic changes in *kiss1* mRNA levels were not statistically different among the groups as revealed by real-time PCR. However, T strongly decreased *kiss2* expression levels in the hypothalamus, which was documented in the MBH and the nucleus of the lateral recess (NRLd) in GDX T-treated sea bass males. Conversely, it appears that E_2 evokes low *kiss1* mRNA in the CH, while there were cells expressing *kiss2* in the MBH and NRLd in these ovariectomized females. These results demonstrate that kisspeptin neurons are presumably sensitive to the feedback actions of sex steroids in the sea bass, suggesting that the MBH represents a major site for sex steroid actions on kisspeptins in this species. Also, recent data provide evidence that both positive and negative actions occur in key factors involved in sea bass reproductive function including changes in the expression of *gnrh-1*/gonadotropin, *cyp19b*, *er* and *ar* genes and sex steroid and gonadotropin plasma levels in this teleost fish.

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1. Introduction

Kisspeptin has been found to play a key role in mediating gonadal steroid feedback to the GnRH neurons in the hypothalamus (Clarkson et al., 2010; Clarkson, 2013; Roa et al., 2008; Tena-Sempere, 2010). Evidence for this comes from castration and steroid replacement treatments carried out in rodents, sheep and primates and, to a lesser extent, in a few fish species (Kanda and Oka, 2013).

Castration in male rodents (Irwig et al., 2004; Smith et al., 2005a) and rhesus monkeys (*Macaca mulatta*, Zimmermann) (Shibata et al., 2007) is known to increase the expression of *Kiss1* mRNA in the arcuate nucleus (ARC) of the hypothalamus. Conversely, the transcriptional activity of *Kiss1* is decreased by testosterone (T), suggesting that *Kiss1* neurons play an active role in the negative feedback regulation of gonadotropin-releasing hormone (GnRH) and, in turn, gonadotropin secretion (luteinizing hormone, LH and follicle-stimulating hormone, FSH). Of note, in mice (*Mus musculus*, Linnaeus) (but not rats *Rattus rattus*, Linnaeus), *Kiss1* expressing neurons located in the hypothalamic anteroventral periventricular nucleus (AVPV) are reduced after castration and increase with T treatment (Kauffman et al., 2007; Smith et al., 2005a). Furthermore, estrogens influence the preovulatory LH surge observed in rodent females, indicating a positive feedback action of estradiol (E_2) that is mediated by *Kiss1* neurons at the AVPV (Adachi et al., 2007; Kauffman et al., 2007; Smith et al., 2005b, 2006; Smith, 2009). Importantly, it appears to be mediated through kisspeptins fibers that make connections to GnRH neurons, thus allowing that kisspeptin regulates GnRH secretion and finally, the stimulation of LH secretion (Smith, 2009). Of note, it has been suggested that the regulation of *Kiss1* mRNA in mice occurs via androgen (AR) and estrogen (ER) receptors (Smith et al., 2005a). In the case of ewes, *Kiss1* neurons in the ARC participate in both the negative and positive feedback mechanisms exerted by sex steroids, and in-turn on the control of GnRH-LH secretion. Ovariectomy stimulates *Kiss1* expression, while estrogen replacement after castration prevents this effect. On the other hand, an elevated *Kiss1* expression in the ARC is observed prior to the preovulatory GnRH/LH surge, thus showing that kisspeptin cells are able to respond to the estrogen positive feedback signals during this stage of the follicular phase (Estrada et al., 2006; Smith et al., 2007).

Little information regarding kisspeptin gene regulation is available for fish; however, it is known that *kiss1* (but not *kiss2*) neurons are positively regulated by ovarian estrogen at the nucleus ventral tuberis (NVT) in medaka (*Oryzias latipes*,

Temminck and Schlegel) (Kanda et al., 2009; Mitani et al., 2010). Furthermore, *kiss2* neurons are sensitive to E_2 in zebrafish (*Danio rerio*, Hamilton) at the dorsal (Hd) and ventral (Hv) hypothalamus (Servili et al., 2011), and up-regulation of *kiss2* neurons by ovarian estrogens was found to occur in the preoptic area (POA) of goldfish (*Carassius auratus*, Linnaeus) (Kanda et al., 2012). In ovariectomized female striped bass (*Morone saxatilis*, Walbaum), T replacement reduces the expression of kisspeptins, although this steroidal feedback effect apparently depends on the maturational stage of the animals (Klenke et al., 2011).

Since the reproductive physiology of European sea bass (*Dicentrarchus labrax*, Linnaeus.) is well documented (Carrillo et al., 1995, 2009), this species has emerged as an interesting teleost model to investigate the differential involvement of the two kisspeptin systems in controlling fish reproduction (Alvarado et al., 2013; Escobar et al., 2013a,b; Felip et al., 2009, 2015; Migaud et al., 2013). This species exhibits a clear stimulation of gonadotropin release following systemic kisspeptin administration (Felip et al., 2009), and more recently, intracerebroventricular injections of Kiss2 have demonstrated the involvement of this peptide in neuroendocrine regulation of gonadotroph activity (Espigares et al., 2015). Furthermore, a very close correlation between the wide distribution of Kiss2-positive fibers and that of *kiss-R2*-expressing cells (also known as *gpr54-2b* or *kissr3*) has been observed in the hypothalamus (Escobar et al., 2013b). So far, the majority of *kiss-1* neurons in the rostral mediobasal hypothalamus (MBH) have been shown to express estrogen receptors, while *kiss-2* neurons have been detected very close to *erβ2* expressing cells, although no cells coexpressing *kiss-2* and *erβ2* mRNA were detected (Escobar et al., 2013a). In addition, it has been reported that neurons expressing somatostatin (SRIF), tyrosine hydroxylase (TH), neuropeptide Y (NPY) and neuronal nitric oxide (nNOS) synthase are targets for kisspeptins, but not Gnrh-1 neurons, at least as direct targets of kisspeptins (Escobar et al., 2013b). In this context, the aim of the present work was to test the impact of T or E_2 implantation in early recrudescent male and female sea bass, respectively, on the expression of kisspeptin genes during the advanced recrudescence, depending on the physiological status of animals. Changes in the plasma levels of reproductive hormones and the expression of certain reproduction-related genes were also evaluated in groups of gonad-intact and castrated fish, as well as in castrated fish receiving steroid replacement.

2. Material and Methods

2.1. Animals

Adult male sea bass (n = 54) of 2 years of age with an average body weight (BW) of 152.37 g and females (n = 48) of 3-4 years of age with an average BW of 1034.5 g were obtained from stocks at the Instituto de Acuicultura de Torre de la Sal (IATS). All fish were individually tagged using passive integrative tags and maintained under natural photoperiod and temperature conditions at our facilities (IATS, Castellón, Spain, 40ºN 0ºE), where they were fed once a day.

2.2. Castration and steroid treatments

Fish were deeply anesthetized with 2-phenoxyethanol (0.5 ml/l of seawater) and the two gonads were dissected out through a 4-5-cm longitudinal incision in the abdominal cavity and sutured with a non-absorbable silk thread according to Crespo et al. (2013). For sham-operated fish (control), all steps were followed, except for the removal of the gonads. Operated fish were allowed to recover for 15 days, the sutures were removed and solid silastic implants (DowCorning, Midland, MI) prepared as previously described for sea bass (Zanuy et al., 1999) were administered via a small 2-3-mm incision in the abdomen in order to maintain high circulating steroid levels during seasonal gonadal development. Testosterone and estradiol were purchased from Sigma (St. Louis, MO). Males received one silastic implant, either empty or containing a T dose of 100 μg per gram of fish, while 50 μg of E_2 per gram of fish were administered to females. The day on which the implants were inserted was considered to be the start of the experiments (day 0). All experimental procedures involving the care and use of live animals were carried out according to the guidelines for animal experiments established by Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU. The local ethics committee (REGA-ES120330001055) approved this study.

2.3. Experimental design

Experiment 1. In mid October, coinciding with early testicular recrudescence (Carrillo et al., 1995), male sea bass (gonadosomatic index, GSI = 0.07±0.01%, n = 18 fish per group) were either subjected to a sham-operation (testis intact, Control group), gonadectomized (GDX group), or gonadectomized with T replacement (GDX+T group) (Fig. 1A). From October to February, blood was periodically sampled for hormone

analysis (see below). After 105 days of the treatment (February), male fish (n= 6-8 per group) were sacrificed. Examination of the gonads in the control fish indicated that males showed signs of gonadal recrudescence (GSI = 1.56±0.72%). Brains were collected and the pituitary was separated from the brain to permit dissection of the hypothalamus and telencephalon. All tissues were frozen on dry ice and stored at -80ºC until total RNA extraction. Changes in kisspeptin expression levels were evaluated by quantitative real-time PCR (qRT-PCR) in the whole hypothalamus and pituitary. Moreover, brains (n = 2 per group) were collected for *in situ* hybridization (ISH). In addition, changes in kisspeptin receptor mRNA levels and other reproductive-related genes were also analyzed.

