

# Profile of Gene Expression Changes During Estrodiol-17 $\beta$ -Induced Feminization in the *Takifugu Rubripes* Brain

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## Research Article

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# Abstract

**Background:** As the critical tissue of the central nervous system, the brain has been found to be involved in gonad development. Previous studies have suggested that gonadal fate may be affected by the brain. Identifying brain-specific molecular changes that occur during estradiol-17 $\beta$  (E<sub>2</sub>) -induced feminization is crucial to our understanding of the molecular control of sex differentiation by the brains of fish.

**Results:** In this study, the differential transcriptomic responses of the *Takifugu rubripes* larvae brain were compared after E<sub>2</sub> treatment for 55 days. Our results showed that 514 genes were differentially expressed between E<sub>2</sub>-treated-XX (E-XX) and Control-XX (C-XX) *T. rubripes*, while 362 genes were differentially expressed between E<sub>2</sub>-treated-XY (E-XY) and Control-XY (C-XY). For example, the expression of *cyp19a1b*, *gnrh1* and *pgr* was significantly up-regulated, while *st*, *sl*, *tsh $\beta$* , *prl* and *pit-1*, which belong to the growth hormone/prolactin family, were significantly down-regulated after E<sub>2</sub> treatment, in both sexes. The *arntl1*, *bhlbe*, *nr1d2*, *per1b*, *per3*, *cry1*, *cipc* and *ciart* genes, which are involved in the circadian rhythm, were also found to be altered. Differentially expressed genes (DEGs), which were identified between E-XX and C-XX, were significantly enriched in neuroactive ligand-receptor interaction, arachidonic acid metabolism, cytokine-cytokine receptor interaction and the calcium signaling pathway. The DEGs that were identified between E-XY and C-XY were significantly enriched in tyrosine metabolism, phenylalanine metabolism, arachidonic acid metabolism and linoleic acid metabolism.

**Conclusion:** By using transcriptome sequencing of XX and XY brains of torafugu larvae showed that many genes and pathways were altered by E<sub>2</sub> exposure. They provide the opportunity to further study the possible involvement of networks in the brain-pituitary-gonadal axis in sex differentiation in *T. rubripes*.

## Background

Sex determination and differentiation are the most essential processes for species reproduction [1]. Sex determination is defined as the developmental process by which the sex is established. Gonadal sex differentiation is defined as the process during which the undifferentiated gonad develops into either an ovary or a testis after the determination of sex [2]. Since sexual dimorphism (such as body size and growth rate) is common in fish, elucidating the mechanism involved in sex determination and differentiation, which may lead to the development of a sex control technique, is of great commercial interest in the aquaculture industry [3]. Moreover, as the largest group of vertebrates, fish display the greatest diversity of sexual phenotypes and are considered excellent models for the investigation of mechanisms involved in sex determination, differentiation, and sexual plasticity. Unlike mammals, sex determination and differentiation are tremendously complex and flexible in fish and the sexual fate of fish has been proven to be affected by exogenous factors (such as social dynamics, temperature, light conditions, density, pH, stress and hormones) [3–6]. Among those factors, estrogens are conserved and are known to be required for ovarian differentiation and maintenance of the female phenotype [6]. Prior to sexual differentiation, the administration of estradiol-17 $\beta$  (E<sub>2</sub>) can induce sex reversal (male-to-female) in fish [7–8]. In fish, estrogens are synthesized by the aromatization of androgens, through cytochrome P450 aromatase, which is mainly encoded by *cyp19a1a/b*

[9]. Previous studies have shown that treatment with an aromatase inhibitor and knockout of *cyp19a1a* can result in sex reversal in the female [10–12].

*Takifugu rubripes*, which is commonly known as the tiger puffer or torafugu, is one of the most popular marine-cultured species in Asia. It is famous for its umami taste and has been available in local Chinese markets since 2016. Over half of the yearly *T. rubripes* production is exported overseas [13]. Since *T. rubripes* testis is a delicacy, male torafugu are more expensive and popular than female torafugu. Hence, monosex male production is desirable in aquaculture. Moreover, *T. rubripes* is considered as an ideal model for investigating the molecular mechanisms that underlie sex determination and differentiation as it has a relatively small and compact genome, when compared to other vertebrates [14–15]. The process of gonadal development in torafugu and the expression profiles of genes related to the sex differentiation process have been described in previous studies [16–18]. It has been demonstrated that treatment of genetically XY torafugu with E<sub>2</sub>, prior to morphological sex differentiation, can induce feminization. Treatment of XX torafugu with aromatase inhibitor (fadrozole or letrozole) results in the inhibition of ovarian cavity formation [8, 17, 19]. Several genes involved in E<sub>2</sub>-induced feminization in the gonads of torafugu have been characterized in our previous study [19].

Prior to differentiation of the gonads, sexual differences exist in non-gonadal tissues as well as the germline. Thus, these differences are created up-stream of gonadal differentiation [20–22]. As the critical tissue of the central nervous system, the brain has been found to be involved in gonad development, and the differential development of the two sexes could be the result of differential gene expression in the brain, prior to the formation of the gonads [21–26]. Several sexually dimorphic markers or genes have been identified in the brains of vertebrates [27–33]. Although sex differences in the brain are often presumed to be a consequence of gonadal sex, rather than the cause [34], sex differentiation in the brain is a highly complicated process in lower vertebrates. Previous studies have suggested that gonadal fate may be affected by the brain. For example, forebrain transplants between male and female Japanese quail embryos, before sexual differentiation, disrupted testis function [35–36]. Perceived social changes can also induce sexual transitions via intersection of the hypothalamic-pituitary-interrenal pathway and hypothalamic-pituitary-gonad pathway before sex differentiation in some sequentially hermaphroditic coral reef fish [37]. In zebrafish, luteinizing hormone beta subunit (*lhβ*)/follicle stimulating hormone beta subunit (*fshβ*) double knock out resulted in all male fish, whilst gene disruption of *fshr*, but not *lhcg*, resulted in masculinization into males and a complete failure of follicle activation [38–39]. Until now, unlike gonadal sex differentiation, the mechanisms that underlie the sexual differentiation of the brain have not been completely defined. Identification of genes related to sex differentiation in the brain may facilitate studies of gene interaction between the gonads and brain, which control sex differentiation. However, few studies have focused on gene expression changes in the brain during the process of sex differentiation, particularly during the process of sex steroid hormone-induced feminization or masculinization in fish species such as the *T. rubripes* [26].

We previously reported the sexually dimorphic expression profile of genes in torafugu brain [40]. In this study, *T. rubripes* larvae were treated with 100 µg/L E<sub>2</sub>, from 25 to 80 days after hatching (dah). Transcriptomic analysis of brains from the control and E<sub>2</sub>-treated groups was then performed. This study aimed to identify target genes and pathways that are involved in the development of torafugu brains and that responded to E<sub>2</sub>

administration. The data may provide new insights into the mechanism of sex differentiation in the brain and may indicate how estrogen affects gene expression in the brain.

