



# Activation of the brain-pituitary-gonadotropic axis in the black porgy *Acanthopagrus schlegelii* during gonadal differentiation and testis development and effect of estradiol treatment



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## ABSTRACT

Previous studies revealed an estradiol (E2)-dependent peak in brain activity, including neurosteroidogenesis and neurogenesis in the black porgy during the gonadal differentiation period. The brain-pituitary-gonadotropic axis is a key regulator of reproduction and may also be involved in gonadal differentiation, but its activity and potential role in black porgy during the gonadal differentiation period is still unknown. The present study analyzed the expression of regulatory factors involved in the gonadotropic axis at the time of gonadal differentiation (90, 120, 150 days after hatching [dah]) and subsequent testicular development (180, 210, 300 dah). In agreement with previous studies, expression of brain aromatase *cyp19a1b* peaked at 120 dah, and this was followed by a gradual increase during testicular development. The expression of gonadotropin subunits increased slightly but not significantly during gonadal differentiation and then increased significantly at 300 dah. In contrast, the expression of brain *gnrh1* and pituitary *gnrh receptor 1 (gnrhr1)* exhibited a pattern with two peaks, the first at 120 dah, during the period of gonadal differentiation, and the second peak during testicular development. *Gonad fshr* and *lhcr* increased during gonadal differentiation period with highest transcript level in prespawning season during testicular development. This suggests that the early activation of brain *gnrh1*, pituitary *gnrhr1* and *gths*, and gonad *gths* might be involved in the control of gonadal differentiation. E2 treatment increased brain *cyp19a1b* expression at each sampling time, in agreement with previous studies in black porgy and other teleosts. E2 also significantly stimulated the expression of pituitary gonadotropin subunits at all sampling times, indicating potential E2-mediated steroid feedback. In contrast, no significant effect of E2 was observed on *gnrh1*. Moreover, treatment of AI or E2 had no statistically significant effect on brain *gnrh1* transcription levels during gonadal differentiation. This indicated that the early peak of *gnrh1* expression during the gonadal differentiation period is E2-independent and therefore not directly related to the E2-dependent peak in brain neurosteroidogenesis and neurogenesis also occurring during this period in black porgy. Both E2-independent and E2-dependent mechanisms are thus involved in the peak expression of various genes in the brain of black porgy at the time of gonadal differentiation.

## 1. Introduction

Black porgy is a hermaphroditic protandrous teleost, with functional males for the first two years of life and sex change to females during the third year. Previous studies showed that during the period of gonadal differentiation (90–150 days after hatching [dah]), the brain exhibited increased estradiol (E2) synthesis, cell proliferation, and neurogenesis. The expression of neurosteroidogenesis-related genes, including *star*,

brain-type aromatase *cyp19a1b*, and nuclear estrogen receptors, peaked at 120 dah (Tomy et al., 2009; Tomy et al., 2007; Wu et al., 2010). Exogenous E2 treatment stimulated neurosteroidogenesis and neurogenesis, while aromatase inhibitor (AI) decreased brain cell proliferation, which indicated that E2 played important roles in the induction of early brain development (Lin et al., 2016). In contrast, no significant differences in brain gene expression or neurogenesis were found in castrated fish compared to controls, further supporting the idea that the peak in

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early brain activity was controlled by locally produced brain E2 and was independent of the gonads (Lin et al., 2016).

In teleosts, as in mammals, the neuropeptide gonadotropin-releasing hormone (GnRH) acts on pituitary gonadotrope cells via binding to the GnRH receptor (GnRH-R) and stimulates the biosynthesis and release of GTHs (gonadotropin hormones; luteinizing hormone [LH] and follicle-stimulating hormone [FSH]). GTHs binds to GTH receptors (GTH-R; follicle-stimulating hormone receptor [FSH-R] and luteinizing hormone/chorionic gonadotropin receptor [LHCG-R]) on gonad and directly control reproductive function by inducing gonadal steroidogenesis and gametogenesis. The gonadal steroids exert feedback on the brain-pituitary-gonadotropic axis (Amano et al., 1997).

Steroid hormones, particularly E2, play important roles in sex determination and reproduction. Brain steroids may be originally synthesized in the gonad and reach the brain via the general circulation. The brain may also produce steroids locally including E2 via neurosteroidogenesis (Diotel et al., 2011). Brain aromatase expression in teleosts is exclusive in radial glial cells and is found in olfactory bulbs, telencephalon, ventral diencephalon and pituitary gland, with the highest expression at preoptic area and hypothalamus (Forlano et al., 2001; Menuet et al., 2003; Strobl-Mazzulla et al., 2005). The expression levels of brain aromatase activity in teleosts is higher than any other vertebrates, which has been suggested a mechanism of continual neurogenesis found in fish (Forlano et al., 2001; Pellegrini et al., 2007). Gene expression of *cyp19a1b* in black sea bass was analyzed in brain and pituitary during reproductive development and sex change; elevated *cyp19a1b* expression was found in the brain at stage of female vitellogenic growth and in the pituitary at stage of male spermiating (Breton et al., 2015). In female ricefield eel, *Cyp19a1b* colocalized with *Lhb* in pituitary was observed by immunohistochemistry (Zhang et al., 2014). The immunoreactive levels and colocalization frequency increased during gametogenesis toward maturation, which suggested a relationship between pituitary *Cyp19a1b* and *Lhb* during vitellogenesis (Zhang et al., 2014). Pituitary *cyp19a1b* but not brain *cyp19a1b* involved in the regulation of social behavior in cichlid fish (Ramallo et al., 2017). Higher levels of E2 or *cyp19a1b* were detected in brain of male pejerrey and rainbow trout during gonadal differentiation (Strobl-Mazzulla et al., 2008; Vizziano-Cantonnet et al., 2011). But studies about pituitary *cyp19a1b* during gonadal differentiation is limited.

The brain-pituitary-gonadotropic axis is well known for its role in controlling reproductive functions and may also play a role in gonadal differentiation in fish (Senthilkumaran et al., 2015). In European sea bass, for example, higher expression levels of brain *gnrh* genes and pituitary *fshb* were observed during gonadal differentiation period (Moles et al., 2007). As shown by immunostaining in pejerrey, *Gnrh* neurons and *Fshb*- and *Lhb*-positive pituitary cells were detectable before gonadal differentiation, which suggested a role for the brain-pituitary-gonadotropic axis in this period (Miranda et al., 2003; Miranda et al., 2001). In contrast, in Nile tilapia, according to results from qPCR and immunostaining, *fshb* and *lhb* in the pituitary were not related to gonadal differentiation (Yan et al., 2012).

Previous studies from our group investigated the brain-pituitary-gonadotropic axis in black porgy. Annual profiles analyzed by radioimmunoassay showed an increase in *Gnrh1* and *Lh* levels during sexual maturation in 1- and 2-yr-old fish (Du et al., 2005). In 2+ - to 3-yr-old black porgy, higher levels of plasma *Lh* during the nonspawning season were detected in male fish than in fish that sex-changed to females later in the spawning season (Du et al., 2005). Two types of GnRH receptors, *gnrhr1* and *gnrhr2*, were cloned and analyzed in 2+ - and 3-yr-old black porgy (Lin et al., 2010). The tissue distribution revealed that *gnrhr1* was strongly expressed in the pituitary, and *gnrhr2* (40 cycles) was expressed at very low level as compared to *gnrhr1* (30 cycles) and was mainly expressed in gonad and kidney by semi-quantitative PCR. Sex steroids (E2 and T) and LHRH analog increased the expression of *gnrhr1*, but not *gnrhr2*, in pituitary *in vivo* or pituitary cells *in vitro* (Lin et al., 2010). These results indicated that *gnrhr1* may play a more

important role in the pituitary than does *gnrhr2*. An increase in the pituitary expression of *gnrhr1* was found during sexual maturation in 2+ -yr-old black porgy (Lin et al., 2010). E2 treatment stimulated *Lh* levels in pituitary and plasma in 2-yr-old fish (Chang et al., 1991; Du et al., 2001; Lee et al., 1999; Lee et al., 2000; Yen et al., 2002). Increased levels of plasma *Lh* and plasma E2 during the prespawning and spawning seasons were correlated with the natural sex change in 3-yr-old black porgy (Chang et al., 1994; Lee et al., 2000). Higher transcript levels of *fshr* and *lhcg* in the bisexual testicular tissue compared with that of ovarian tissue was found in 1+ - and 2+ -yr-old black porgy (Du et al., 2005; Wu et al., 2016). Whereas many studies have analyzed the brain-pituitary-gonadotropic axis during sexual maturation and sex change, data during gonadal differentiation are limited: only one study reported the profile of brain *Gnrh* by radioimmunoassay during this period (Du et al., 2005), with no data on gonadotropins.

The present study aimed to investigate the activity of the brain-pituitary-gonadotropic axis in black porgy during gonadal differentiation. Quantitative real-time PCRs were used to analyze gene expression of *fshb* and *lhb* subunits, common glycoprotein alpha subunit (*gpa*), GnRH receptor 1 (*gnrhr1*), and brain-type aromatase (*cyp19a1b*) in the pituitary and of *gnrhr1*, *gnrhr2*, *gnrhr3*, and *cyp19a1b* in anterior brain at the time of gonadal differentiation (90–150 dah). Comparisons were made with their expression levels during subsequent testicular development in sexually maturing males (up to 300 dah). The effects of E2 treatment on these brain and pituitary gene expression levels during the periods of gonadal differentiation and testicular development were also investigated to infer potential E2-dependent regulatory mechanisms.

## 2. Materials and methods

### 2.1. Animals

Juvenile (75 dah) black porgy were obtained from an aquaculture farm and acclimated in seawater (salinity 33 ppt) to the ponds at the National Taiwan Ocean University culture station (NTOU; Keelung, Taiwan). These experimental fish were kept in indoor ponds with natural temperature until 90 dah before starting the experiment. Water temperatures ranged from 19 °C to 26 °C. Samples were collected from May (90 dah) to Dec (300 dah). The fish were fed twice a day (8% of their body weight in total feed per day) with commercial feed (Fwa Sou Feed, Taichung, Taiwan). All the procedures and experiments were approved by the NTOU Institutional Animal Care and Use Committee and were conducted in accordance with the animal experimentation procedure guidelines.