Experiment 2. In mid October, coinciding with the onset of vitellogenesis (Prat et al., 1999), female sea bass (GSI = 0.77±0.15%, n = 16 fish per group) were either subjected to a sham-operation (ovary intact, Control group), ovariectomized (OVX group), or ovariectomized with E_2 replacement (OVX+E_2 group) (Fig. 1B). From October to December, blood was periodically sampled for subsequent hormone analysis, except for Lh levels, which were only measured at days 0, 30 and 60 after E_2 implantation. After 60 days of treatment, female fish (n= 4-6 per group) were sacrificed. Examination of the gonads in the control fish indicated that females showed signs of gonadal recrudescence (GSI = 4.88±0.89%). Brains were collected and processed as previously described in Experiment 1. Changes in kisspeptin expression levels were evaluated by quantitative real-time PCR (qRT-PCR) in hypothalamus and pituitary, while whole brains (n = 2 per group) were collected for ISH. Changes in kisspeptin receptor mRNA levels and other reproduction-related genes were also analyzed. In this study, we observed that some changes in mRNA levels were not statistically different among groups. They are described throughout the text for comparison, although they were not included in the data shown.

2.4. Hormone analysis

Blood samples were collected during the light phase (at 10:00 ± 1 hour) from the caudal vein. Plasma was separated by centrifugation at 4ºC and stored at -20ºC until analysis. Hormonal levels were measured by conventional enzyme immunoassays as described by Rodríguez et al. (2000) for T, Rodríguez et al. (2001) for 11-KT, Molés et al. (2008) for E_2, Mateos et al. (2006) for Lh and Molés et al. (2012) for Fsh.

2.5. RNA isolation and reverse transcription for qRT-PCR

The procedure for RNA isolation and reverse transcription for qRT-PCR has been previously described by Alvarado et al. (2013). The gene-specific primers and Taqman fluorogenic probes used in this study to evaluate changes in the mRNA levels of the two kisspeptin genes and their receptors, as well as *gnrh-1*/*gnrhr-II-1a*, were those described in Alvarado et al. (2013). Changes in mRNA levels of gonadotropin genes (*fshβ, lhβ*) were evaluated using the procedures described by Felip et al. (2008), while changes in expression levels of reproductive-related genes were measured according to the method used by Blázquez et al. (2008) for *cyp19b* (i.e., the gene that encodes P450 aromatase that is expressed in brain, also named *cyp19A2*) and García-López et al. (2011) for *erα* and *erβ1*. Specific primers for *ar* (M.J. Mazón, Estudio de la función de la hormona estimuladora del folículo de la lubina: su implicación en la espermatogénesis y sus rutas de señalización intracelular/Analysis of the function of follicle stimulating hormone in the sea bass: Its involvement in the spermatogenesis and signalling pathway, PhD Thesis, University of Murcia, Spain 2014; http://digitum.um.es/xmlui/handle/10201/42046) and *erβ2* (R. Rodríguez, A. Felip, S. Zanuy and M. Carrillo, unpublished data) were as follows: *ar* forward, CGG CTG AGG AGG TGT TTT GAA (200 nM); *ar* reverse, GTT TTT CTG TTG TCC AAT CTT CTT TAG TT (200 nM); *erβ2* forward, GTG GAC TCC AGA CTC GGG AC (200 nM); *erβ2* reverse, ATC ATG CTA GCC TCG GTG AAG (200 nM). PCR efficiency and amplicon size were 0.94 and 78 bp for the *ar* gene and 0.91 and 246 bp for the *erβ2* gene, respectively. All standards and experimental samples were run in duplicate. The sea bass elongation factor-1α (*ef1α*) gene was used as a control gene (Alvarado et al., 2013; Rocha et al., 2009). Data were expressed as relative values of mRNA for each target gene/mRNA *ef1α* (starting quantity mean ± standard error of the mean, SEM). Negative controls were also run for each real-time experiment.

2.6. *In situ* hybridization (ISH)

The ISH procedure has been previously described by Servili et al. (2011) and Escobar et al. (2013a). The whole brain was sectioned at 8 µm in 2 series of adjacent sections. All the slides of the two series were hybridized with probe for *kiss1* or *kiss2* mRNA detection, thus considering that one complete series of slides was representative of the all regions of the brain. The atlas of the European sea bass brain was used for the localization of *kiss1* and *kiss2*-expressing cells (Cerdá-Reverter et al., 2001a,b).

Micrographs were taken using an epifluorescence microscope (Olympus Provis) equipped with a DP71 digital camera. Images were then processed with Olympus Analysis Cell software and Photoshop CS4 (Adobe Systems, San Jose CA).

2.7. Statistical analysis

Data are represented as the mean ± SEM. Hormonal and gene expression levels were analyzed by a two- and one-way ANOVA, respectively, and a Holm-Sidak test, which was used for multiple comparison tests. Prior to analysis, values for gene expression levels were ln-transformed to meet normality and homoscedasticity requirements. All analyses were conducted using SigmaStat version 3.0 (SYSTAT Software Inc., Richmond, CA). Differences were considered to be significant when $P < 0.05$ (Sokal and Rohlf, 1981).

3. Results

3.1. Effects of castration and T replacement on plasma hormone levels in males

As expected, T plasma levels differed among the groups (Fig. 2A). While the control and GDX groups exhibited low circulating levels of T, castrated testosterone-implanted fish displayed higher levels, with a significant elevation 3 days after implantation and maximum values at day 7 (48.4 ± 4.73 ng ml^{-1}). Levels subsequently decreased on day 15 and remained constant until day 90, before returning to basal levels at the end of experiment (day 105). Plasma 11-KT levels in the controls exhibited a significant increase on day 60 (December) and remained high during spermatogenesis (January), before dropping significantly on day 105 (February), which corresponds to full spermiation in this species (Fig. 2B). The GDX group exhibited low levels of circulating 11-KT (≤ 2.06 ng ml^{-1}), while the GDX+T group showed a significant elevation after 3 and 7 days of implantation, as compared to the control and GDX groups. Levels increased again on days 60 and 90, although they remained significantly lower than in the control animals. Conversely, plasma Lh levels in control males showed maximum values on day 15, during mid November (3.32 ± 0.30 ng ml^{-1}), although they were not statistically different throughout the experiment (Fig. 2C). In GDX and GDX+T groups, the observed Lh levels were higher than those in the control group on day 0, they peaked on day 3 and then significantly dropped on day 7, to levels comparable to those of the control fish. These Lh levels remained low, with values around 1.23 ± 0.20 ng ml^{-1}, while T replacement maintained Lh levels fluctuating

around 1.89 ± 0.37 ng ml^{-1}. Changes in plasma levels of Fsh throughout the experiment were bimodal in the controls. Plasma Fsh levels were high during early October (day 3), followed by a significant decline on day 7. A second increase in plasma Fsh levels was observed at the end of November (day 30), coinciding with mid-testicular growth in this species. These levels remained high in December and then decreased until low levels were reached at full spermiation (January-February) (Fig. 2D). It is interesting to note that the observed Fsh levels in the GDX and GDX+T groups showed a significant decrease after 3 days of implantation, as compared to the control group. Along the same lines, Fsh levels in these two groups significantly decreased on day 30, coinciding with the second increase in Fsh levels in the control group. The results showed that castrated sea bass reached circulating Fsh values of up to 34.38 ± 1.60 ng ml^{-1}. Changes in plasma levels of Fsh in GDX+T showed an increase after 7 and 15 days of implantation, after which they proceeded to decrease.

3.2. Effects of castration and T replacement on the expression of *kiss/kissr* and *gnrh-1*/*gnrhr-II-1a* and gonadotropin genes in the brain of males

Changes in hypothalamic *kiss1* mRNA levels were not statistically different among the groups after 105 days of implantation (Fig. 3A). However, T replacement after castration evoked a dramatic decrease in *kiss2* expression in the hypothalamus between GDX+T and GDX groups, while castrated males showed *kiss2* mRNA levels that were comparable to those of the control animals (Fig. 3B). An *in situ* hybridization was performed to evaluate whether castration and T replacement treatment had impacted the distribution of kisspeptin-expressing neurons in specific areas of the brain in male sea bass. Populations expressing *kiss1* could be identified in the mediobasal hypothalamus (MBH) (Fig. 4A-C) and caudal hypothalamus (CH) (Fig. 4D-F). Although *in situ* hybridization results were not statistically analysed because of the low number of processed samples per group, it is interesting to report that a decrease in the number of *kiss1* positive cells were constantly noted in all the brain sections containing the MBH and CH of GDX animals (see Fig. 4B and 4E for representative pictures, respectively). Of note, *kiss1* cells in the dorsal habenular nucleus (NHd) did not show sex steroid sensitivity. Conversely, cells expressing *kiss2* mRNA were observed in the MBH (Fig. 4G-I) and the dorsal part of the nucleus of the lateral recess (NRLd) (Fig. 4J-O). Interestingly, the counting of the total number of the *kiss2* expressing cells visualized by *in situ* hybridization in all the sections containing the MBH (Fig. 4I) as

well as the anterior (Fig. 4L) and posterior NRLd (Fig. 4M-O) would suggest a decrease of *kiss2* expression in these brain regions of the GDX+T group compared to control. *kiss2* expression in the preoptic area (POA) was unaffected by T. In addition, relative expression levels of kisspeptin receptors in the hypothalamus failed to show significant differences among groups. Neither *kiss2* nor kisspeptin receptor mRNA levels in the pituitary showed steroid sensitivity, while *kiss1* transcripts were undetected in this study. Furthermore, no changes in mRNA levels of hypothalamic *gnrh-1* (Fig. 3C) or pituitary *gnrhr-II-1a* genes were observed. No significant changes were detected in mRNA levels of *lhβ* (Fig. 3D), but *fshβ* mRNAs significantly decreased in GDX+T (Fig. 3E).