## Results

### Histological evaluation of gonadal development

Figure 1 shows the results of the histological analysis of the gonads. As reported by Yan et al., sex reversed larvae were not observed in the control groups [19]. Gonads from the C-XX group occupied the ovarian cavities, which were filled with a small number of oocytes and a large number of oogonia, closely arranged on the oviposition plate. Gonads from the C-XY group were filled with spermatogenic cells at different developmental stages (Fig. 1a and b). However, gonads from the E<sub>2</sub>-immersed group were smaller than those from the control group. In addition, exposure to E<sub>2</sub> obviously induced the feminization of testes and an ovarian cavity was observed in all E<sub>2</sub>-treated XY torafugu (Fig. 1c and d).

### Illumina Sequencing and mapping, and identification of global DEGs, in response to E<sub>2</sub> treatment

Transcriptomic analysis generated 44,523,690 (C-XX\_1), 45,264,242 (C-XX\_2), 66,606,798 (C-XX\_3), 43,978,518 (C-XY\_1), 46,201,180 (C-XY\_2), 46,409,586 (C-XY\_3) 44,537,526 (E-XX\_1), 40,345,586 (E-XX\_2), 49,202,476 (E-XX\_3), 47,352,9109 (E-XY\_1), 43,506,024 (E-XY\_2) and 45,728,118 (E-XY\_3) raw reads from each library, respectively (Table 2). After data filtering, 43,622,952 (C-XX\_1), 44,436,616 (C-XX\_2), 42,850,608 (C-XX\_3), 42,851,086 (C-XY\_1), 45,072,134 (C-XY\_2), 45,005,800 (C-XY\_3), 43,442,122 (E-XX\_1), 39,629,744 (E-XX\_2), 48,253,210 (E-XX\_3), 46,193,034 (E-XY\_1), 42,700,218 (E-XY\_2) and 44,804,906 (E-XY\_3) clean reads were obtained from each library, respectively (Table 2).

As shown in Figs. S1, 3, only eight DEGs were identified in the C-XY versus (vs) C-XX comparison. These included three DEG that was up-regulated and one that were down-regulated, such as aryl hydrocarbon receptor interacting protein-like 1 (*aip1*), serine protease hepsin-like, retinol dehydrogenase 11-like (*rdh11*), and nucleoprotein TPR-like (Table 3). In the E-XX vs C-XX comparison, 514 DEGs were identified, of which 85 were up-regulated (Fig. 2a, 3-4). These included gonadotropin-releasing hormone 1 (*gnrh1*), cytochrome P450 aromatase (*cyp19a1b*), progesterone receptor (*pgr*), solute carrier family 6 (*slc6a20*) and cytochrome P450 1A1-like (*cyp1a1*). There were 216 down-regulated DEGs in this comparison, which included prolactin (*prl*), thyroid stimulating hormone (*tshb*), somatolactin-like (*sl*), glycoprotein hormones (*cga*) and pro-opiomelanocortin-like (*pomc*) (Table 3). Moreover, 362 DEGs were identified in the E-XY vs C-XY comparison, of which 52 were up-regulated (Fig. 2b, 3-4), such as vitellogenin-2-like (*vtg2*), *pgr*, *gnrh1*, *cyp19a1b*, zona pellucida sperm-binding protein 4-like (*zp3*) and *cyp1a1*. 172 down-regulated DEGs were observed in this comparison. These included potassium channel (*kcnk18*), WD40 repeat-containing protein (*wd40*), basic helix-loop-helix family (*bhlhe41*) and forkhead box protein O1-a (*foxoa*) (Table 3).

## GO enrichment analysis of DEGs

In the C-XY vs C-XX, E-XX vs C-XX and E-XY vs C-XY comparisons, genes were mainly enriched in biological processes, followed by molecular function and cellular component GO terms (Fig. S2, 5). In the C-XY vs C-XX comparison, the DEGs were mainly significantly enriched in microtubule-based movement and movement of cell or subcellular component, in the biological process category. In the molecular function category, they were enriched in serine-type exopeptidase activity and exopeptidase activity (Fig. S2). In the E-XX vs C-XX comparison, DEGs were mainly enriched in response to oxygen-containing compound, response to drug and proteolysis, for biological process, in hormone activity, sequence-specific DNA binding and serine-type peptidase activity, for molecular function, and in calcium ion binding, myosin complex and actin cytoskeleton, for cellular component. The up-regulated genes were mainly clustered in proteolysis, for the biological process category, extracellular region, for the cellular component category, and hormone activity, for the molecular function category. The down-regulated genes were mainly clustered in cell cycle arrest, for the biological process category, myosin complex, for the cellular component category, and protein kinase regulator activity, for the molecular function category (Fig. 5a). In the E-XY vs C-XY comparison, response to oxygen-containing compound, response to chemical and response to extracellular stimulus were highly represented for the biological process category. Integral component of plasma membrane, intrinsic component of plasma membrane and plasma membrane part were highly represented for the cellular component category. Sequence-specific DNA binding, heme binding and tetrapyrrole binding were highly represented for the molecular function category. The up-regulated genes were mainly clustered in response to extracellular stimulus and response to nutrient levels, in the biological process category, integral component of plasma membrane, for the cellular component category, and sequence-specific DNA binding, for the molecular function category. The down-regulated genes were clustered in lipid transport and lipid localization in the biological process category (Fig. 5b).

## KEGG enrichment analysis of DEGs

The most enriched KEGG pathways in the E-XX vs C-XX comparison were neuroactive ligand-receptor interaction, arachidonic acid metabolism, cytokine-cytokine receptor interaction and the calcium signaling pathway (Fig. 6a). The KEGG pathways most enriched by down-regulated DEGs were neuroactive ligand-receptor interaction, steroid hormone biosynthesis, retinol metabolism, calcium signaling pathway and GnRH signaling pathway. Eight pathways, which included neuroactive ligand-receptor interaction, notch signaling pathway, cytokine-cytokine receptor interaction, PPAR signaling pathway, steroid biosynthesis, calcium signaling pathway, metabolism of xenobiotics by cytochrome P450 and GnRH signaling pathway, were the most enriched by up-regulated DEGs. In the E-XY vs C-XY comparison, the most enriched KEGG pathways were tyrosine metabolism, phenylalanine metabolism, arachidonic acid metabolism and linoleic acid metabolism (Fig. 6b). The pathways most enriched by down-regulated DEGs were steroid hormone biosynthesis, retinol metabolism, PPAR signaling pathway, carbon metabolism, metabolism of xenobiotics by cytochrome P450, calcium signaling pathway and neuroactive ligand-receptor interaction. The tyrosine metabolism, phenylalanine metabolism, arachidonic acid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism, steroid biosynthesis, histidine metabolism, metabolism of xenobiotics by

cytochrome P450, tryptophan metabolism and calcium signaling pathways were those most enriched by up-regulated DEGs.