### 2.2. Experimental design

Black porgy is a protandrous fish with gonadal differentiation at 120–150 dah followed by testis development until the stage of functional testes and spawning (at approximately 1 year old). Previous studies in black porgy have reported that the expression levels of neurosteroidogenesis-related genes, including *star*, *cyp19a1b*, and *nuclear estrogen receptors*, peak in the early brain during gonadal differentiation (Tomy et al., 2009; Tomy et al., 2007; Wu et al., 2010). Furthermore, we already know that the brain changes in this period are controlled by locally produced E2 and are independent from gonadal factors (Lin et al., 2016).

In the present study, brain, pituitary and gonadal samples were collected from 90 to 300 dah fish during the gonadal differentiation period (at 90, 120 and 150 dah) and the testicular development period (at 180, 210 and 300 dah) to analyze the expression profiles of genes involved in the brain-pituitary-gonadotropic axis. The effects of E2 on gene expression were also examined. To investigate the effects of E2, fish were treated *in vivo* with E2 from 90 dah until the end of the experiment. The E2-treated group received 6 mg E2 (Sigma-Aldrich, St. Louis, MO)/kg feed, according to the dosage previously established in

black porgy (Lee et al., 2000). The control group was fed the same diet but without E2. Brain and pituitary samples from E2-treated fish were collected along with control fish during the gonadal differentiation period (at 120 and 150 dah) and the testicular development period (at 180, 210 and 300 dah). Gonad samples from control and E2-treated fish were collected during the testicular development period (at 180, 210, 240 and 300 dah). For fish at 90 dah, a single group of initial control fish was collected. To further confirm the effects of E2 on gene expression, we tested the effect of aromatase inhibitor (AI; 1,4,6-androstatrien- 3,17-dione; Steraloids, Newport, RI) during the gonadal differentiation period. Fish at 90 dah were divided into three groups: E2, AI, and control groups. The control group was fed the same diet but without E2 (6 mg/kg feed) or AI (20 mg/kg feed). The E2 and AI dosages were established previously in black porgy (Lee et al., 2000; Lee et al., 2002). Brain samples were collected at 90, 120 and 150 dah.

### 2.3. Biometry and brain and pituitary sampling

Fish were anesthetized with 0.1% 2-phenoxyethanol in seawater and were sacrificed by decapitation. The biometric parameters body weight (BW) and body length (BL) were measured and are reported in Supporting Information Table S1. No statistically significant differences in BW or BL were found between the control and E2-treated groups (NS).

The brain and pituitary were dissected out. The anterior brain ( $n = 8$  individuals per group and sampling time) was further dissected into two parts: the telencephalon, including olfactory bulbs (TE), and the di-/mesencephalon, including the hypothalamus, preoptic area and thalamus (DME). The schematic drawings of brain dissection are shown in Fig. 1. Pituitaries were pooled into groups of ten (90, 120 and 150 dah fish) or of five (180, 210 and 300 dah fish), and 4 pools were collected per group and sampling time. Gonads were also collected and analyzed: samples were pooled into groups of eight (3 pools for 90, 120 and 150 dah fish), four (4 pools for 180 dah fish) or two (5 pools for 210 and 240 dah fish); and 10 individual gonads for 300 dah fish. The samples were stored at  $-80^{\circ}\text{C}$  until qPCR analysis.

### 2.4. Histology and immunocytochemistry of gonads

Gonad samples from control fish or E2-treated fish were collected to examine the gonadal status by histological analysis. The gonad developmental stages were defined according to previous reports (Tomy et al., 2007; Wu et al., 2008a; Wu et al., 2008b), both E2 and AI treatments resulted in the regression of testicular tissue, development of oogonia/primary oocytes and growth of ovarian tissue. Samples were quickly removed, fixed for 16 h with 4% paraformaldehyde in 0.1 M sodium phosphate buffer with saline (PBS, pH 7.4), and then dehydrated through a graded methanol series, embedded in paraffin, and subjected to serial sectioning into  $6\ \mu\text{m}$  transverse sections for H&E staining and immunostaining.

After dewaxing and rehydrating, gonad sections were immunostained for PcnA (S phase-related proliferating cell nuclear antigen) as a marker of cell proliferation using the standard avidin-biotinylated-peroxidase complex kit (ABC kit; Vector Laboratories, Burlingame, CA). The gonad sections were incubated for 10 min in 3% H<sub>2</sub>O<sub>2</sub>, for 1 h in 5% skim milk, and overnight at  $4^{\circ}\text{C}$  with primary antibodies (PcnA; 1:200; DakoCytomation, Glostrup, Denmark). After washing with PBS with 0.1% Tween-20, the sections were incubated with a biotinylated goat IgG antibody (1:2,000; Vector Laboratories) and finally incubated in ABC solution. The peroxidase reaction was visualized with a solution of 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and then observed and photographed under a microscope (BX51; Olympus, Tokyo, Japan).

### 2.5. Measurement of gene transcripts by qPCR analysis

TRIzol reagent (Invitrogen) was used, and total RNA was extracted from pituitaries and dissected brain tissues. Total RNA was reverse transcribed to first-strand cDNA using Superscript III (Invitrogen) with oligo(dT)<sub>12–18</sub> primers (Promega, Madison, WI) according to the manufacturer's protocol. First-strand cDNA was used for qPCR analysis. qPCR was conducted using a 7500 Real-Time PCR System (Applied Biosystems, CA) and SYBR Green as described in our previous study (He et al., 2003).

The pituitary transcript levels of the gonadotropin beta subunits *fshb*, *lhb*, and of the common glycoprotein alpha subunit *gpa* were analyzed. Two types of GnRH receptors, *gnrhr1* and *gnrhr2*, were also analyzed. However, the gene expression of pituitary *gnrhr2* was very low (Ct > 37.0 to Undetectable), so that only the results for *gnrhr1* are presented.

The transcript levels of the three types of GnRH present in the black porgy, *gnrh1*, *gnrh2*, and *gnrh3*, were analyzed in dissected brain parts. *Gnrh1* transcripts could be measured in both the telencephalon and di-/mesencephalon, while *gnrh2* and *gnrh3* transcripts were measurable in the di-/mesencephalon and telencephalon, respectively.

The transcript levels of *cyp19a1b* were analyzed in the pituitary and dissected brain parts. Previous data from tissue distribution in 1+ yr-old black porgy showed that the “gonad form of aromatase”, *cyp19a1a*, was expressed only in gonad tissue and not in the brain (Wu et al., 2016). Conversely, we demonstrated that the “brain form of aromatase”, *cyp19a1b*, was only expressed in the brain and pituitary (Supporting Information Fig. S1). Therefore, the brain form of aromatase (*cyp19a1b*) was analyzed by qPCR in pituitary and brain parts in the present study.

The transcript levels of gonadotropin receptors *fshr* and *lhcg* were analyzed in the gonadal samples of black porgy.

As an internal control, *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) was used to normalize the gene expression level. No significant changes in the expression levels of *gapdh* were found among the groups ( $P > 0.05$ ).

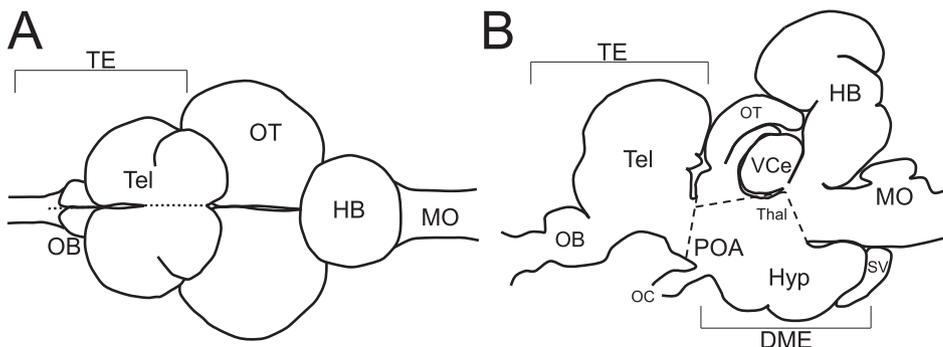


Fig. 1. Schematic drawings of black porgy brain in dorsal and lateral views. Brain tissues were collected and dissected into two parts, the telencephalon (TE) and di-/mesencephalon (DME), for analysis in real-time PCR. TE includes both the telencephalon and olfactory bulbs; DME includes the hypothalamus, preoptic area and thalamus. HB, hindbrain; Hyp, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; OC, optic chiasm; OT, optic tectum; POA, preoptic area; SV, sacculus vasculosus; Tel, telencephalon; Thal, thalamus; VCe, valvula cerebelli.

The accession numbers of the sequences analyzed in this study, and gene-specific primers and annealing temperatures are described in Supporting Information Table S2. The primer specificity is shown in Supporting Information Fig. S2.

## 2.6. Statistics

The data are presented as the mean  $\pm$  SEM (standard error of the mean). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Duncan's test using Statistical Package for the Social Sciences (SPSS). Student's *t*-test was also conducted to determine significant differences between treatments. A value of  $P < 0.05$  or  $P < 0.01$  was considered statistically significant.

## 3. Results

### 3.1. Gonadal differentiation and testicular development

Undifferentiated gonad tissue with early germ cells was observed in 90 dah fish (Fig. 2A1). Differentiated gonads with ovarian cavities were found in 120 dah fish (Fig. 2B1). Early spermatogonia (type A) began to appear in 120 dah (Fig. 2B1) and 150 dah fish (Fig. 2C1). Lobular testicular tissue with late spermatogonia (type B) was observed in 180 dah (Fig. 2D1) and 210 dah fish (Fig. 2E1). The characteristics of the different male germ cells (type A and type B spermatogonia) are shown in Supporting Information Fig. S3. To further examine the cell activity, PcnA was used as a cell proliferation marker to confirm the gonadal status. According to the PcnA immunostaining, undifferentiated gonads had fewer PcnA-positive cells in 90 dah fish (Fig. 2A2 and A2'). Proliferating activity of gonadal cells was dramatically increased during gonadal differentiation in 120 dah and 150 dah fish (Fig. 2B2, B2', C2, and C2'). Large numbers of PcnA-positive germline cells were observed in 180 dah (Fig. 2D2) and 210 dah fish (Fig. 2E2).