3.3. Effects of castration and T replacement on the expression of *cyp19b*, *ar* and *er* genes in males

While no changes in hypothalamic *cyp19b* mRNA levels were detected among groups (data not shown), expression levels in the telencephalon were significantly increased by T after castration (Fig. 5A). Furthermore, hypothalamic *ar* expression significantly increased in GDX, while T replacement after GDX showed similar mRNA levels to those of the control group (Fig. 5B). Interestingly, we observed that *erβ1* mRNA levels (Fig. 5C) significantly increased in the hypothalamus in castrated males after T treatment, although no changes in *erα* and *erβ2* mRNAs were observed. At the pituitary level, GDX decreased *cyp19b* expression, while T replacement after GDX stimulated its expression to a level that was comparable to that of the control fish (Fig. 5D). Castration caused a significant decrease in *erα* expression that was restored by T (Fig. 5E). No differences in pituitary *ar*, *erβ1* and *erβ2* mRNAs were observed among the groups. It should be noted that *er* gene expression levels in the pituitary were higher than those in the hypothalamus (10^3- to 10^4-fold).

3.4. Effects of castration and E_2 replacement on plasma hormone levels in females

Circulating E_2 levels differed among the groups (Fig. 6A). While the control and OVX groups exhibited low circulating levels of E_2, post-ovariectomy estradiol-implanted fish displayed higher levels of this steroid, with an increase 3 days following implantation and maximum values at day 7 (15.4 ± 2.04 ng ml^{-1}). These levels remained high on day 15 and then decreased to circulating levels of around 1.87 ± 0.37

ng ml^{-1}. Lh levels measured on day 30 and 60 after treatment showed no significant differences among the groups (Fig. 6B). Conversely, control females exhibited plasma Fsh levels of around 25.4 ± 0.79 ng ml^{-1}, and ovariectomy significantly increased circulating Fsh levels up to 108.79 ± 11.46 ng ml^{-1}. Treatment with E_2 significantly decreased Fsh (Fig. 6C).

3.5. Effects of castration and E_2 replacement on the expression of *kiss/kissr* and *gnrh-1*/*gnrhr-II-1a* and gonadotropin genes in the brain of females

Changes in hypothalamic *kiss1* and *kiss2* mRNA levels were not statistically different among the groups after 60 days of implantation (Fig. 7A-B). *In situ* hybridization in females revealed *kiss1* expression in the MBH and the CH. Although E_2 replacement after OVX had no effect on *kiss1* expression in the MBH (data not shown), the counting of the total number of the *kiss1* expressing cells visualized by *in situ* hybridization in the CH (Fig. 8A-C) would suggest an decrease of *kiss1* expression in this brain region of female sea bass. On the other hand, cells expressing *kiss2* mRNA were detected in the MBH (Fig. 8D-F) and NRLd (Fig. 8G-I). In OVX animals the number of *kiss2* expressing cells were constantly reduced in all setions including the regions of the MBH (Fig. 8E) and NRLd (Fig. 8H), while estrogen replacement after OVX prevented this effect in both MBH (Fig. 8F) and NRLd (Fig. 8I) regions. E_2 had no effect on *kiss2* expression in the POA in ovariectomized sea bass. Furthermore, no changes in kisspeptin receptor mRNA levels were observed in any of the groups. Neither pituitary *kiss2* nor kiss receptor mRNA levels showed steroid sensitivity and *kiss1* transcripts were undetectable. We found that OVX elicited a significant elevation of *gnrh-1* expression in the hypothalamus (Fig. 7C), while OVX+E_2 and controls showed comparable mRNA levels. No significant variations in pituitary *gnrhr-II-1a* and *lhβ* (Fig. 7D) expression levels were observed among the groups, although *fshβ* transcripts significantly increased in OVX fish as compared to the OVX+E_2 group (Fig. 7E).

3.6. Effects of castration and E_2 replacement on *er* mRNA expression in females

OVX increased hypothalamic *erα* (Fig. 9A), *erβ1* (Fig. 9B) and *erβ2* (Fig. 9C) mRNA levels as compared to the control group, although not to levels that were statistically different from OVX+E_2. In the pituitary, castration significantly increased *erβ2* expression and E_2 replacement after OVX prevented this effect (Fig. 9D), but no

changes in *er*α and *er*β*1* were observed among groups. As in males, pituitary estrogen receptor mRNA levels were higher than those in the hypothalamus (10^3- to 10^4-fold) in females.

4. Discussion

This study examined the impact of castration and steroid replacement on the expression of the kisspeptin genes, as well as the potential relationships with other key reproductive parameters in adult sea bass (Table 1). After the gonads were properly removed, steroid analysis revealed the expected steroid plasma elevations following implantation. In order to establish the influence of long-term castration and understand the brain level at which steroids act, its potential role in the hypothalamus and pituitary was explored by analyzing changes in the expression levels of two kisspeptin genes, as well as of key reproductive-related genes (*gnrh-1*/*gnrhr-II-1a* pair, gonadotropin, *ar*, *er* and *cyp19b*) at one endpoint, once the implants were exhausted coinciding with recrudescence. The distribution and steroid regulation of kisspeptin-expressing neurons was also explored in this teleost fish species. Our findings show that kisspeptin neurons apparently exhibit sensitivity to sex steroids in sea bass, thus supporting the idea that this property is conserved among vertebrates (Oakley et al., 2009; Kanda and Oka, 2013). However, in order to better interpret these results, a number of issues should be considered for future experiments, including the possibility of using a second implant or distinct doses that might maintain plasma elevations of the expected steroid throughout the time course of the experiment, the time of sampling and a larger sample size to confirm these findings. Accordingly, due to circulating levels of T and E_2 were maximum 7 days after implantation in this study, in further studies would be interesting to analyze the impact of steroid administration at this sampling point, at least on the expression levels of two kisspeptin genes, in the experimental groups for comparison with the results of this work. All in all, to the extent of our knowledge, this is the first time that a negative feedback action of T on hypothalamic *kiss2* expression has been reported in a male adult fish. Here, we show that T replacement after castration decreases *kiss2* mRNA expression in the MBH and NRLd of male sea bass, while distribution of *kiss2* mRNA was found readily identifiable in these regions of the hypothalamus in GDX animals. According these findings, further studies will be needed to elucidate if T effects are or not due to its aromatization to E_2 in GDX+T by using aromatase inhibitors and/or non-aromatizable androgens. On the other hand, the

number of hypothalamic *kiss1* positive cells visualized by *in situ* hybridization in all the sections containing the CH and MBH of GDX+T would suggest a positive steroid feedback in these brain regions, although these observations were not statistically confirmed by relative mRNA expression levels. One possible explanation for the differences between qRT-PCT and ISH analysis could be a dilution effect due to the use of whole hypothalamus for qRT-PCR method, as ISH only reveals those specific kisspeptin cells in restricted hypothalamic nuclei. Our results reveal that MBH presumably represents a major site for sex steroid actions on kisspeptins in fish, as this brain region shows a noticeable sex steroid sensitivity of kisspeptin cells. Furthermore, the MBH appears to be responsible for both positive and negative feedback actions, at least in sea bass males. In this context, steroid-induced regulation of kisspeptin expression in the MBH of sea bass would be comparable to steroidal control of *Kiss1* expression in the ARC of female sheeps (ewes), it may also mediate both feedback signals (Estrada et al., 2006; Smith et al., 2007). Conversely, kisspeptin cells in the POA appear to lack sex steroid sensitivity in sea bass. The interpretation of the results in females was not so straightforward and several causes, as previously discussed, probably contributed to this situation. In medaka, only *kiss1* neurons in the nucleus ventralis tuberis (NVT) are positively regulated by ovarian estrogens via their coexpressed *er*α (Kanda et al., 2009), while estrogen treatment of juvenile zebrafish causes an increase in *kiss1* expression, although the effects on *kiss2* neurons were much more pronounced (Servili et al., 2011). However, it should be noted that *kiss2* expression is up-regulated by E_2 only in the POA of goldfish, showing colocalisation with all three types of *er* genes (Kanda et al., 2012). Recent work in female striped sea bass suggested that the kisspeptin system is not under gonadal control during early recrudescence; however, T treatment during mid-vitellogenesis reduces *kiss1* and *kiss2* expression, suggesting a negative steroidal feedback effect on this system in females (Klenke et al., 2011). Studies in mammals have established that only *Kiss1* neurons (the *Kiss2* gene was lost during the evolution of vertebrates (Akazome et al., 2010; Felip et al., 2009) mediate both negative and positive sex steroid feedback effects involving distinct hypothalamic nuclei, which are practically confined to the ARC and AVPV nuclei, respectively (Kanda and Oka, 2013). Regarding to steroid feedback actions on kisspeptin receptors, this study provides that sea bass kisspeptin receptor expression levels in the hypothalamus are unaffected by steroids. However, it has been reported that estradiol causes an increase in kiss receptors in zebrafish (Servili et al.,

2011), while T-replacement reduces the transcript levels in striped sea bass (Klenke et al., 2011). Comparisons between primates and rodents show that a lack of response to sex steroids occurs at the kiss receptor in the male monkeys (Shibata et al., 2007), while in rats, T influences *Kissr* mRNA levels following castration and steroid replacement (Navarro et al., 2004). Accordingly, some factors such as sex, gonadal stage and species (among others) are likely to influence the regulatory response of kisspeptin gene (Kitahashi and Parhar, 2013).