## qPCR

The RNA-Seq data were completely consistent with the qPCR results (Fig. 7). In the control group, no significant difference in mRNA level of *gnrh1*, *cyp1a1* and *cyp19a1b* was found between the XY and XX groups ( $p < 0.05$ ). In the E<sub>2</sub> treatment group, the expression of *gnrh1*, *cyp1a1* and *cyp19a1a*, in XX and XY larvae, was significantly higher than in the control group. The level of *cyp19a1b* in the E<sub>2</sub>-treated XY group was significantly higher than in the E<sub>2</sub>-treated XX group. In the control group, no significant difference was observed in the expression level of *prph*, *per1b*, *per3*, *cipc* and *ciart*, between XY and XX larvae, whilst the level of *nart1* was significantly lower in XY larvae than in XX larvae. The expression levels of *bh1be*, *nr1d2*, *per1b*, *per3*, *cipc* and *ciart*, in E<sub>2</sub>-treated larvae brains, were significantly lower than in the control group ( $p < 0.05$ ), whilst the expression levels of *arntl1a* and *cry1* were significantly higher than in the control group ( $p < 0.05$ ).

## Discussion

To date, the use of E<sub>2</sub> for gender control has been reported in at least 56 bony fish genera from 24 families. These fish include rainbow trout, fighting fish and tilapia [43–48]. In *T. rubripes*, previous studies have also found that treatment with E<sub>2</sub>, during early sex differentiation, can induce feminization [8, 47–48]. Our previous study used the transcriptomic analysis of gonads to identify a large number of DEGs and pathways that may be involved in the process of E<sub>2</sub>-induced feminization in torafugu [19]. For example, the expression of *cyp19a1a*, *foxl2*, *gsdf*, *dmrt1*, *cyp11a1*, *cyp17a1*, *hsd3b1*, *hsd17b1* and *cyp11c1* changed dramatically in the gonad when torafugu were treated with E<sub>2</sub>. In this study, we analyzed the brain transcriptome in order to better clarify the effects of estrogen on gonadal differentiation. The number of DEGs (eight DEGs at 80 dah) identified in the brain in the C-XX vs C-XY comparison was fewer than that identified at 30 dah (250 DEGs) and 40 dah (499 DEGs), in our previous study [40]. This could be attributed to the use of different differentiation stages of the torafugu that were employed in the two transcriptomic analyses. In addition, more DEGs were identified in the brain in both the E-XX vs C-XX comparison (514 DEGs) and E-XY vs C-XY comparison (362 DEGs). This suggests that the exogenous E<sub>2</sub> triggered significant alteration of gene expression profiles in the brains of both XX and XY torafugu.

Various effects of exposure to E<sub>2</sub> have been observed in teleosts. These effects include changes in brain development, endocrine regulation, gonadal development, growth, bone development, rhythm, feeding behavior and absorption, which are closely related to fish brain modulation [49–50]. Aromatase (CYP19A1) catalyzes the biosynthesis of estrogens from androgens. In contrast to most mammalian species, which possess a single *Cyp19* gene, most teleosts possess duplicated copies of *cyp19a1*, namely *cyp19a1a* and *cyp19a1b* [51–52]. The *cyp19a1a* gene is predominantly expressed in the ovary, while *cyp19a1b* is predominantly expressed in the brain and is critical for E<sub>2</sub> biosynthesis [52–53]. After E<sub>2</sub> treatment, the expression level of *cyp19a1b* was up-regulated in the torafugu brain, which indicates that the E<sub>2</sub> synthesized

in the brain can be influenced by circulating E<sub>2</sub> levels. In zebrafish, up-regulation of *cyp19a1b* gene expression by estrogenic compound administration has also been observed and estrogen-responsive element (ERE) half sites, in the region of the *cyp19a1b* promoter, have been identified by sequence analysis [54]. Menuet et al., (2005) also found that full induction of the *cyp19a1b* promoter, by E<sub>2</sub>, requires only low estrogen receptor (ER) concentrations in zebrafish [55]. Indeed, in silico analyses of the torafugu *cyp19a1b* promoter have identified putative ERE binding sites (5'-TGACC-3', 5'-GGTCAG-3'), similar to those identified in stickleback and medaka [56]. Thus, ERE may be necessary for E<sub>2</sub>-regulated *cyp19a1b* expression in torafugu.

The GnRH neurons are the principal output neurons from the hypothalamus and control reproduction [57]. Three paralogous forms of these neurons exist, termed GnRH1, GnRH2 and GnRH3 [58]. Pulsatile secretion of GnRH1 is essential for reproduction in all vertebrates. It induces follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion in the pituitary, which triggers gonadal steroidogenesis [59]. In *T. rubripes*, the levels of *gnrh1* expression in the GnRH signaling pathway were up-regulated in the brain. The use of E<sub>2</sub> has also been shown to induce *gnrh1* up-regulation in other vertebrates. In vivo experiments in mice showed that E<sub>2</sub> rapidly acts as a hormone-activated transcription complex, to increase GnRH1 neuronal activity via ER [60]. However, ERs have not been identified as DEGs between our control and E<sub>2</sub> treatment groups. This may be due to the limitation of the transcriptomic analysis being performed at a coarse anatomical scale (such as the whole brain). Thus, we cannot exclude the possibility that the up-regulation of *gnrh1*, by exogenous estrogen, occurs via ER in the torafugu brain.

In addition, the progesterone receptor (*pgr*) was up-regulated in the torafugu brain after E<sub>2</sub> treatment. Like other members of the steroid receptor superfamily, progesterone receptors are vertebrate intracellular, ligand-inducible transcription factors [61] that are activated in the absence of their ligands by alterations in phosphorylation status [62]. Estradiol exerts positive or negative feedback on the hypothalamic-pituitary system [63–65]. Bashour et al., (2012) found that progesterone can act directly on GnRH neurons, through Pgr [66], and McCartney et al., (2009, 2010) found that the progesterone-sensitive mechanism is influenced by gonadal steroids [67, 68]. In rats, a previous study indicated that Pgr is a downstream mediator of the estradiol/ER $\alpha$  action in kisspeptin neurons [69]. Therefore, *pgr* may be involved in the mediation of E<sub>2</sub>-induced *gnrh1* expression in the torafugu brain.