After E2 administration, development of ovarian tissue was found in protandrous black porgy. Primary oocytes and oogonia appeared in gonadal tissues during gonadal differentiation stage (Fig. 2F1 and F1'). During gonadal development stage, large numbers of primary oocytes were observed in these ovarian tissues at 180 dah fish (E2 administration for 3 months) (Fig. 2G1 and G1'). PcnA-positive oogonia were found in the gonads of E2-treated fish during gonadal differentiation and development period (Fig. 2F2, F2', G2 and, G2'). The E2-induced development of ovarian tissue in black porgy at young age also described in our previous study (Wu et al., 2008a).

Combining these data with our previous studies (Lee et al., 2008; Wu et al., 2008a), gonadal differentiation occurs at approximately 120 dah in black porgy. In protandrous black porgy, the period from the completion of gonadal differentiation to the first spawning season is considered the testicular development stage.

### 3.2. Expression profiles of pituitary gonadotropin subunits, *gnrh* receptor 1 and aromatase

Gene expression profiles were measured by qPCR during the period of gonadal differentiation (from 90 dah to 150 dah) and during the subsequent period of testicular development (from 180 dah to 300 dah) for comparison (Fig. 3).

The transcript levels of *lhb* were slightly but not significantly increased at the time of gonadal differentiation (120 dah) compared to those at 90 dah (2.1-fold,  $P > 0.05$ ). *Lhb* transcript levels increased markedly during testicular development to reach a 26.0-fold-higher value at 300 dah than at 90 dah ( $P < 0.01$ ). *Fshb* transcript levels did not vary during gonadal differentiation but were markedly increased at 300 dah (26.4-fold increase compared to 90 dah,  $P < 0.05$ ). The pituitary *gpa* transcript levels were slightly but significantly increased at 300 dah (4.1-fold increase compared to 90 dah,  $P < 0.05$ ).

The relative expression of *gnrh1* peaked at the time of gonadal

differentiation at 120 dah (3.9-fold compared to 90 dah,  $P < 0.01$ ). A further increase was observed during testicular development at 180 dah (6.6-fold compared to 90 dah,  $P < 0.01$ ), but transcript levels then decreased to reach values at 300 dah (3.8-fold compared to 90 dah,  $P < 0.05$ ) similar to those at 120 dah.

We also measured *cyp19a1b* pituitary transcript levels and observed a gradual increase during the period of gonadal differentiation (1.6-fold at 120 dah,  $P > 0.05$ ; 3.4-fold at 150 dah,  $P < 0.05$ ; compared to 90 dah) and further increase during testicular development (3.6-fold at 180 dah,  $P < 0.01$ ; 4.4-fold at 210 dah,  $P < 0.05$ ; 4.8-fold at 300 dah,  $P < 0.01$ ; compared to 90 dah). However, there was no peak expression of *cyp19a1b* in the pituitary as compared to a peak expression in the brain during gonadal differentiation.

### 3.3. Expression profiles of brain *gnrh* genes and aromatase gene

qPCR was performed on telencephalon and di-/mesencephalon from 90 dah to 300 dah fish to determine the gene expression profiles during the periods of gonadal differentiation and testicular development (Fig. 4).

The transcript levels of *gnrh1* increased significantly during the period of gonadal differentiation, with a peak at 120 dah in the telencephalon (2.8-fold compared to 90 dah,  $P < 0.05$ ) and di-/mesencephalon (2.5-fold compared to 90 dah,  $P < 0.01$ ). An increase was also observed during testicular development in both the telencephalon and di-/mesencephalon, with levels at 300 dah similar to those at 120 dah (2.6-fold at 300 dah,  $P < 0.01$  for telencephalon; 3.1-fold at 300 dah,  $P < 0.01$  for di-/mesencephalon; compared to 90 dah telencephalon and 90 dah di-/mesencephalon, respectively).

Transcript levels of *gnrh2* in the di-/mesencephalon gradually increased during the period of gonadal differentiation with a peak at 150 dah (2.4-fold compared to 90 dah,  $P < 0.01$ ) and then gradually decreased during testicular development such that the level at 300 dah was similar to that at 90 dah.

Concerning *gnrh3*, no significant variation was observed during gonadal differentiation, with only a slight increase (1.4-fold compared to 90 dah,  $P < 0.05$ ) at one sampling time (180 dah) during testicular development.

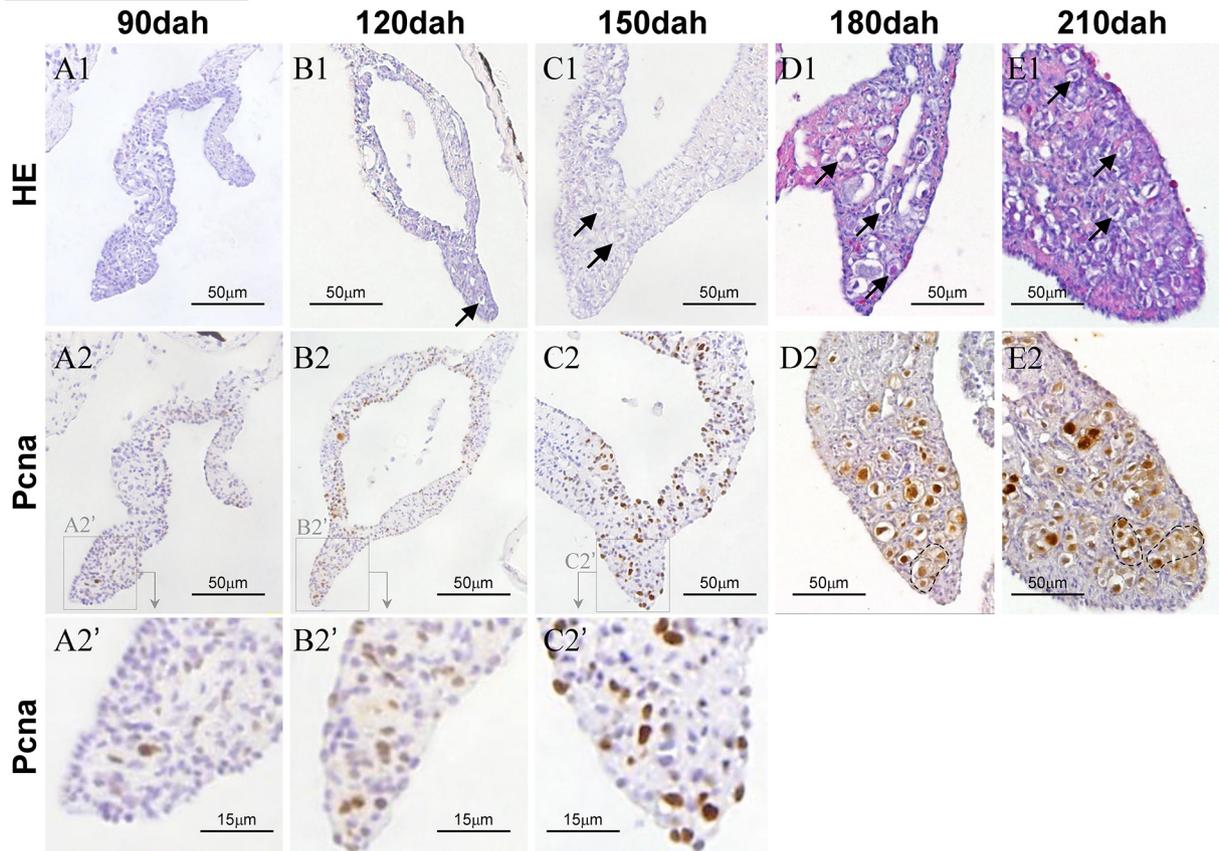
We also measured *cyp19a1b* brain transcript levels and observed peaks of expression at 120 dah in the telencephalon (2.2-fold compared to 90 dah,  $P < 0.01$ ) and di-/mesencephalon (2.3-fold as compared to 90 dah,  $P < 0.01$ ) during gonadal differentiation. These results were in agreement with our previous study (Lin et al., 2016). A large, gradual increase was observed in telencephalon during testicular development, with transcript levels at 300 dah reaching values 22.9-fold higher than at 90 dah ( $P < 0.01$ ), while a more moderate but also significant increase was observed in di-/mesencephalon (5.2-fold compared to 90 dah,  $P < 0.01$ ).

### 3.4. Expression profiles of gonadal gonadotropin receptors

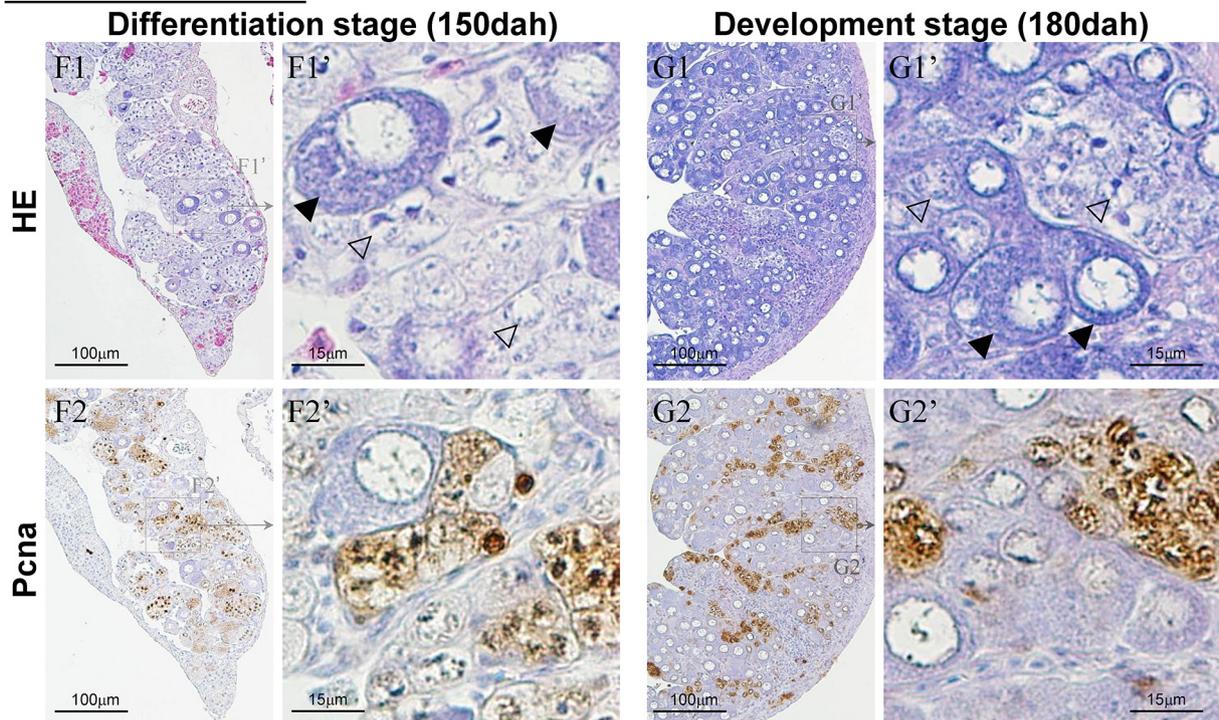
qPCR was performed on gonad from 90 dah to 300 dah fish to determine the gene expression profiles during the periods of gonadal differentiation and testicular development (Fig. 5).