On the other hand, studies in mammals have also documented the close anatomical relationships between the *Kiss1* and *Gnrh-1* neurons, leading to the notion that Kiss1 neurons mediate the feedback signal from the gonad to GnRH (Bellefontaine et al., 2011; Oakley et al., 2009; Prevot, 2002; Radovick et al., 2012). However, in teleost fish, with the exception of one cichlid fish (Parhar *et al*. 2004), data from zebrafish (Servili et al., 2011), medaka (Kanda et al., 2012) and sea bass (Escobar et al., 2013b) all failed to confirm the presence of kiss receptors in *gnrh* neurons. Importantly, this situation suggests that other neurons distinct to those of Gnrh-1 are direct targets for kisspeptins. The present work shows that testosterone does not modify *gnrh-1*/*gnrhr-II-1a* transcript levels in GDX, suggesting the lack of any effect of sex steroids on the *gnrh-1* gene expression, at least, at the hypothalamus level during recrudescence in male sea bass. These results are in line with those recently reported on this species, which show that *gnrh-1* mRNA levels in the hypothalamus of males fluctuate during different gonadal stages (Alvarado et al., 2013), most likely in order to maintain several batches of gametes in the testes undergoing spermatogenesis during a long spawning season in this teleost fish (Carrillo et al., 1995). Interestingly, changes in kisspeptin expression in this species are not apparently associated with those of gnrh system and gonadotropins when the whole brains (Migaud et al., 2013) or the hypothalamus (Alvarado et al., 2013) have been analyzed. Recently, *in vivo* studies in males of this fish species have demonstrated that intracerebroventricular injection of Kiss2 evokes a marked effect on *gnrh-1* expression levels at the forebrain-midbrain, while it does not affect the expression of this *gnrh* form in the hypothalamus, thus suggesting that, at leasts, a differential involvement of the neuroendocrine areas of the forebrain-midbrain and the hypothalamus exists in the control of the reproductive axis via Kiss2/Gnrh1 in male sea bass (Espigares et al., 2015). Additionally, this work has provided evidence of Kiss2/Gnrh-1 system modulates the activity of gonadotrophs involving the neuroendocrine areas of the forebrain-midbrain (Espigares et al., 2015). Since

testosterone is an aromatizable androgen and fish present the highest levels of brain aromatase activity in vertebrates (Callard et al., 1990; Diotel et al., 2010), changes in *cyp19b* expression levels were analyzed. The high *cyp19b* mRNA levels in the brain of male sea bass parallel reduced *ar* mRNA expression in the hypothalamus and increased transcriptional activity of hypothalamic *erβ1* and pituitary *erα* in GDX+T. Although it cannot be determined whether T exerts its effects by itself or after aromatization into estradiol, these data suggest that T effects could be aromatase-dependent in this teleost fish species. The effects of E_2 showed that hypothalamic and pituitary estrogen receptors (*era* and *erβ*) were sensitive to estrogens. Currently, no information exists about the presence of *ar* coexpression in sea bass kisspeptin neurons, although it has been reported that most *kiss1* mRNA-containing cells in the MBH express both *erα* and *erβ2* (Escobar et al., 2013a). Furthermore, it seems that *kiss2*-expressing cells are close to *erβ2*-expressing cells in the MBH, although no co-expression has been observed with *erα* and *erβ1* (Escobar et al., 2013a). Thus, further studies will be needed to determine whether these effects are directly mediated in kisspeptins neurons or other steroid-sensitive neurons are involved or certain chemical transmitters interact with hypothalamic neuropeptides, such as kisspeptins. In this context, an increasing body of evidence supports the idea that GnRH neurons in mammals are regulated by distinct neuronal networks and interactions via specific cell-cell signalling molecules, which may be affected by the modulatory influence of gonadal steroids (Bellefontaine et al., 2011; Prevot, 2002; Radovick et al., 2012). In mice, nitric oxide (NO) is likely involved in mediating the estrogenic positive feedback of *Kiss1* onto GnRH neurons (Hanchate et al., 2012). Recent data in sea bass have shown that *kissr2* (also referred to as *gpr54-2b* or *kissr3*) is expressed in NO positive cells (Escobar et al., 2013b), although its potential interaction with kisspeptins and the mediation of steroid feedback is still unknown.

Finally, studies in salmonids provide evidence that both positive and negative actions occur in the control of gonadotropins after gonadectomy (Antonopoulou et al., 1999; Borg et al., 1998; Larsen and Swanson, 1997). The present study shows that an inhibitory action of T and E_2 occurs on *fshβ* mRNA levels in sea bass, as previously reported in this species (Mateos et al., 2002). Thus, a combination of several mechanisms of action of sex steroids on the gonadotropic function might be considered including, the effect of steroids at the level of the pituitary, the hypothalamus or both. In addition, these results indicate that while hypothalamic *kiss2* mRNA levels are

significantly suppressed in males, they show a positive trend in females. This suggests that transcriptional regulation of the *kiss2* gene can be presumably exerted through several functional mechanisms. Moreover, no changes in mRNA *lhβ* levels were observed in adult sea bass, although they are known to slightly increase at rest (Mateos et al., 2002). Thus, it confirms that steroids exert different effects, depending on the gonadal stage of the animals. Finally, the fact that T and E_2 decrease the baseline levels of circulating Fsh (~ 3-4 fold) and, to a lesser extent, Lh in castrated fish, demonstrates a negative action of steroids on gonadotropin release in adult sea bass, as occurs in other fish (Antonopoulou et al., 1999; Saligaut et al., 1998). These findings therefore provide evidence of the involvement of hypothalamic kisspeptin neurons in long steroidal regulatory feedback on pituitary function in sea bass, adding further evidence of the role of kisspeptins in controlling vertebrate reproduction.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All co-authors have checked and confirmed their contribution to manuscript. A.F., M.C. and M.V.A conceived and designed the study. M.V.A., A.F. M.C. and G.M. collected and analyzed the data. M.V.A., A.S., M.M.G. and O.K. analyzed the ISH data. M.V.A. and A.F. interpreted the data and wrote the manuscript.

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Figures

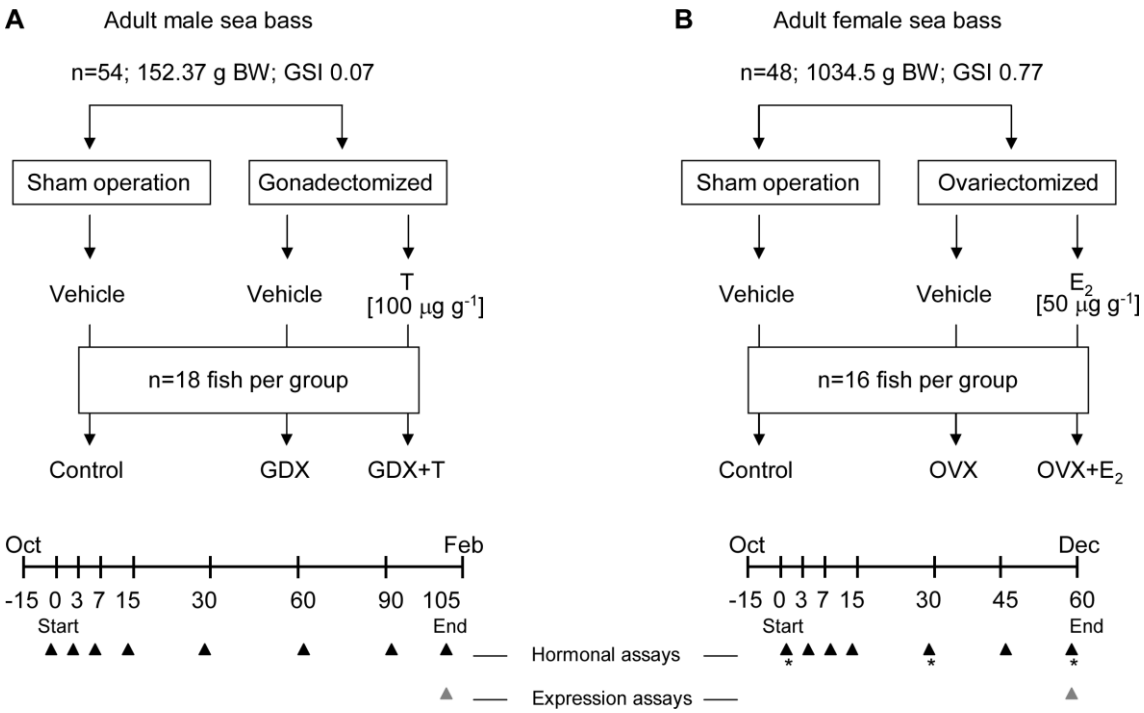

Figure 1. Time chart of the experimental procedure to evaluate the effects of castration and testosterone (T) and estradiol (E_2) implantation in adult male (A) and female (B) sea bass, respectively, during gonadal recrudescence. Males received one silastic implant, either empty or containing a T dose of 100 µg per gram of fish (Experiment 1), while females were administered 50 µg of E_2 per gram of fish (Experiment 2). The day on which implants were inserted was considered to be the start of the experiments (day 0), which lasted until day 105 (February, Experiment 1) and day 60 (December, Experiment 2) post-treatment, coinciding with advanced gametogenesis in this species. Samples for hormonal analysis were collected on the days after implantation indicated by black arrowheads. Asterisks represent blood collection and analysis for plasma levels of Lh in females. Samples for mRNA expression assays are indicated by grey arrowheads.