Our previous study proved that E<sub>2</sub> significantly inhibits growth, survival and gonad development in torafugu larvae [19]. The results were consistent with data from both tilapia [70] and rainbow trout [71], in addition to results from another study on torafugu [47]. However, the mechanisms that underlie the effects are not clear. In mammals, it has been demonstrated that there is a close interdependence among the factors that regulate growth and reproduction, which involve the interactions between multiple growth peptides and estrogens, with their receptors [72–73]. In our transcriptome data, somatotropin (*st*), thyroid stimulating hormone beta (*tshb*), somatolactin-like (*sl*), prolactin (*prl*) and pou1f1 (*pit-1*) were down-regulated in the *T. rubripes* brain after E<sub>2</sub> treatment. The *st*, *sl* and *prl* genes belong to the growth hormone/prolactin family and have an important role in the regulation of growth and reproduction [74–75]. Somatotropin is a pituitary hormone that promotes growth by stimulating plasma IGF-I release, to support the role of IGF-I in growth regulation [76]. Studies have revealed IGF-I mediated effects on gonad cells in fish [77]. For example, IGF-I is essential for the

induction of steroidogenesis and cell proliferation [78–80]. In torafugu, E<sub>2</sub> may down-regulate the GH/IGF-I signal pathway, to inhibit fish growth and gonad development. The *tshb* gene encodes a nuclear hormone receptor for triiodothyronine, used to mediate the biological activities of the thyroid hormone [81]. Its transcription levels may represent thyrotropin expression activity from the pituitary [82]. Thyroid hormones are important for development, growth and reproduction in vertebrates [83]. The somatolactin-like pituitary hormone has been characterized in fish species and its reduction in the plasma of rainbow trout is associated with a decrease in circulating growth hormone (GH) and thyroid hormone levels [84]. Prolactin is a multifunctional polypeptide hormone that acts in many physiological processes [85]. In fish, injections of ovine Prl have been shown to antagonize the stimulatory effect of triiodothyronine (T<sub>3</sub>) on the resorption of the Japanese flounder dorsal fin rays. Prolactin works in a synergistic manner with HCG and androgens can stimulate seminal vesicle growth and secretion, to save the rapidly atrophic seminal vesicles of castrated catfish (*Heteropneustes fossilis*), caused by hypophysectomy [86]. The *pit-1* gene is one of the pou gene family and has a role as a transcription factor for *gh*, *prl*, *tshb* and *transforming growth factor-β genes (tgfb)* [87–88]. Mice that are *pit-1* gene-deficient have the combined deletion of *gh*, *prl* and *tsh*. Taken together, these results indicate that there is a close interaction in the torafugu brain between estrogens and genes that are involved in the growth and development of both sexes.

Circadian rhythm is essential for living organisms to regulate a wide array of behavior and physiology, such as sleep, activity, reproduction, feeding and endocrine functions [89]. It exists in most life forms, from unicellular bacteria to higher organisms [90]. Although the basic regulatory mechanisms and functions follow the same general design, the conservation of expression of genes involved in the circadian rhythm, throughout the kingdom, is limited [91]. In fish, the lack of conservation may allow spontaneous transitions between circadian activity, as well as dramatic seasonal changes in activity patterns [92]. This means that the system is complex, with competing central and peripheral circadian oscillators and a large number of circadian clock genes [93]. The Circadian Locomotor Output Cycles Kaput (Clock) and Brain and Muscle ARNT-like protein 1 (Arntl1/Bmal1) transcription factors promote the transcription of target genes, such as the Period (*per1*, *per2* and *per3*) and Cryptochrome (*cry1* and *cry2*) gene families. Here, we showed that the core regulators of gene expression in the brain, involved in the circadian rhythm, were also affected by E<sub>2</sub> in both sexes. For example, *arntl1a* and *cry1* were up-regulated and *bhlbe*, *nr1d2*, *per1*, *per3*, *cipc* and *ciart* were down-regulated after E<sub>2</sub> treatment. The *cry1*, *bhlbe*, *nr1d2*, *per1b*, *per3*, *cipc* and *ciart* genes are all transcriptional suppressors that play a critical role in rhythm generation via different mechanisms [94]. The *cry1*, *per1* and *per3* genes form core components of the circadian clock [95], whilst *bhlhe*, *nr1d2*, *cipc* and *ciart* inhibit Clock-Arntl/BMAL1 heterodimers, which are core clock components used to coordinate the circadian rhythm [96]. Recently, there has been increasing evidence to suggest that estrogens can alter circadian clock gene expression in mammals [97–102]. Therefore, the significant alteration of the expression levels of circadian clock genes indicates that estrogen may also interfere with the biological clock in torafugu.

The DEGs observed between the E<sub>2</sub>-treated and control groups in both sexes were significantly enriched in KEGG pathways such as neuroactive ligand-receptor interaction, calcium signaling pathway and cell adhesion molecules (CAMs). The neuroactive ligand-receptor interaction pathway comprised all ligands and receptors in the cell membrane for signal transduction [102]. Cell adhesion molecules are proteins located on

the cell surface and are required for the assembly and interconnection of various cellular functions, maintenance of tissue integration and wound healing [103–104]. Our results suggest that exogenous stimulating hormones can interfere with signal transduction. In the E-XX group, the cytokine-cytokine receptor interaction pathway was the most significantly affected, when compared with E-XY. Cytokines can act in the CNS as immunoregulators and neuromodulators during health and disease [105–106]. During cytokine-cytokine interactions, convergence of signaling pathways and divergence of the cytokine signal to activate other cytokine systems are involved in synergistic activities [107]. In the olive flounder, all 11 pathways were enriched in the brains of E<sub>2</sub>-treated phenotypic females, for example, circadian rhythm, circadian entrainment, dopaminergic synapse, calcium signaling, glutamatergic synapse, long-term depression, and taste transduction pathways, ect [26]. The circadian rhythm, calcium signaling and glutamatergic synapse pathways were also enriched in our study. These results suggest that cell adhesion, transport, circadian rhythm and the calcium signaling pathway may be affected by exposure to E<sub>2</sub> in teleosts.

In conclusion, by using transcriptomic sequencing of XX and XY brains of torafugu larvae to show that many genes and pathways were altered by E<sub>2</sub> exposure. The genes and pathways identified here will help to elucidate the genetic basis behind the E<sub>2</sub>-induced feminization process. The data also open the possibility of investigating networks in the brain-pituitary-gonadal axis in torafugu.

## Materials And Methods

### Animals

Twenty days after hatching (dah), torafugu larvae of  $6.40 \pm 0.1$  mm body length were purchased from Dalian Fugu Aquatic Product Co., Ltd., Dalian, China.

### Treatment of *T. rubripes* larvae

After a short period of acclimatization (five days), 5,700 larvae were randomly divided into two groups (control and E<sub>2</sub>-treated groups) and three replicates were created for each group (950 larvae/tank (approximately 100 L)). E<sub>2</sub> powder of  $\geq 99\%$  purity (Sigma, St. Louis, MO, USA) was dissolved in 95% ethanol to form a solution for the experimental treatment. The E<sub>2</sub> administration method was as described in our previous study [19]. In brief, from 25 to 80 dah, larvae were immersed in the 100  $\mu\text{g/L}$  E<sub>2</sub> solution for two hours each day. The larvae were fed six to eight times per day with a commercial diet (SanTong Company, Weifang, China). The experiments were carried out at  $\sim 21.0$  °C, under a natural photoperiod. Approximately 200 L water was changed after the two-hour E<sub>2</sub> exposure.