The transcript levels of *lhcr* were increased during gonadal differentiation period (2.4-fold at 120 dah,  $P > 0.05$ ; 2.6-fold at 150 dah,  $P < 0.01$ ; compared to 90 dah). Transcript levels of *lhcr* gradually increased during the period of testicular development with a peak at 240 dah (4.3-fold compared to 90 dah,  $P < 0.01$ ). The relative expression of *fshr* significantly increased during gonadal differentiation period (2.9-fold at 120 dah,  $P < 0.01$ ; 3.0-fold at 150 dah,  $P < 0.01$ ; compared to 90 dah). A gradual increase of *fshr* was observed during testicular development to reach a 3.7-fold-higher value at 240 dah than at 90 dah ( $P < 0.01$ ). Similar expression patterns of gonadotropin receptors were found, both the transcript levels of *fshr* and *lhcr* gradually increased during the period of testicular development and then

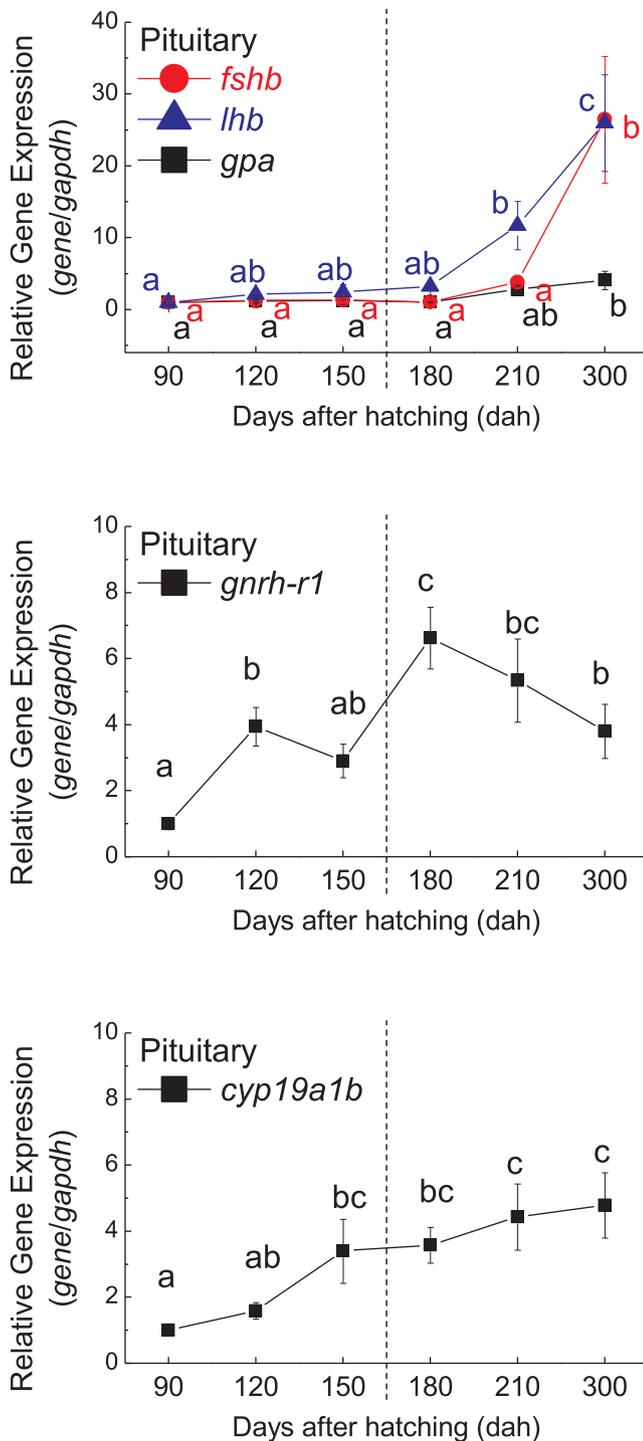
## Control fish



## E2-treated fish



**Fig. 2.** Characterization of gonadal features during the periods of gonadal differentiation (90, 120 and 150 dah) and of testicular development (180 and 210 dah) in control and estradiol (E2)-treated fish. Transverse sections (6 µm) of gonad tissues were stained by hematoxylin-eosin (A1-G1) and immunohistochemical staining of Pcna, a cell proliferation marker (A2-G2). High-magnification images are shown in F1'-G1' (H&E staining) and A2'-G2' (Pcna-immunostaining). The arrow indicates spermatogonia, and the dashed circle indicates the lobular structure of the testicular cyst in control fish. For E2-treated fish, the arrowhead indicates primary oocytes and the open arrowhead indicates oogonia.



**Fig. 3.** Gene expression profiles in the pituitary of black porgy at 90–300 dah, as determined by real-time PCR analysis. The relative gene expression levels of *fshb*, *lhb*, *gpa*, *gnrhr1* and *cyp19a1b* were analyzed during the sex differentiation period (90–150 dah) and testicular development period (180–300 dah). The transcript values of genes were calibrated with *gapdh* as an internal control and then normalized to the value of the pituitary at 90 dah, which was defined as 1. The results are expressed as the mean  $\pm$  SEM (n = 4 pools/group for pituitary with 10 individual pituitaries/pool for 90 to 150 dah or with 5 individual pituitaries/pool for 180 to 300 dah). Lowercase letters indicate significant differences ( $P < 0.05$ ) in gene expression in the pituitary between groups according to one-way ANOVA followed by Duncan's test.

significantly decreased at 300 dah that the level was similar to that at 90 dah.

### 3.5. Effect of E2 on transcript levels

Based on our previous study that demonstrated E2-dependent activation of brain neurogenesis and neurosteroidogenesis during the period of gonadal differentiation (Lin et al., 2016), we investigated the effect of E2 treatment on the expression of our genes of interest in the present study.

#### 3.5.1. Effect of E2 on pituitary and brain transcript levels of aromatase

qPCR was performed on fish at 90 dah to 300 dah to investigate the effect of E2 treatment on *cyp19a1b* transcript levels during periods of gonadal differentiation and testicular development (Fig. 6).

E2 showed a positive effect on *cyp19a1b* expression in the brain, with significant increases observed in telencephalon and di-/mesencephalon at each sampling time, during both gonadal differentiation and testicular development periods. In telencephalon, expression levels ranging from 5.5-fold to 15.3-fold higher than the respective control group were observed ( $P < 0.01$  to  $P < 0.01$ ). A similar positive effect of E2 was observed in di-/mesencephalon, which showed expression levels 3.0-fold to 9.6-fold higher than in each respective control group ( $P < 0.01$  to  $P < 0.01$ ). The significant stimulatory effect of E2 on *cyp19a1b* during gonadal differentiation in dissected brain parts is in agreement with our previous study (Lin et al., 2016).

The effect of E2 on the pituitary *cyp19a1b* was different from brain *cyp19a1b*. The E2 effect on pituitary *cyp19a1b* was not significant at all sampling times and was limited to 120 dah and 300 dah. The *cyp19a1b* expression after E2 treatment in the pituitary was 2.7-fold higher than that in the control at 120 dah ( $P < 0.05$ ) and was 2.9-fold higher at 300 dah ( $P < 0.01$ ).

#### 3.5.2. Effect of E2 on pituitary transcript levels of gonadotropin subunits and *gnrhr1*

Transcript level analysis was performed by qPCR to investigate the E2 effect during the period of gonadal differentiation and testicular development (Fig. 7).

Stimulatory effects of E2 on pituitary *fshb* and *lhb* subunit expression were observed at all sampling times during the gonadal differentiation and testis development periods. Pituitary *fshb* showed a strong response to E2, with expression levels 3.0-fold to 20.0-fold higher than each respective control group ( $P < 0.05$  to  $P < 0.01$ ). *Lhb* showed a smaller response to E2 compared to *fshb*: 1.5-fold to 3.9-fold increases were observed ( $P < 0.05$  to  $P < 0.01$ ).

A significant response of *gpa* to E2 was observed only during the testicular development period, with a 2.1-fold to 5.9-fold increase compared to the respective control groups ( $P < 0.05$  to  $P < 0.01$ ). E2 had no significant effect on *gpa* during the gonadal differentiation period.