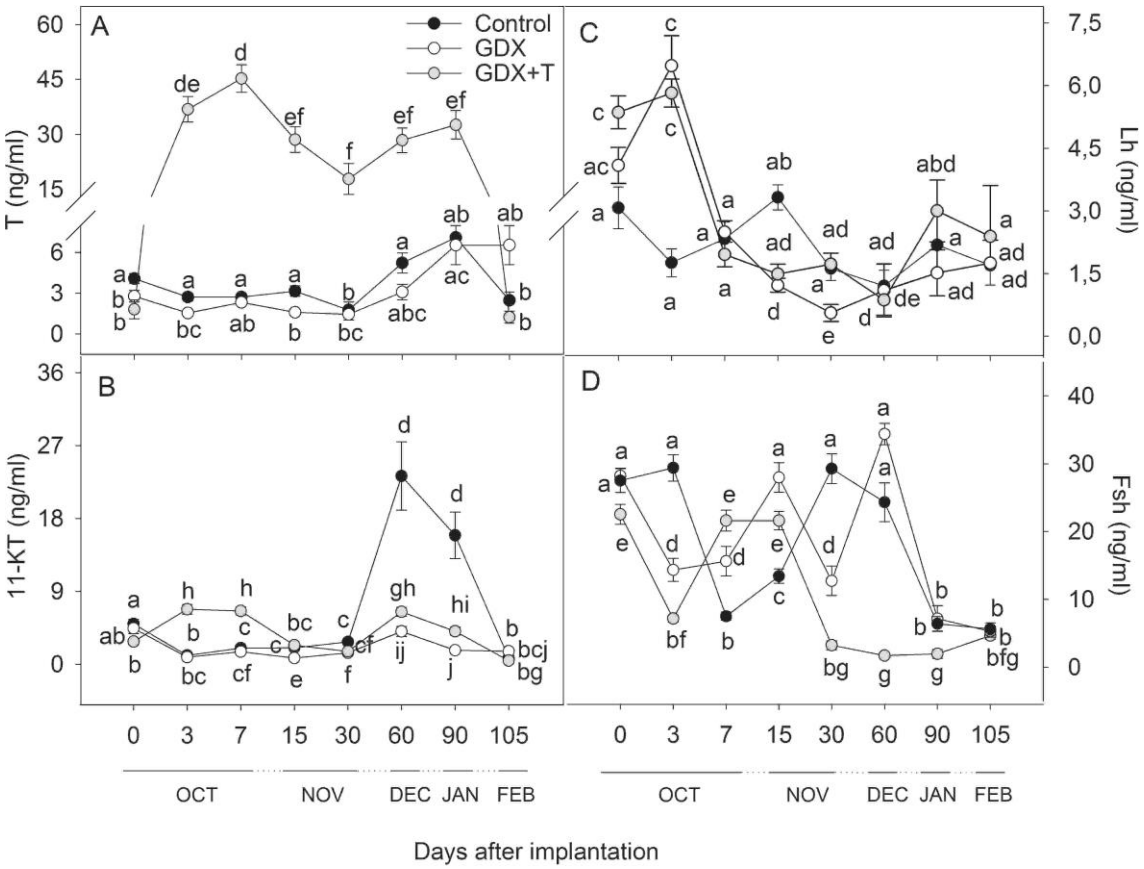

Figure 2. Time courses of the mean (± SEM) concentration of (A) testosterone (T), (B) 11-ketosterone (11-KT), (C) luteinizing hormone (Lh) and (D) follicle-stimulating hormone (Fsh) in the circulation of gonad-intact (Control, black points), castrated (GDX, white points) and castrated males receiving T replacement (100 µg g^{-1}) (GDX+T, grey points) in sea bass. Different lowercase letters indicate significant differences ($P<0.05$) among experimental groups or within the same group throughout the experiment (n = 6-18 per group and sampling point).

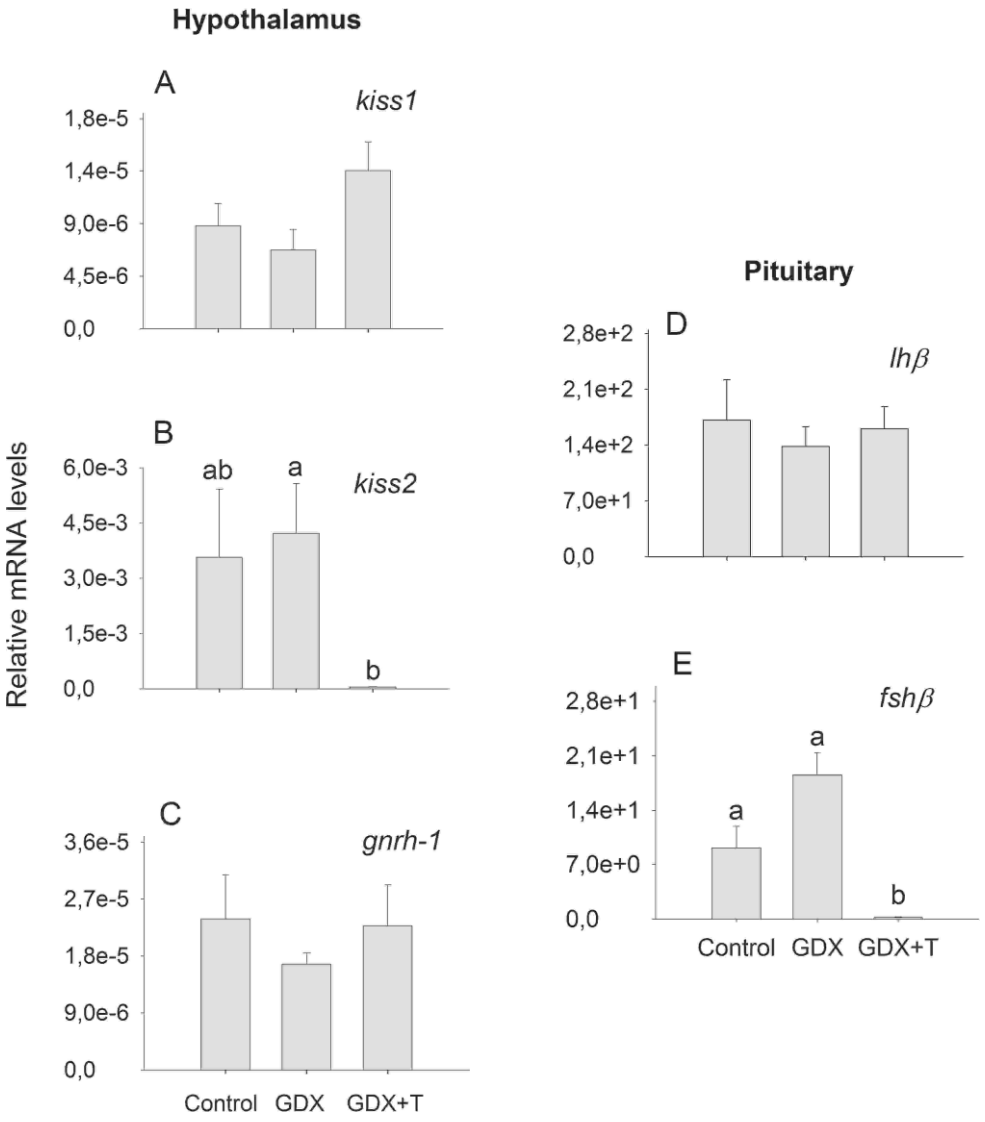

Figure 3. Effect of castration and testosterone (T) replacement treatment on hypothalamic *kiss1* (A), *kiss2* (B) and *gnrh-1* (C) mRNA levels and pituitary *lhβ* (D) and *fshβ* (E) mRNA levels in male sea bass, as determined by qRT-PCR. Changes in expression levels were evaluated on day 105 (February) after treatment, coinciding with advanced gametogenesis in males (n = 6-8 per group). Males were either subjected to sham-operation (testis intact, Control group), gonadectomized (GDX group) or gonadectomized and received T replacement (100 μg g^{-1}) (GDX+T group). Different letters indicate significant differences ($P<0.05$) among experimental groups.