### Tissue sampling

At the end of the experiment (55 days after treatment (dat)), larvae were anesthetized in ice water. Gonads to be used for histological analysis were dissected and fixed in 4% paraformaldehyde for 24 h. They were then stored in 70% ethanol. Sampling of brains was performed using 90 torafugu from each treatment (30 larvae

per tank). Brains were stored individually in RNAlater reagent (Thermo Fisher Scientific, Baltics, USA), in a 1.5 mL plastic tube on ice. They were then snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction and sequencing. A fin of each individual to be used for genetic sex verification was placed into a single tube.

## Histological analysis, genetic gender verification and RNA preparation

Histological analyses were conducted as described previously [19]. After histological observation, in order to identify  $\text{E}_2$ -induced feminized torafugu, genomic DNA from the paraffin-block tissue was extracted in accordance with the manufacturer's instructions (TIANamp FFPE DNA kit, Tiangen, China). Genetic gender verification for each individual was performed before RNA extraction from brains. The gender was verified using an *amhr2* gene SNP, through PCR and Sanger sequencing, as described previously [15,18-19].

Total RNA from brain sample from each individual was prepared in accordance with a previously described protocol [19].

## RNA-Seq

Total RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared as follows; for each replicate, a pool of 6  $\mu\text{g}$  of RNA from genetic XX or XY torafugu (1  $\mu\text{g}$  of purified brain RNA from each individual, six individuals were mixed together) was regarded as a single sample and 1  $\mu\text{g}$  of RNA per sample was used as input material. Twelve sequencing libraries, which included the control XX (C-XX\_1, C-XX\_2, C-XX\_3), control XY (C-XY\_1, C-XY\_2, C-XY\_3),  $\text{E}_2$ -treated XX (E-XX\_1, E-XX\_2, E-XX\_3), and  $\text{E}_2$ -treated XY (E-XY\_1, E-XY\_2, E-XY\_3), were generated using a NEBNext Ultra RNA Library Prep kit for Illumina (NEB, Ipswich, MA, USA) [41]. The products were purified using an AMPure XP system (Beckman Coulter, Beverly, USA) to obtain a region of approximately 250 to 300 bp. The library preparations were conducted as previously described [19].

The reference genome and gene model annotation file were downloaded directly from NCBI ([ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF\\_000180615.1\\_FUGU5](ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000180615.1_FUGU5)) and Hisat2 v2.0.5 was used for the alignment analysis of clean data from each library. The most common method, fragments of kilobase per exon model per million reads mapped (FPKM) was used to calculate gene expression levels. Differential expression analysis for the control or  $\text{E}_2$ -treated groups was conducted using the DESeq2 R package. The  $p$ -values were adjusted in accordance with methods that have been reported previously [42]. The threshold for significant differential expression was a  $p$ -value of 0.05 and  $\log_2$  (fold-change) of 1 (Anders et al., 2010; Love et al., 2014). Subsequently, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to categorize differentially expressed genes (DEGs). The GO terms and pathways with a corrected  $p$ -value of less than 0.05 were considered significantly enriched.

## qPCR verification

The *gnrh1*, *cyp19a1b*, *cyp1a1*, *prph*, *nart1*, *arntl1a*, *bhlbe*, *nr1dd2*, *per1b*, *per3*, *cipc*, *cry1* and *ciart* genes were randomly selected for RNA-seq validation by qPCR, using an Applied Biosystems 7900 HT Real-Time PCR system, as described previously [18]. The reference gene used for the qPCR analysis was *β-actin*. Primers for the reference gene and other genes selected for validation were designed using the Primer Premier 5.0 program (Table 1). The relative expression of genes was calculated using the  $2^{-\Delta\Delta CT}$  method. Data are expressed as the mean  $\pm$  SEM. Statistical significance analysis between the treatment and control groups was conducted using one-way ANOVA ( $p$ -value  $< 0.05$ ) in the IBM SPSS software.

## Declarations

## Ethics approval and consent to participate

All experiments including torafugu procedures described here were approved by the lab animal protection regulations and guidelines of the Key Laboratory of Environment Controlled Aquaculture, Ministry of Education at Dalian Ocean University, Dalian, China. All methods were carried out in accordance with relevant Liaoning province and China's guidelines and regulations. All methods of this study also adhere to the ARRIVE Guidelines for reporting animal research.

## Consent for publication

Not applicable.

## Availability of data and materials

The data sets supporting the results of this article are available at the SRA database of NCBI (<https://www.ncbi.nlm.nih.gov/>) under project accession number PRJNA760675.

## Competing interests

The authors declare that they have no conflict of interest.

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# Authors' contributions

SX dealt with the experimental materials and wrote the manuscript. YH designed the research. JJ, LW performed the experiments, interpreted the results, and analyzed the data. XY, LQ, and LY revised the paper. All authors have read and approved the manuscript.

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## Tables

**Table 1****Primers used for qPCR of  $\beta$ -actin and sex-biased genes**

Name	Primer	Sequence (5'→3')	Length (bp)
<i><math>\beta</math>-actin</i>	Forward	CAGATGTGGATCAGCAAGCA	245
	Reverse	GCTGAAGTTGTTGGGCATTT	
<i>gnrh1</i>	Forward	GCTGGTCGGGAGTCTGATGT	155
	Reverse	AACCCAGAAGAGCGGAGGA	
<i>cyp19b</i>	Forward	AACAAGTACGGCAGCCTGG	153
	Reverse	TCCCTCCATCCCGATACT	
<i>cyp1a1</i>	Forward	ATGGCACCGAGGTCAACAA	119
	Reverse	CAGGATTGCCAGGAAGAGGTA	
<i>prph</i>	Forward	AAGCCATAGGAAAGGAGAGGG	137
	Reverse	GCGGAAGGCAATCAGGTTA	
<i>nart1</i>	Forward	TTCCCACAATAACCAGCATCA	147
	Reverse	CACGCTTACACTTTCAGCAACA	
<i>arntl1a</i>	Forward	TCCTGTTTGTGGTCGGTTGT	181
	Reverse	CTCTCTCGTGGGGCTGTATCT	
<i>bhlbe</i>	Forward	GCGACGGCAAAGATAAAGATAC	200
	Reverse	CTGTCCCACGCTGCTTATTC	
<i>nr1d2</i>	Forward	CGCCACATCAACAAGGA	187
	Reverse	ATGTGCGTAGGTGGGAGTGT	
<i>per1b</i>	Forward	CACCCTCAACGCACTCAA	175
	Reverse	GTCGGTGTTTTTCAGGGTGTA	
<i>per3</i>	Forward	ACAATGGTTCCAGCGGTTAT	109
	Reverse	TGCGAGTCCTCCCACAGA	
<i>cipc</i>	Forward	ACAGGGTCAAAGGAAGGGTG	105
	Reverse	GTTGGTGATGCTGATGCTTGT	
<i>cry1</i>	Forward	AGGCGGGTGTAGAGGTCATT	110
	Reverse	GGTCTGGAAACGCTTGTAGGT	
<i>ciart</i>	Forward	CGCTCCCTCCAAGATTCCT	145