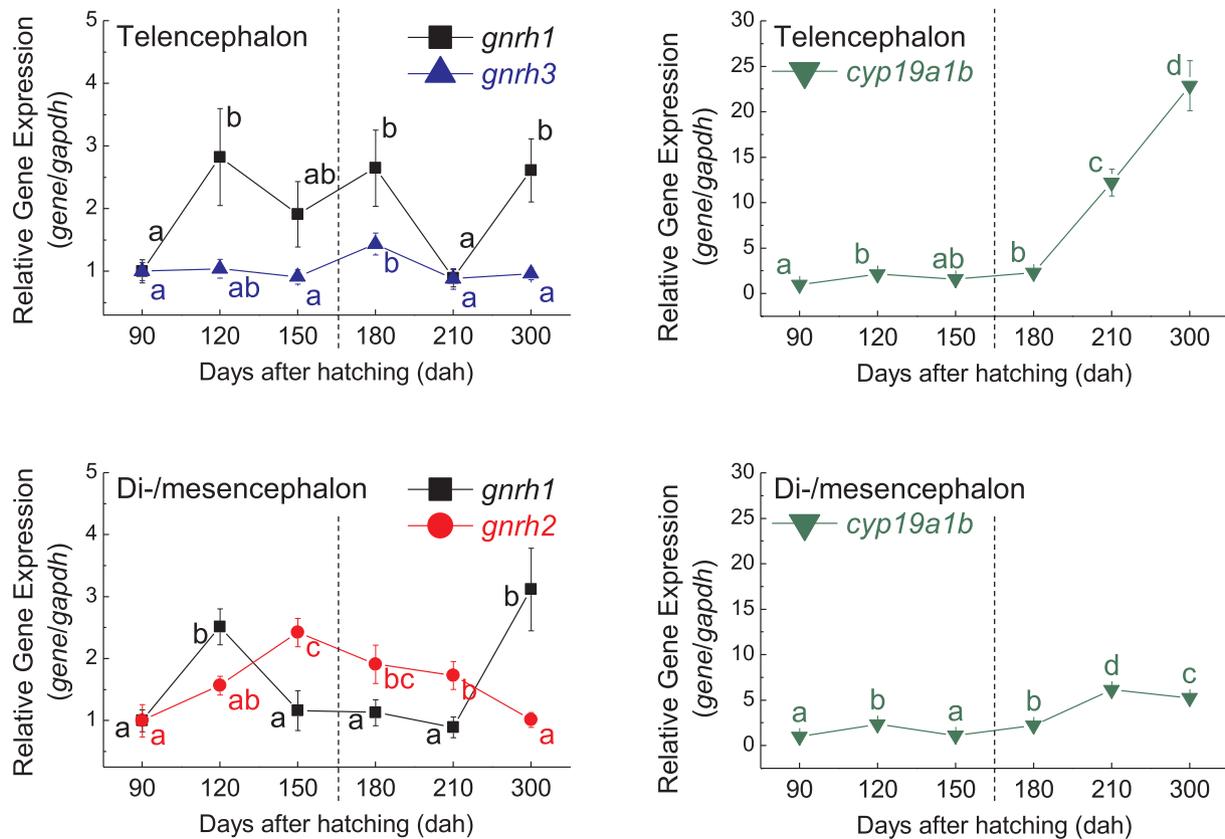
A slight but significant stimulatory effect of E2 on *gnrhr1* transcript levels was observed during gonadal differentiation, with a 2.3-fold increase at 120 dah ( $P < 0.05$ ) and a 1.8-fold increase at 150 dah ( $P < 0.05$ ), as well as a slight response with a 1.8-fold increase at 300 dah ( $P < 0.05$ ), compared to their respective control groups.

Altogether, E2 treatment significantly stimulated pituitary *fshb*, *lhb*, and *gnrhr1* expression during the gonadal differentiation period, while there was no significant effect on *gpa* at that time. During testicular development, E2 showed positive effects on gonadotropin subunits (*fshb*, *lhb*, *gpa*) and *gnrhr1* but only at a limited sampling time (300 dah).

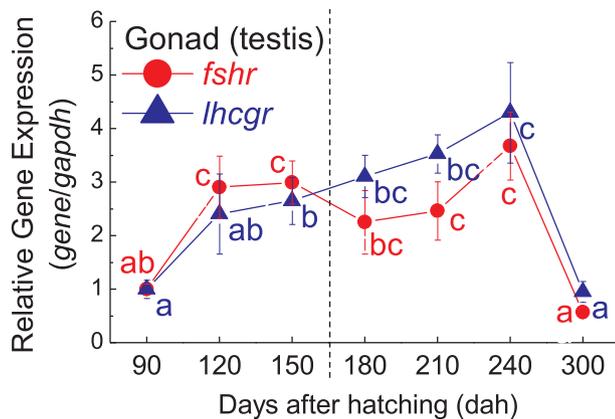
#### 3.5.3. Effect of E2 on brain transcript levels of *gnrh* genes

qPCR was performed on telencephalon and di-/mesencephalon to investigate the E2 effect on *gnrh* genes in fish from 90 dah to 300 dah (Fig. 8).

E2 had no effect on *gnrh1* expression in the telencephalon at any



**Fig. 4.** Gene expression profiles in the telencephalon and di-/mesencephalon of black porgy at 90–300 dah, as determined by real-time PCR analysis. The relative gene expression levels of *gnrh1*, *gnrh2*, *gnrh3* and *cyp19a1b* were analyzed during the sex differentiation period (90–150 dah) and testicular development period (180–300 dah). Based on their brain expression distribution, *gnrh1* was measured in telencephalon and di-/mesencephalon, *gnrh2* in di-/mesencephalon, and *gnrh3* in telencephalon. The transcript values of genes were calibrated with *gapdh* as an internal control and then normalized to the value of the indicated part of the brain at 90 dah, which was defined as 1. The results are expressed as the mean  $\pm$  SEM ( $n = 8$  individuals/group). Lowercase letters indicate significant differences ( $P < 0.05$ ) in gene expression in different brain parts between groups according to one-way ANOVA followed by Duncan's test.



**Fig. 5.** Gene Expression profiles in the gonad of black porgy at 90–300 dah, as determined by real-time PCR analysis. The relative gene expression of *fshr* and *lhcr* were analyzed during sex differentiation period (90–150 dah) and testicular development period (180–300 dah). The transcript values of gene were calibrated with *gapdh* as an internal control and then normalized to the value of the gonad at 90 dah, which was defined as 1. The results are expressed as the mean  $\pm$  SEM ( $n = 3$  pools/group for gonad with 8 individual gonads/pool for 90 to 150 dah,  $n = 4$  pools/group for gonad with 4 individual gonads/pool for 180 dah,  $n = 5$  pools/group for gonad with 2 individual gonads/pool for 210 and 240 dah,  $n = 10$  individual gonads/group for 300 dah). Lowercase letters indicate significant difference ( $P < 0.05$ ) in gene expression in gonad between groups according to the one-way ANOVA followed by Duncan's test.

sampling time during gonadal differentiation and testicular development. Similarly, E2 did not affect *gnrh1* expression in the di-/mesencephalon, except at one sampling time (210 dah: 2.0-fold compared to the respective control group,  $P < 0.05$ ).

A slight but significant stimulatory effect of E2 on *gnrh3* was observed in the telencephalon at most sampling times (1.6-fold to 1.8-fold higher than each respective control group,  $P < 0.05$ ) but not at 300 dah (1.3-fold compared to the respective control group,  $P > 0.05$ ).

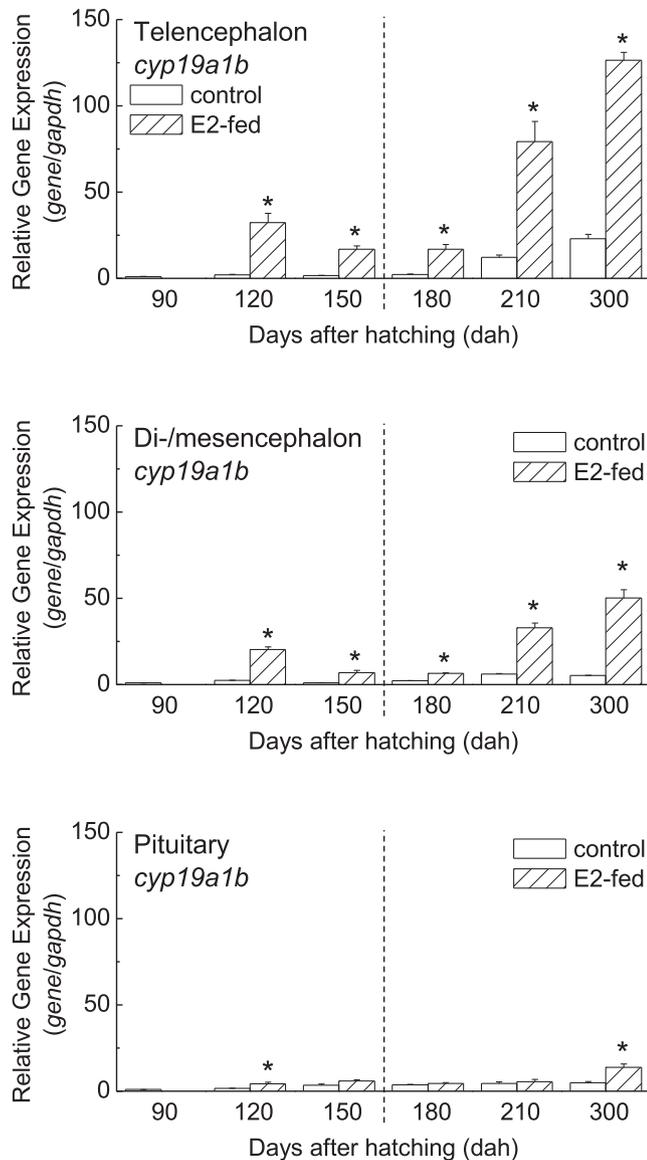
No effect of E2 on *gnrh2* was found in di-/mesencephalon, except for the samples taken at 120 dah, which showed a slight increase of 1.4-fold ( $P < 0.05$ ) compared to the respective control group.

**3.5.4. Effect of E2 on gonadal transcript levels of gonadotropin receptors**  
qPCR was performed on gonad to investigate the E2 effect on *fshr* and *lhcr* genes in fish from 180 dah to 300 dah (Fig. 9).

Inhibitory effects of E2 on gonadotropin receptors expression were observed at most sampling times but not at 300 dah (*fshr*, 1.5-fold compared to the respective control group,  $P > 0.05$ ; *lhcr*, 1.9-fold compared to the respective control group;  $P > 0.05$ ). *Fshr* showed a strong response to E2, with expression levels 0.1-fold lower than each respective control group ( $P < 0.05$  to  $P < 0.01$ ). *Lhcr* showed a smaller response to E2 compared to *fshr*: 0.5-fold to 0.2-fold decreases were observed ( $P < 0.05$  to  $P < 0.01$ ).