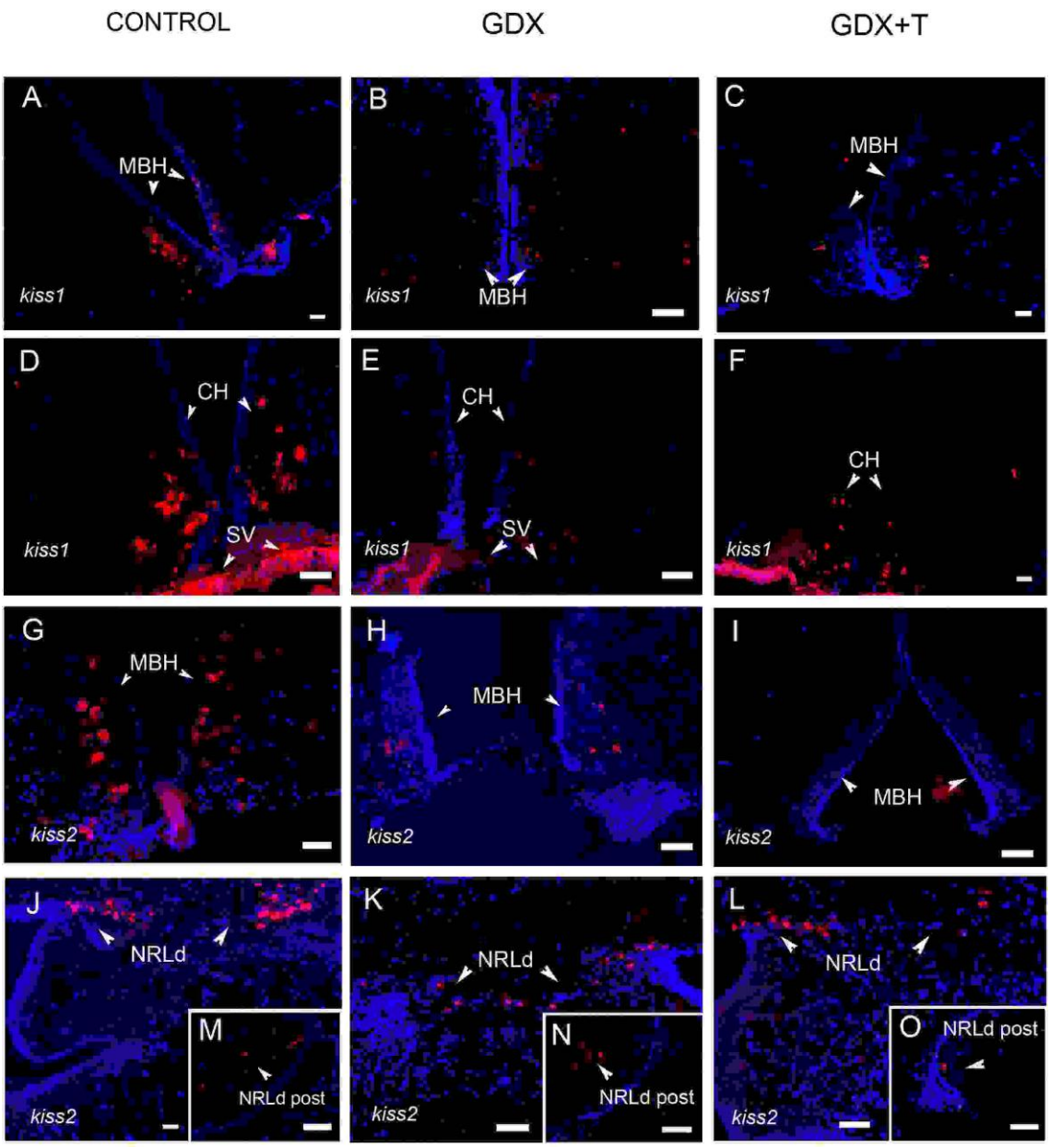

Figure 4. Representative pictures of the *in situ* hybridization results showing *kiss1* and *kiss2* mRNA-expressing cells (in red) on transverse sections in the brain of male sea bass (n = 2 per group), according to the physiological status of the animals. ISH analysis was performed on day 105 (February) post-treatment, coinciding with advanced gametogenesis in this species. Males were either subjected to sham-operation (testis intact, Control group), gonadectomized (GDX group) or gonadectomized and received T replacement (100 µg/g) (GDX+T group). Expression of *kiss1* mRNAs in the mediobasal hypothalamus (MBH) of control **(A)**, GDX **(B)** and

GDX+T **(C)**, and in the caudal hypothalamus (CH) of control **(D)**, GDX **(E)** and GDX+T **(F)** fish. Expression of *kiss2* mRNAs in the MBH of control **(G)**, GDX **(H)** and GDX+T **(I)**, and in the nucleus of the lateral recess (NRLd) and posterior part of NRLd of control **(J-M)**, GDX **(K-N)** and GDX+T **(L-O)** fish, respectively. SV, saccus vasculosus. Scale bars = 30 µm.

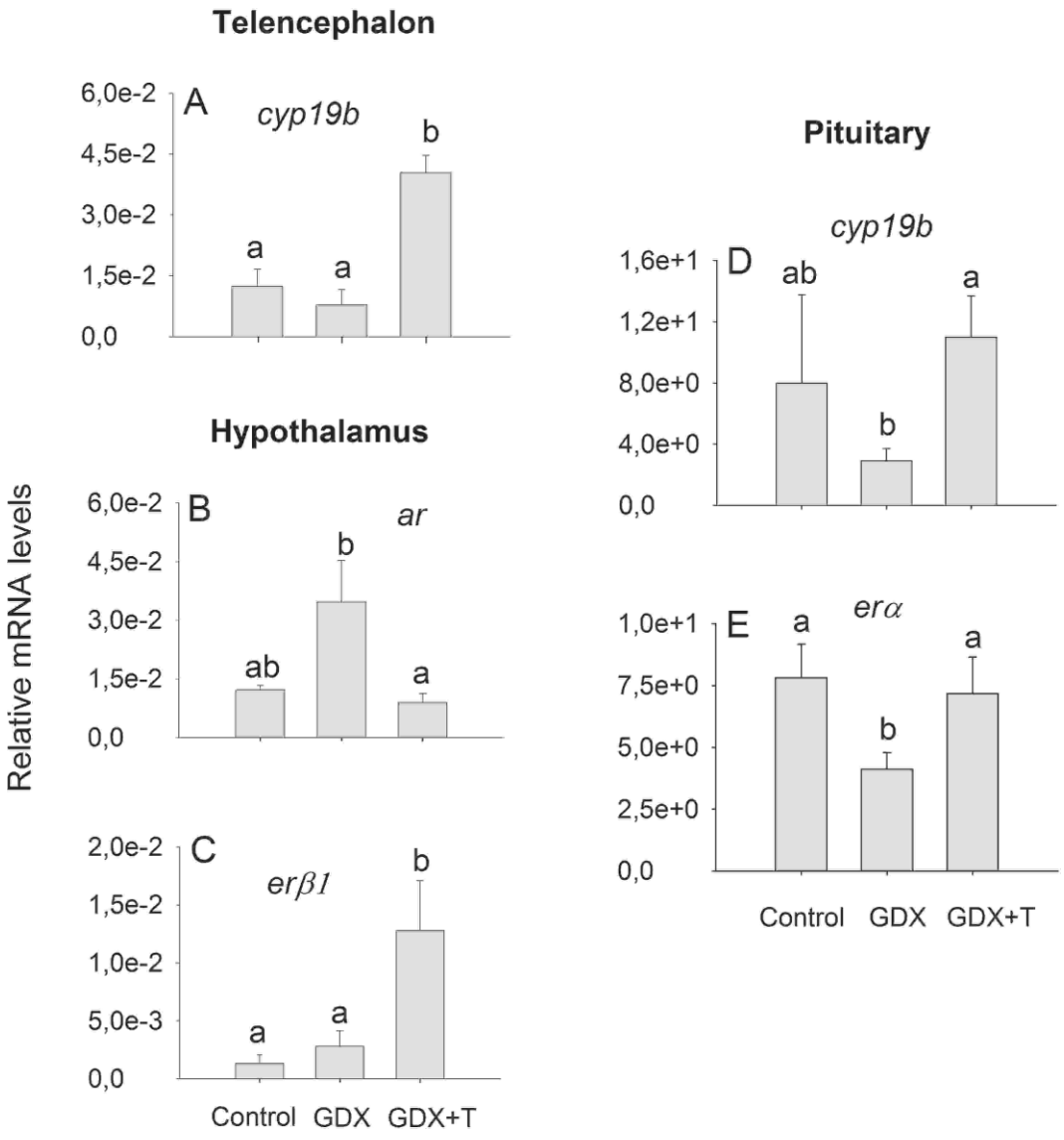

Figure 5. Effect of castration and testosterone (T) replacement treatment on mRNA levels of brain aromatase (*cyp19b*) in the telencephalon (A), androgen receptor (*ar*) in the hypothalamus (B), estrogen receptor β1 (*erβ1*) in the hypothalamus (C) and *cyp19b* (D) *and erα* (E) in the pituitary, as determined by qRT-PCR (see Fig. 3 legend).