Table 2

Summary statistics of the transcriptome sequencing and mapping in *Takifugu rubripes*

Sample	Raw data	Clean_reads	Total_map	Unique_map	Multi_map
C-XX_1	44,523,690	43,622,952	40,982,945(93.95%)	39,573,936(90.72%)	1,409,009(3.23%)
C-XX_2	44,523,690	44,436,616	41,718,759(93.88%)	40,266,124(90.61%)	1,452,635(3.27%)
C-XX_3	66,606,798	42,850,608	40,099,291(93.58%)	38,741,203(90.41%)	1,358,088(3.17%)
C-XY_1	43,978,518	42,851,086	39,724,413(92.7%)	38,336,011(89.46%)	1,388,402(3.24%)
C-XY_2	46,201,180	45,072,134	42,198,181(93.62%)	40,661,639(90.21%)	1,536,542(3.41%)
C-XY_3	46,409,586	45,005,800	42,144,064(93.64%)	40,678,707(90.39%)	1,465,357(3.26%)
E-XX_1	44,537,526	43,442,122	40,708,228(93.71%)	39,264,335(90.38%)	1,443,893(3.32%)
E-XX_2	40,345,586	39,629,744	37,253,090(94.0%)	35,927,359(90.66%)	1,325,731(3.35%)
E-XX_3	49,202,476	48,253,210	45,656,235(94.62%)	44,078,863(91.35%)	1,577,372(3.27%)
E-XY_1	47,529,109	46,193,034	43,403,167(93.96%)	41,703,371(90.28%)	1,699,796(3.68%)
E-XY_2	43,506,024	42,700,218	40,001,299(93.68%)	38,698,894(90.63%)	1,302,405(3.05%)
E-XY_3	45,728,118	44,804,906	42,008,134(93.76%)	40,598,629(90.61%)	1,409,505(3.15%)

Table 3

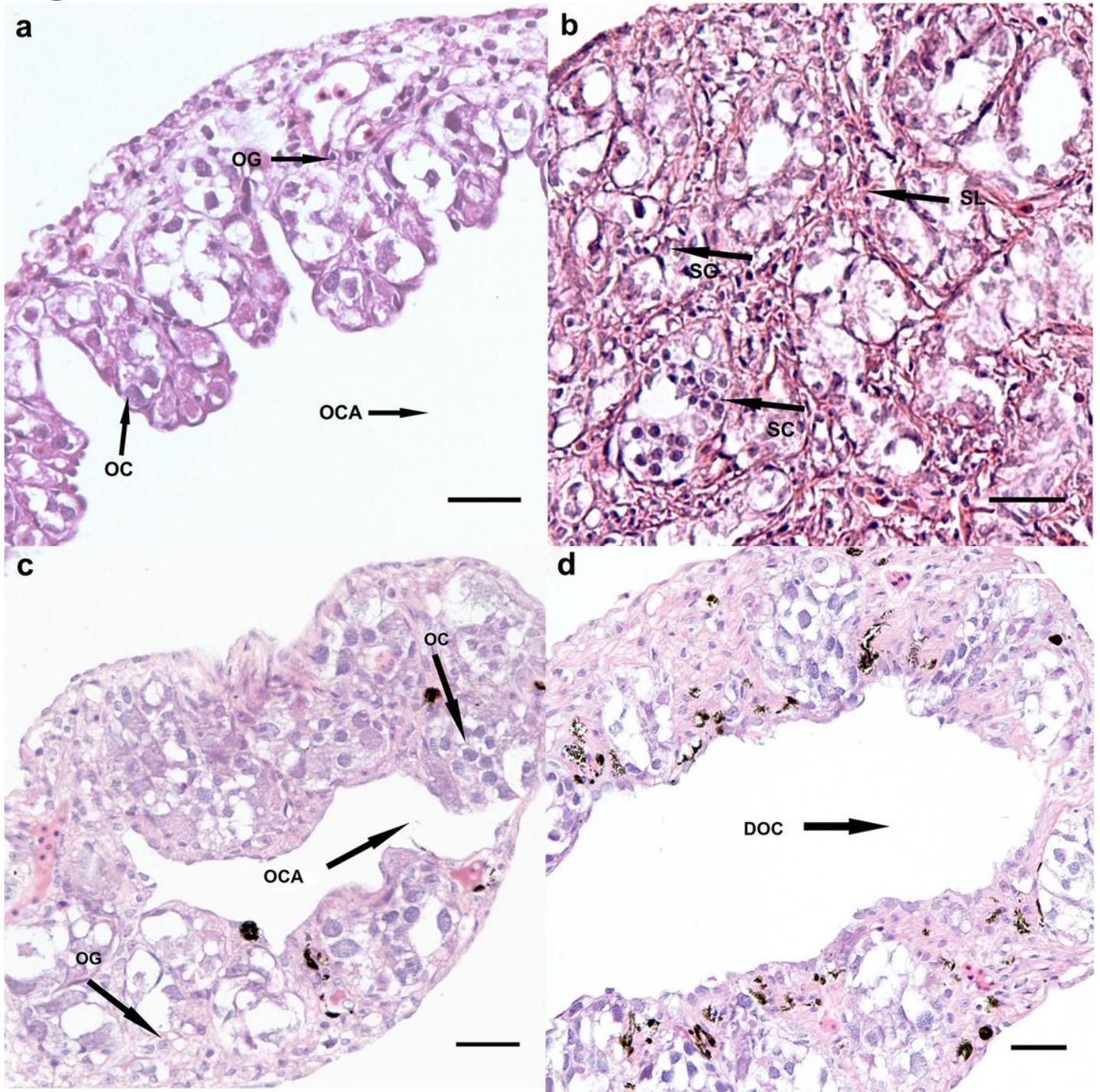
## Representative DEGs in C-XYvsC-XX, E-XXvsC-XX, and E-XYvsC-XY