**3.6. Effect of AI on brain transcript levels of *gnrh1* and *cyp19a1b***

Our previous studies reported that AI-feeding treatment reduced brain aromatase enzyme activity by 70–80% (Lee et al., 2002). We investigated the effect of E2 and AI treatment on the expression of our



**Fig. 6.** Real-time PCR analysis of *cyp19a1b* in the telencephalon, di-/mesencephalon and pituitary of control fish or estradiol (E2)-treated fish. E2-treated fish were fed with an estradiol diet (6 mg/kg feed) from 90 dah. Control and E2-treated samples were collected during the sex differentiation period (90–150 dah) and testicular development period (180–300 dah). The transcript values of *cyp19a1b* were calibrated with *gapdh* as an internal control and then normalized to the value of 90 dah control fish, which was defined as 1. The results are expressed as the mean  $\pm$  SEM ( $n = 8$  individuals/group for telencephalon and di-/mesencephalon;  $n = 4$  pools/group for pituitary with 10 individual pituitaries/pool for 90 to 150 dah, or with 5 individual pituitaries/pool for 180 to 300 dah). Asterisks indicate significant differences ( $P < 0.05$ ) between control and E2-treated groups at each age, according to Student's *t*-test.

genes of interest in the present study. qPCR was performed on telencephalon and di-/mesencephalon to investigate the AI effect on *gnrh1* and *cyp19a1b* in fish during gonadal differentiation period (Fig. 10).

No statistically significant ( $P > 0.05$ ) differences on *gnrh1* expression were found in the telencephalon or di-/mesencephalon between the control and AI-treated groups. Similarly, E2 had no effect on *gnrh1* expression in the telencephalon or di-/mesencephalon at any sampling time.

As an experimental positive control, AI reduced significantly *cyp19a1b* expression in telencephalon, which showed expression levels with 0.5-fold ( $P < 0.01$ ) and 0.6-fold ( $P < 0.01$ ) lower than the respective control group at 120 and 150 dah. A similar inhibitory effect of

AI on *cyp19a1b* in di-/mesencephalon with expression levels of 0.2-fold ( $P < 0.01$ ) at 120 dah and 0.5-fold ( $P < 0.05$ ) at 150 dah lower than the each respective control group were observed. Positive effect of E2 on *cyp19a1b* was observed in di-/mesencephalon and telencephalon, which showed expression levels 2.2-fold to 14.0-fold higher than the respective control group ( $P < 0.05$  to  $P < 0.01$ ).

## 4. Discussion

### 4.1. Activation of gene expression in the brain-pituitary-gonadotropic axis during gonadal differentiation and testicular development in black porgy

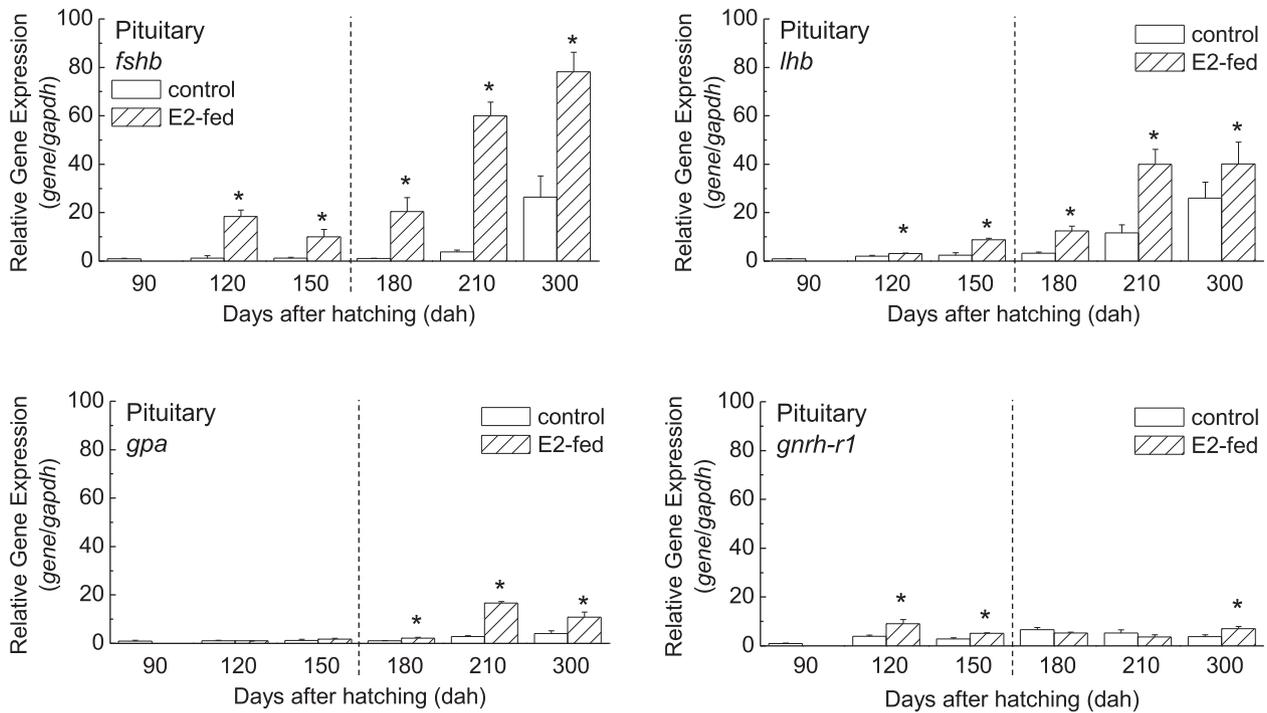
In previous studies, we revealed a peak in the brain activity of neurosteroidogenesis and neurogenesis during the gonadal differentiation period in the black porgy (Tomy et al., 2009; Tomy et al., 2007; Wu et al., 2010). We further demonstrated that this peak expression in the early brain was controlled by locally produced brain E2 and was independent of the gonads (Lin et al., 2016).

In the present study, we observed a significant peak in the expression of brain aromatase *cyp19a1b* at 120 dah, during the period of gonadal differentiation. This result is in agreement with our previous studies supporting the role of aromatase and locally produced brain E2 in the peak of brain activity and neurogenesis at this time (Lin et al., 2016). Comparison with the subsequent period of testicular development revealed a further large increase in brain aromatase expression, supporting further roles of aromatase during testis maturation, including mediation of feedback activities exerted by aromatizable gonadal androgens (Lee et al., 2001; Lee et al., 2000; Lee et al., 2002).

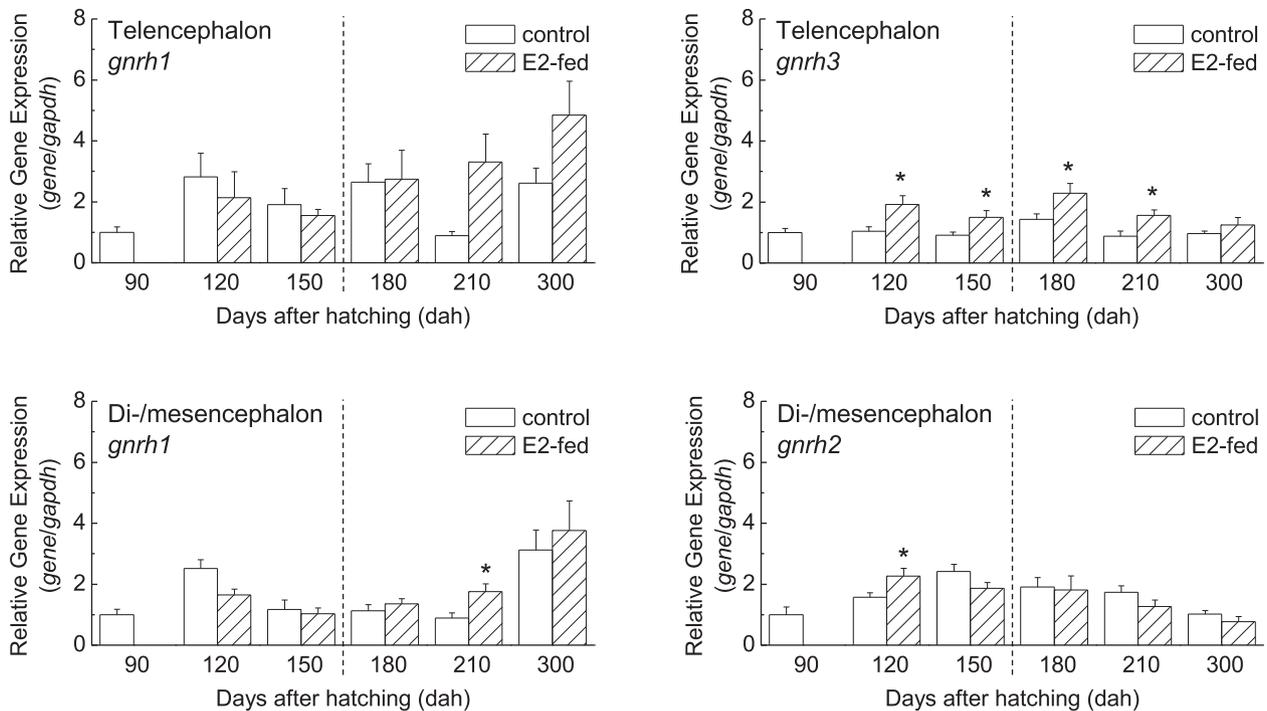
Concerning the gonadotropic axis, a small but not significant increase in pituitary gonadotropin subunit transcript levels was observed during the gonadal differentiation period, while a large increase was measured during subsequent testicular development. This large increase in transcript levels is in agreement with the involvement of gonadotropin hormones in the control of gonadal maturation and reproduction. Interestingly, pituitary *gnrh1* exhibited a very different pattern, with two significant peak increases, at 120 dah and at 300 dah, instead of a high increase during maturation. A similar pattern of increases at 120 dah and at 300 dah was also observed for brain *gnrh1*. For the gonadotropin receptors analyzed in gonads, the gene expressions of gonadal *fshr* and *lhcg*r increased during gonadal differentiation. Gradual increase of gonadal *gthrs* was measured during subsequent testicular development, but significant decrease of *gthrs* gene expression was found from 240 dah (Oct) to 300 dah (Dec). Similar decreased patterns were also found in 1+ -yr-old black porgy (Wu et al., 2016): significantly higher expression was found in prespawning season (Sep) and drop dramatically in spawning season (Dec) (15.7-fold decrease for *fshr*; 23.5-fold decrease for *lhcg*r).