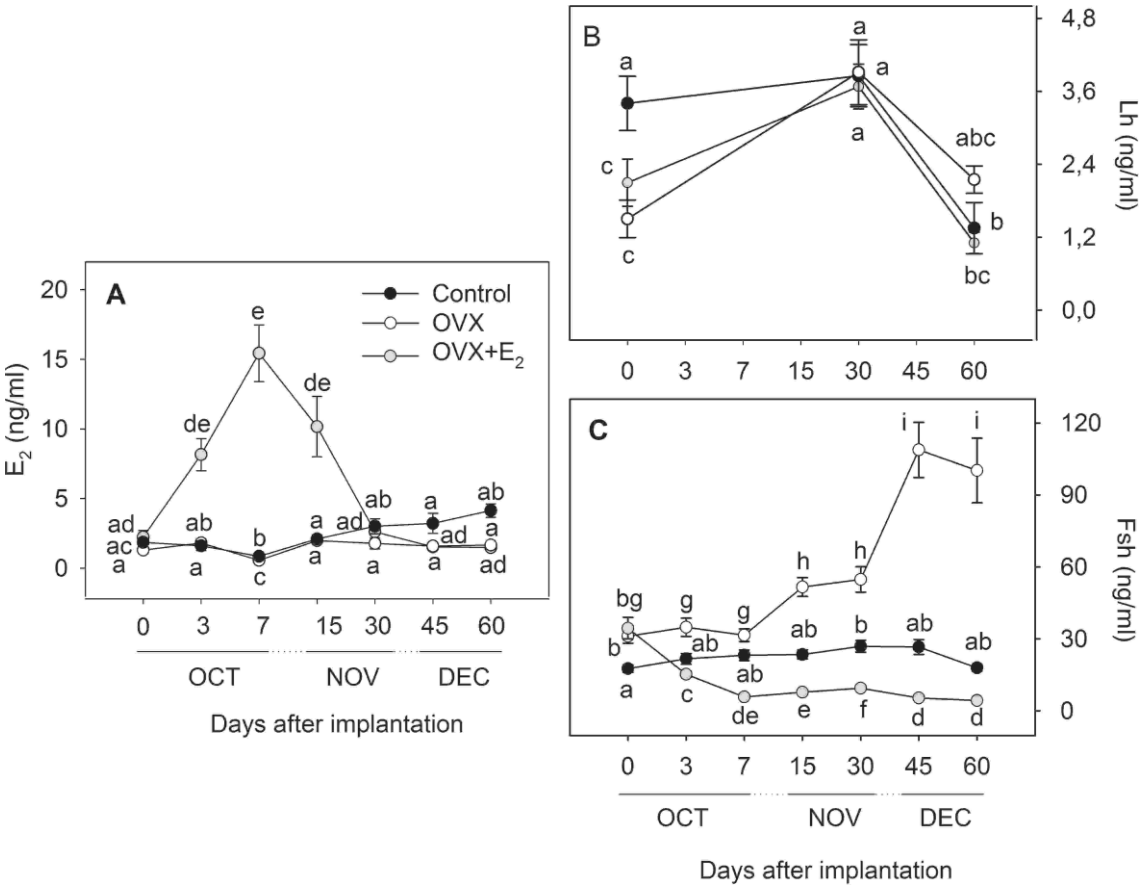

Figure 6. Time courses of the mean (± SEM) concentration of (A) estradiol (E_2), (B) luteinizing hormone (Lh) and (C) follicle-stimulating hormone (Fsh) in the circulation of gonad-intact females (Control, black points), ovariectomized (OVX, white points), and ovariectomized and receiving E_2 replacement (50 µg g^{-1}) (OVX+ E_2, grey points) in sea bass. Different lowercase letters indicate significant differences ($P<0.05$) among experimental groups or within the same group throughout the experiment (n = 4-15 per group and sampling point).

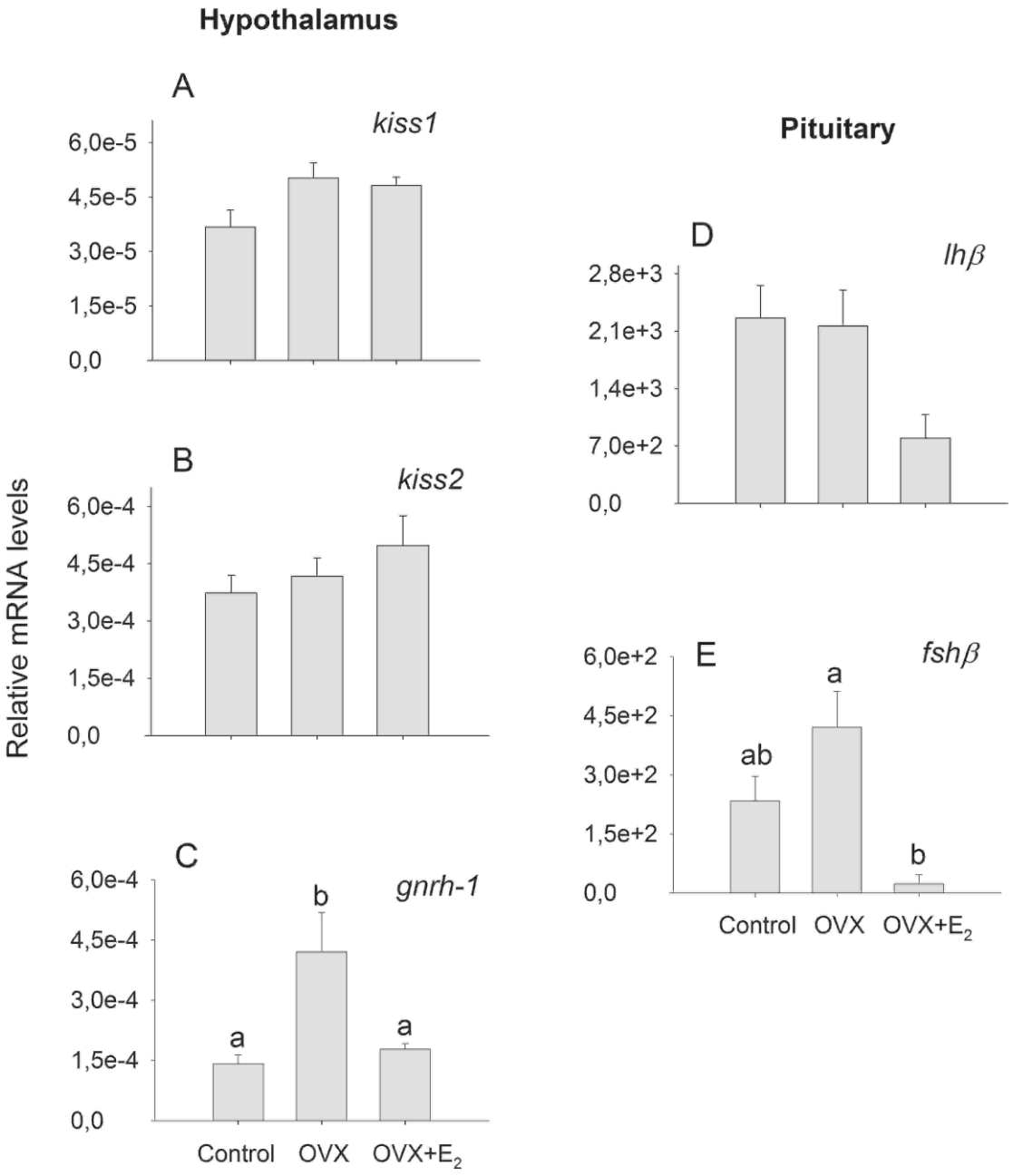

Figure 7. Effect of castration and estradiol (E_2) replacement treatment on hypothalamic *kiss1* (A), *kiss2* (B) and *gnrh-1* (C) mRNA levels and pituitary *lhβ* (D) and *fshβ* (E) mRNA levels in female sea bass, as determined by qRT-PCR. Changes in expression levels were evaluated on day 60 (December) after treatment, coinciding with advanced gametogenesis in females (n = 4-6 per group). Females were either subjected to sham-operation (ovary intact, Control group), ovariectomized (OVX group) or ovariectomized and received E_2 replacement (50 μg g^{-1}) (OVX+E_2 group).