Gene name	log2 Fold change	Description
<b>(C-XYvsC-XX)</b>		
<i>aip1</i>	1.38	aryl hydrocarbon receptor interacting protein-like 1
<i>rdh11</i>	1.55	retinol dehydrogenase 11-like
LOC101065721	1.11	serine protease hepsin-like
LOC101063021	-1.15	nucleoprotein TPR-like
<b>(E-XXvsC-XX)</b>		
<i>arntl</i>	1.37	aryl hydrocarbon receptor nuclear translocator-like
<i>bhlhe41</i>	-2.06	basic helix-loop-helix family member e41
<i>cga</i>	-7.19	glycoprotein hormones alpha polypeptide
<i>cipc</i>	-1.96	CLOCK-interacting pacemaker-like
<i>ciart</i>	-1.33	circadian-associated transcriptional repressor-like
<i>cyp19b</i>	2.59	cytochrome P450 aromatase
<i>cyp1a1</i>	2.25	cytochrome P450 1A1-like
<i>gnrh1</i>	3.45	gonadotropin-releasing hormone 1
<i>per1</i>	-1.63	period circadian clock 1
<i>per3</i>	-1.31	period circadian clock 3
<i>pgr</i>	2.55	progesterone receptor
<i>prl</i>	-11.94	prolactin
<i>pomc</i>	-7.17	pro-opiomelanocortin-like
<i>pou1f1</i>	-4.66	POU class 1 homeobox 1
<i>tshb</i>	-11.07	thyroid stimulating hormone beta
<i>sl</i>	-9.9	somatolactin-like
<i>slc6a20</i>	2.12	solute carrier family 6 member 20
<b>(E-XYvsC-XY)</b>		
<i>bhlhe41</i>	-2.25	basic helix-loop-helix family

<i>cyp19b</i>	2.82	cytochrome P450 aromatase
<i>cyp1a1</i>	1.84	cytochrome P450 1A1-like
<i>foxa</i>	-1.41	forkhead box protein O1-A-like
<i>gnrh1</i>	3.35	gonadotropin-releasing hormone 1
<i>kcnk18</i>	-3.05	potassium channel two pore domain subfamily member 18
<i>per3</i>	-1.35	period circadian clock 3
<i>pgr</i>	3.81	progesterone receptor
<i>vtg2</i>	6.54	vitellogenin-2-like
<i>Wd40</i>	-2.89	WD40 repeat-containing protein SMU1-like
<i>zp4</i>	2.11	zona pellucida sperm-binding protein 4-like

## Figures

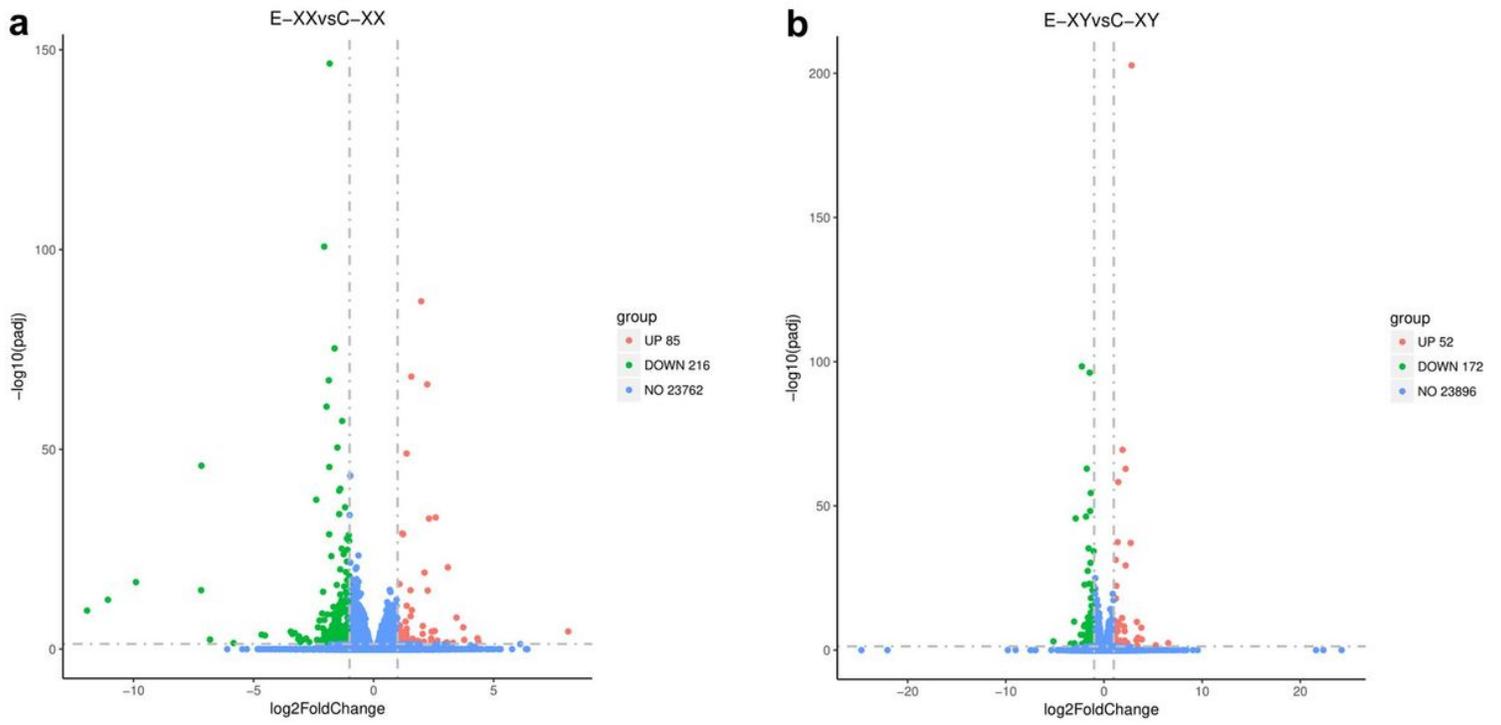
**Fig.1**



**Figure 1**

Hematoxylin-eosin stained gonad development sections from *Takifugu rubripes*. (a) ovary of control group, (b) testis of control group; (c) ovary (genetic XX) of E2 treated group, (d) intermediate gonads (genetic XY) of E2-treated group. OG, oogonia; OC, oocyte; OCA, ovarian cavity; DOC, deformed ovarian cavity; SL, spermatogenic cysts; SG, spermatogonia; SC, spermatocyte. Scale bar, 30  $\mu$ m.