Regarding the brain-pituitary-gonadotropic axis in black porgy, previous studies focused on regulations during gonadal maturation, sex change and reproduction (Chang et al., 1994; Chang and Yueh, 1990; Chang et al., 1991; Du et al., 2001; Du et al., 2005; Lee et al., 1999; Lee et al., 2000; Lin et al., 2010; Yen et al., 2002). Only one study investigated GnRH during the period of gonadal differentiation (Du et al., 2005). This previous study from our group, using HPLC and radioimmunoassay, revealed two similar peaks of GnRH contents, at 120–150 dah and at 300–360 dah, during the nonspawning and spawning seasons, respectively. The *gnrh1* gene expression patterns analyzed in the present study are in good agreement with the peak of radioimmunoassayable GnRH contents reported by Du et al. (2005) at the same times. In particular, they support a peak expression of GnRH1 during the period of gonadal differentiation.

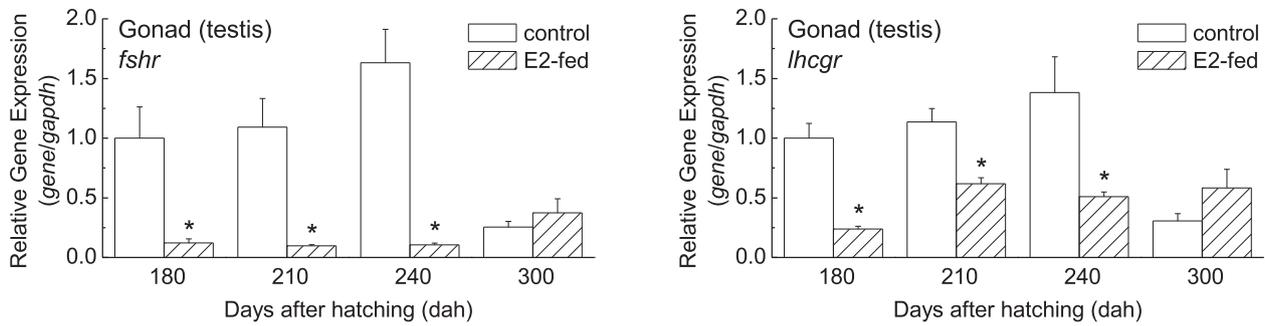
The parallel expression patterns for *gnrh1* and *gnrh1r* observed in the present study might suggest a close relationship between *gnrh1* and *gnrh1r* and their potential activation and roles during gonadal differentiation.



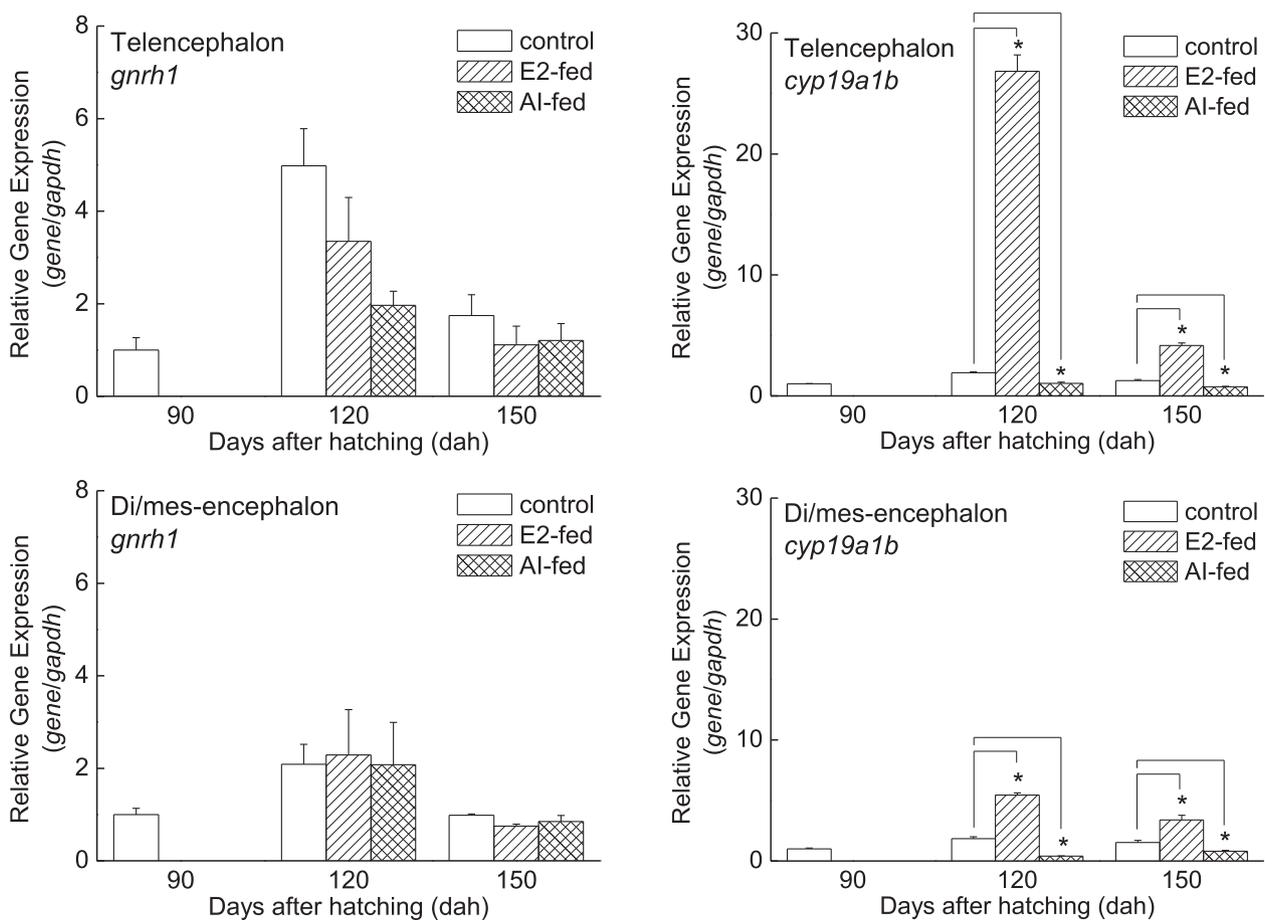
**Fig. 7.** Real-time PCR analysis of *fshb*, *lhb*, *gpa* and *gnhr1* in the pituitary of control fish or estradiol (E2)-treated fish. E2-treated fish were fed with an estradiol diet (6 mg/kg feed) from 90 dah. Control and E2-treated samples were collected during the sex differentiation period (90–150 dah) and the testicular development period (180–300 dah). The transcript values of gene were calibrated with *gapdh* as an internal control and then normalized to the value of 90 dah control fish, which was defined as 1. The results are expressed as the mean  $\pm$  SEM (n = 4 pools/group for pituitary with 10 individual pituitaries/pool for 90 to 150 dah or with 5 individual pituitaries/pool for 180 to 300 dah). Asterisks indicate significant differences ( $P < 0.05$ ) between control and E2-treated groups at each age, according to Student's *t*-test.



**Fig. 8.** Real-time PCR analysis of *gnhr1*, *gnhr2* and *gnhr3* in the telencephalon and di-/mesencephalon of control fish or estradiol (E2)-treated fish. E2-treated fish were fed with an estradiol diet (6 mg/kg feed) from 90 dah. Control and E2-treated samples were collected during the sex differentiation period (90–150 dah) and the testicular development period (180–300 dah). The transcript values of gene were calibrated with *gapdh* as an internal control and then normalized to the value of 90 dah control fish, which was defined as 1. The results are expressed as the mean  $\pm$  SEM (n = 8 individuals/group). Asterisks indicate significant differences ( $P < 0.05$ ) between control and E2-treated groups at each age, according to Student's *t*-test.



**Fig. 9.** Real-time PCR analysis of *fshr* and *lhcr* in the gonad of control fish or estradiol (E2)-treated fish. E2-treated fish were fed with an estradiol diet (6 mg/kg feed) from 90 dah. Control and E2-treated samples were collected during the testicular development period (180–300 dah). The transcript values of gene were calibrated with *gapdh* as an internal control and then normalized to the value of 180 dah control fish, which was defined as 1. The results are expressed as the mean  $\pm$  SEM (n = 4 pools/group for gonad with 4 individual gonads/pool for 180 dah, n = 5 pools/group for gonad with 2 individual gonads/pool for 210 and 240 dah, n = 10 individual gonads/group for 300 dah). Asterisks indicate significant differences ( $P < 0.05$ ) between control and E2-treated groups at each age, according to Student's *t*-test.



**Fig. 10.** Real-time PCR analysis of *gnrh1* and *cyp19a1b* in the telencephalon and di-/mesencephalon of control, estradiol (E2)-treated or aromatase inhibitor (AI)-treated fish. E2- (6 mg/kg feed) or AI- (20 mg/kg feed) treated fish were fed from 90 dah. Samples were collected during the sex differentiation period (90–150 dah). The transcript values of gene were calibrated with *gapdh* as an internal control and then normalized to the value of 90 dah control fish, which was defined as 1. The results are expressed as the mean  $\pm$  SEM (n = 3 individuals/group). Asterisks indicate significant differences ( $P < 0.05$ ) between control and treatment groups at each age, according to Student's *t*-test.

#### 4.2. Possible role of gonadotropic axis in black porgy gonadal differentiation?

The brain-pituitary-gonadotropic axis is well known to regulate sexual maturation and reproduction, and this axis may also play a role in gonadal differentiation in some fish species (Senthilkumaran et al., 2015). A peak in the activity of neurosteroidogenesis and neurogenesis was found in the brain of black porgy during gonadal differentiation.

The factors triggering peak expression are unknown, and whether the gonadotropic axis hormones are involved in gonadal differentiation in black porgy has been unclear.