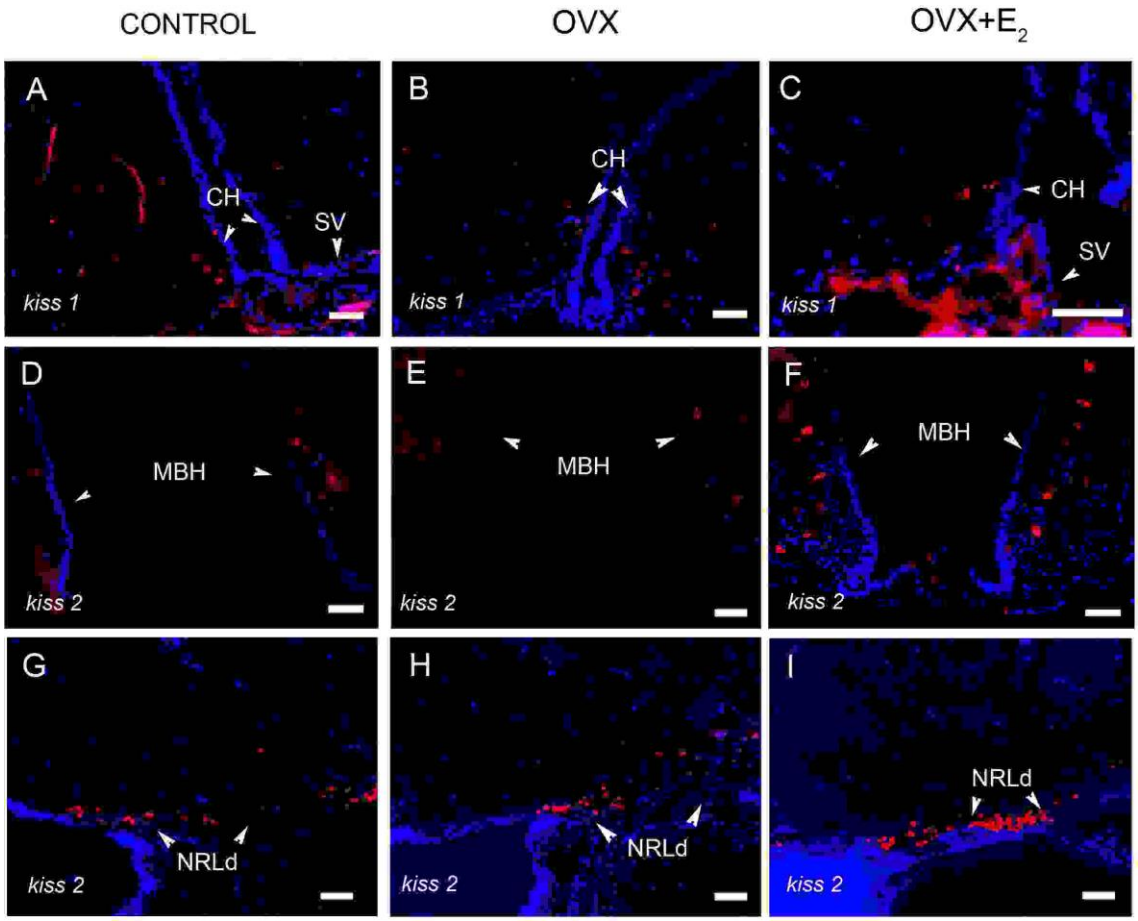

Figure 8. Representative pictures of the *in situ* hybridization results showing *kiss1* and *kiss2* mRNA-expressing cells (in red) on transverse sections in the brain of female sea bass (n = 2 per group), according to the physiological status of the animals. ISH analysis was performed on day 60 (December) after treatment, coinciding with advanced gametogenesis in this species. Females were either subjected to sham-operation (ovary intact, Control group), ovariectomized (OVX group) or ovariectomized and received E_2 replacement (50 µg g^{-1}) (OVX+E_2 group). Expression of *kiss1* mRNAs in the caudal hypothalamus (CH) of control **(A)**, OVX **(B)** and OVX+E_2 **(C)** fish. Expression of *kiss2* mRNAs in the mediobasal hypothalamus (MBH) of control **(D)**, OVX **(E)** and OVX+E_2 **(F)**, and in the dorsal part of the nucleus of the lateral recess (NRLd) of control **(G)**, OVX **(H)** and OVX+E_2 **(I)** fish. SV, saccus vasculosus. Scale bars = 30 µm.

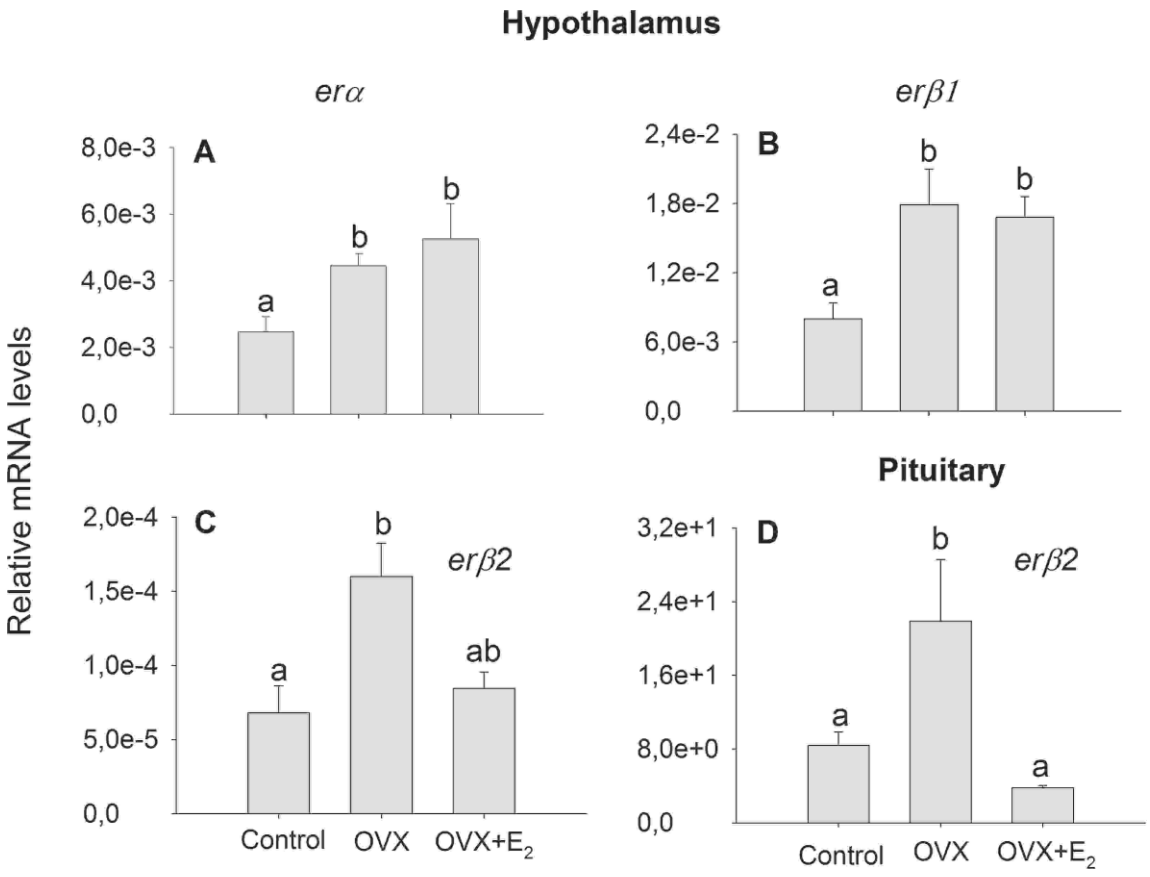

Figure 9. Effect of ovariectomy and estradiol (E_2) replacement treatment on mRNA levels of hypothalamic estrogen receptors (A) *erα*, (B) *erβ1*, (C) *erβ2* and the pituitary *erβ2* (D), as determined by qRT-PCR (see Fig. 7 legend).

Table 1. Summary of the gene expression changes in the brain by RT-PCR after castration and testosterone (T) and estradiol (E_2) replacement in males and females, respectively, in the European sea bass.

		Males			Females		
Tissue	Gene	Control	GDX	GDX+T	Control	OVX	OVX+E_2
Telencephalon	*cyp19b*	- -	- -	+ +	n.a	n.a	n.a
	kiss1	n.s	n.s	n.s	n.s	n.s	n.s
	kiss2	+	+ +	- -	n.s	n.s	n.s
	gpr54-1b	n.s	n.s	n.s	n.s	n.s	n.s
	gpr54-2b	n.s	n.s	n.s	n.s	n.s	n.s
Hypothalamus	*gnrh-1*	n.s	n.s	n.s	- -	+ +	- -
	cyp19b	n.s	n.s	n.s	n.a	n.a	n.a
	erα	n.s	n.s	n.s	- -	+ +	+ +
	erβ1	- -	- -	+ +	- -	+ +	+ +
	erβ2	n.s	n.s	n.s	- -	+ +	-
	ar	-	+ +	- -	n.a	n.a	n.a
	kiss1	n.d	n.d	n.d	n.d	n.d	n.d
	kiss2	n.s	n.s	n.s	n.s	n.s	n.s
	gpr54-1b	n.s	n.s	n.s	n.s	n.s	n.s
	gpr54-2b	n.s	n.s	n.s	n.s	n.s	n.s
	gnrhr-II-1a	n.s	n.s	n.s	n.s	n.s	n.s
	lhβ	n.s	n.s	n.s	n.s	n.s	n.s
Pituitary	*fshβ*	+ +	+ +	- -	+	+ +	- -

cyp19b	+	- -	+ +	n.a	n.a	n.a
erα	+ +	- -	+ +	n.s	n.s	n.s
erβ1	n.s	n.s	n.s	n.s	n.s	n.s
erβ2	n.s	n.s	n.s	- -	+ +	- -
ar	n.s	n.s	n.s	n.a	n.a	n.a

In each sex, the three columns correspond to the experimental groups analysed in this study: Control, castrated (GDX) and castrated males receiving T replacement (100 µg g^{-1}) (GDX+T) and gonad-intact females (Control), ovariectomized (OVX) and ovariectomized and receiving E_2 replacement (50 µg g^{-1}) (OVX+ E_2) in sea bass. The action of sex steroids in each experimental group is indicated as follows: **(**++) positive; (- -) negative; (+) positive but without significant differences; (-) negative but without significant differences; (n.s) not-significant; (n.d) not-detected and (n.a) not-analyzed.