During sex differentiation of European sea bass, increased expression of GnRH1 and GnRH3 was observed in the brain by semi-quantitative PCR and ELISA. The increase in GnRH1 was correlated with *gnrh1* gene expression in the pituitary during this period (Moles et al., 2007). In European sea bass, three GnRHs were detectable in the

brain and pituitary by ELISA during gonadal differentiation (Moles et al., 2007; Rodriguez et al., 2000). However, only *Gnrh1* showed a significant increase during maturation, along with higher plasma Lh levels and plasma testosterone levels in male sea bass (Rodriguez et al., 2000). All three *Gnrhs* were proposed to be involved in achieving gonadal differentiation, while *Gnrh1* may be the most relevant form in the regulation of the first spawning season in male sea bass (Rodriguez et al., 2000). According to immunostaining results in pejerrey, *Gnrh*-, *Fshb*-, and *Lhb*-positive cells appeared in brain or pituitary before gonadal differentiation, which suggested a role for the brain-pituitary-gonadotropic axis in this period (Miranda et al., 2003; Miranda et al., 2001). Increased *Gnrh*-positive neurons were found in pejerrey during the sensitive period of sex determination, which indicated the active activity of brain-pituitary-gonadotropic axis in sex differentiation (Miranda et al., 2003; Somoza et al., 2006). *Gnrh*-positive neurons in Nile tilapia were detected as early as 5 dah before the critical period of sex differentiation (7–21 dah), and showed sexually dimorphic patterns (Swapna et al., 2008). However, in the Nile tilapia and Malabar grouper, qPCR and immunostaining results indicated that changes in pituitary gonadotropin expression levels were not critical for gonadal differentiation (Munakata and Kobayashi, 2010; Yan et al., 2012). The authors suggested that gonadal *fshr* transcript levels in Nile tilapia would be a key factor involved in determining gonadal differentiation (Yan et al., 2012). In zebrafish, gonadotropins or gonadotropin receptors may not involve in gonadal differentiation. The TALEN-disruption of *gonadotropins* and *gonadotropin receptors* delayed spermatogenesis in zebrafish, but not much difference was observed between control and mutant male fish during gonadal differentiation (Zhang et al., 2015a,b).

The role of the brain-pituitary-gonadotropic axis in gonadal differentiation may be species-specific and different among teleost fishes. In the present study, similar but not identical to results from European sea bass, black porgy showed increased expression of *gnrh1* in anterior brain and of *gnrhr1* in the pituitary during gonadal differentiation. Pituitary gonadotropin subunit transcript levels showed a slight but not significant increase during this period. The gene expressions of *lhcg*r and *fshr* in gonads significantly increased during gonadal differentiation. These results support the possibility of the activation of the axis of brain *gnrh1*, pituitary *gnrhr1* and gonadotropin subunits, and gonad *gthrs* at the time of gonadal differentiation. It might be just a correlation, but also that the activation of this axis may involve in the activation of gonadal differentiation in black porgy. We hypothesize that *gnrh1-gnrhr1* might thus involve in stimulating gonadotropin release to achieve gonadal differentiation in black porgy. Future studies may aim at investigating the cell localization and cell number of brain *gnrh1* neurons, pituitary *gnrhr1* and gonadal gonadotropin receptors in relation to the activation of brain-pituitary-gonadal axis during gonadal sex differentiation by *in situ* hybridization or immunohistochemistry.

#### 4.3. Estradiol positive feedback on brain aromatase, and pituitary gonadotropins

E2 exerted positive feedback on brain aromatase gene expression in the anterior brain in the present study, and this result was in agreement with our previous study at the time of gonadal differentiation (Lin et al., 2016). We also observed this stimulatory effect during testicular development. A local upregulation of E2 production via an autoregulatory loop through nuclear estrogen receptors and an ERE (estrogen response element) within the promoter region of the *cyp19a1b* gene has been reported in various teleost species (Balthazart and Ball, 1998; Diotel et al., 2010; Le Page et al., 2011). During the period of gonadal differentiation, this upregulation may enhance the local production of E2 and E2-regulated neurogenesis in the brain. During the period of testicular development, this upregulation may in addition enhance the E2-mediated feedback exerted by aromatizable androgen (testosterone) produced peripherally by the testis.

Teleost brain aromatase presented in radial glial cells, and distributed in brain and pituitary. Expression of *Cyp19a1b* was found in the pituitary of Japanese eel, tilapia, madaka, killifish, and ricefield eel by immunohistochemistry or *in situ* hybridization (Dong and Willett, 2008; Fontaine et al., 2019; Jeng et al., 2012b; Kitahashi et al., 2007; Zhang et al., 2014). Similar increased expression patterns of pituitary *cyp19a1b* and *fshb* were found in roach at the onset of gametogenesis (Trubiroha et al., 2012). The administration of E2 stimulated pituitary *lhb* expression in adult sablefish and ricefield eel (Guzman et al., 2018; Zhang et al., 2014). Colocalized expression of *Cyp19a1b* and *Lhb* in pituitary was determined by immunostaining in female ricefield eel, and the colocalization frequency and immunoreactivity increased during vitellogenesis towards maturation (Zhang et al., 2014). These data suggest a role of pituitary *Cyp19a1b* involved in Gth regulation in teleost gametogenesis and maturation.

Treatment of E2 increased *cyp19a1b* transcripts in the brain, but not in the pituitary *cyp19a1b* of grey mullet (Nocillado et al., 2007). In Japanese eel, E2 and CPH (catfish pituitary homogenates) induced aromatase gene expression in the brain but not in the pituitary (Jeng et al., 2012a). Gene expression of *cyp19a1b* showed different expression and regulation patterns between brain and pituitary of black porgy. The expression of brain *cyp19a1b* but not pituitary *cyp19a1b* reached to a peak level during gonadal differentiation period and higher *cyp19a1b* levels in the brain were observed during testicular development. Pituitary *cyp19a1b* was not strongly regulated by E2 during testicular development as compared to the effects of E2 in the brain *cyp19a1b*. These results indicate the different regulation mechanism of *cyp19a1b* between brain and pituitary. Correlated expression pattern of brain *cyp19a1b* and pituitary *gthrs* was found during testicular development.

A stimulatory effect on gonadotropin subunit transcript levels by E2 was observed in this study. Pituitary *fshb* and *lhb* increased significantly at all sampling times, including the periods of both gonadal differentiation and testicular development. *Fshb* showed a stronger response to E2 than did *lhb*. In previous studies in black porgy, radioimmunoassays for Lh revealed a positive effect of E2 treatment on Lh levels in the pituitary and plasma (Du et al., 2001; Lee et al., 2000; Yen et al., 2002), but no studies had investigated Fsh. The strong stimulatory effect of E2 on gonadotropin subunit transcript levels indicates positive feedback of sex steroids on the gonadotropic axis, a process that may contribute to increased expression during testicular development. E2 significantly stimulated Lh content in the pituitary and plasma, and a GnRH antagonist prevented the E2 stimulation of Lh release in 2-yr-old male adult black porgy, further demonstrating that the effect of E2 on Lh was mediated by *Gnrh* (Du et al., 2001; Lee et al., 2000; Yen et al., 2002). The increase in gonadotropin transcript levels may also result from a direct effect of E2 via the interaction of nuclear estrogen receptors and EREs within the promoter region of the gonadotropin subunit genes. EREs have been found in 5' gene-flanking regions of *fshb* and *lhb* of fish, including Chinook salmon and Nile tilapia (Shupnik and Rosenzweig, 1991; Yaron et al., 2001). Future studies will aim to characterize the promoter regions of the *fshb* and *lhb* genes in black porgy.

For the E2 effect on gonadotropin receptors, E2 decreased gene expressions of *fshr* and *lhcg*r in gonads. In previous study, higher *gth*r gene expression in testis than in ovary was observed in 1+ and 2+-yr-old black porgy (Du et al., 2005; Wu et al., 2016). E2 induced the development of ovarian tissue in protandrous black porgy (Wu et al., 2008a) which may cause the decrease of *gth*r transcript levels in gonads as shown in this study.

#### 4.4. Regulation of *gnrhr* in pituitary

In black porgy, an increase in the pituitary expression of *gnrhr1* was found during sexual maturation in 2+-yr-old fish (Lin et al., 2010). E2 increased pituitary *gnrhr1* transcription levels during gonadal differentiation and during testicular development at 300 dah (Dec) in this

study. Similar stimulation of E2 on pituitary *gnrhr1* also observed in 2+ -yr-old black porgy, both *in vivo* and *in vitro* (Lin et al., 2010). Previous studies showed a positive effect of E2 on Lh release in black porgy (Du et al., 2001; Lee et al., 2000; Yen et al., 2002), thus the increase of *gnrhr1* expression by E2 probably further potentiate the endogenous GnRH action in stimulating pituitary Lh release. In contrast to the positive effects of E2 on pituitary gonadotropin and *gnrhr1* expression (Fig. 7), there was no effect of E2 on brain *gnrh1* expression during gonadal differentiation or during testicular development (Fig. 8).

#### 4.5. Estradiol-independent activation of *gnrh1* during gonadal differentiation

During gonadal differentiation in black porgy, a peak in neurosteroidogenesis and neurogenesis was found in the brain (Tomy et al., 2009; Tomy et al., 2007; Wu et al., 2010). We previously demonstrated that this peak was E2-dependent by applying exogenous E2 or aromatase inhibitor (Lin et al., 2016). E2-dependent mechanisms are thus involved in brain activation, including neurosteroidogenesis, the expression and activity of related genes (*star*, *cyp19a1b*, *nuclear estrogen receptors*, aromatase activity), and neurogenesis (Pcna immunostaining, brain cell marker genes *blbp* and *bdnf*) during the period of gonadal differentiation in black porgy.

Unlike the E2-dependent genes that we examined in previous reports, E2 showed no significant effect on *gnrh1* expression. The E2 effects on *gnrh1* transcript levels were further confirmed by AI treatment, no significant changes were found between the control and the AI- or E2-treated groups. The peaked expression of *gnrh1* during gonadal differentiation was not affected by E2 or AI. Our study indicates that the peak of *gnrh1* expression during the gonadal differentiation period was not induced by E2 and therefore was not directly related to the peak of neurosteroidogenesis during this period in black porgy.

In conclusion, some activation of the gonadotropic axis in black porgy occurs during the gonadal differentiation period, as indicated by the peak expression of *gnrh1* and *gnrhr1*, similar to that observed later during testicular development. This early activation may be involved in the control of gonadal differentiation. The activation of *gnrh1* expression is E2-independent and thus not directly related to the E2-dependent peak in brain neurosteroidogenesis and neurogenesis previously reported during the period of gonadal differentiation. Both E2-independent and E2-dependent mechanisms are thus involved in the peak expression of various genes in the brain of black porgy at the time of gonadal differentiation.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2019.05.008>.

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