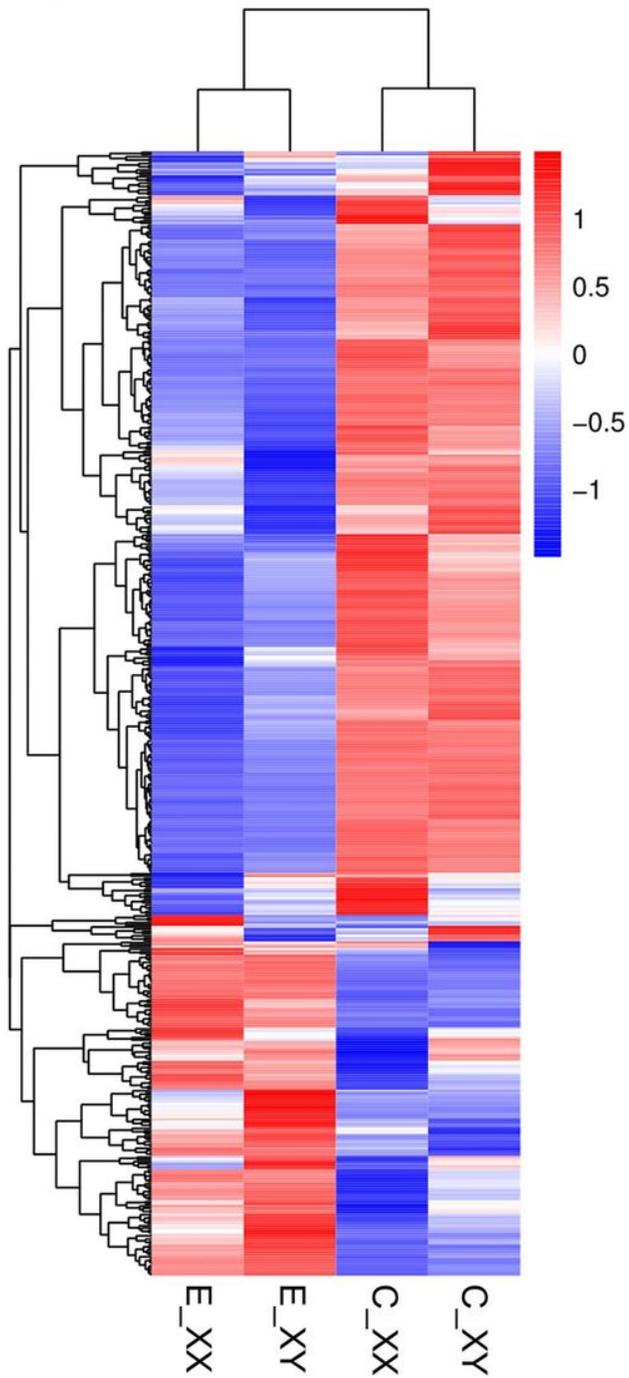
# Fig.2



## Figure 2

Volcano plot of differences in gene expression between control and E2-treated groups. (a) E-XX vs C-XX, (b) E-XY vs C-XY; Up-regulated genes (red), and down-regulated genes (green).

**Fig. 3**



**Figure 3**

Heat map of DEGs observed between the control and E2-treated groups, based on FPKM units. C\_XX, control group XX; C\_XY, control group XY; E2\_XX, E2-treated XX; E2\_XY, E2-treated XY. Red represents genes with high levels of expression and blue represents genes with low levels of expression.

Fig. 4

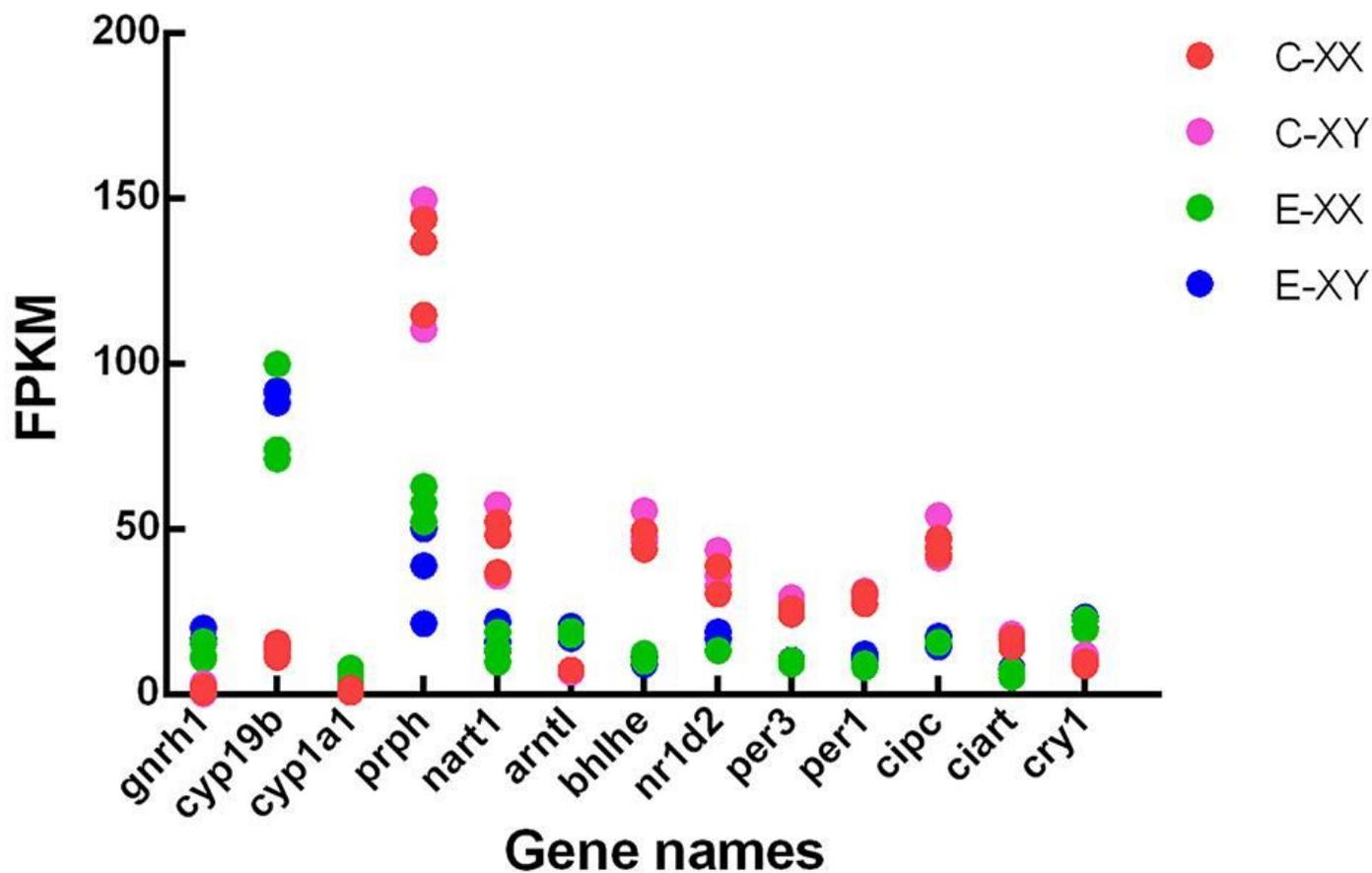


Figure 4

FPKM (reads per kb per million reads) FPKM (reads per kb per million reads) of gnrh1, cyp19a1b, cyp1a1, prph, nart1, arntl1a, bhlbe, nr1d2, per1b, per3, cipc, cry1 and ciart obtained by RNA-seq.



# Fig. 7

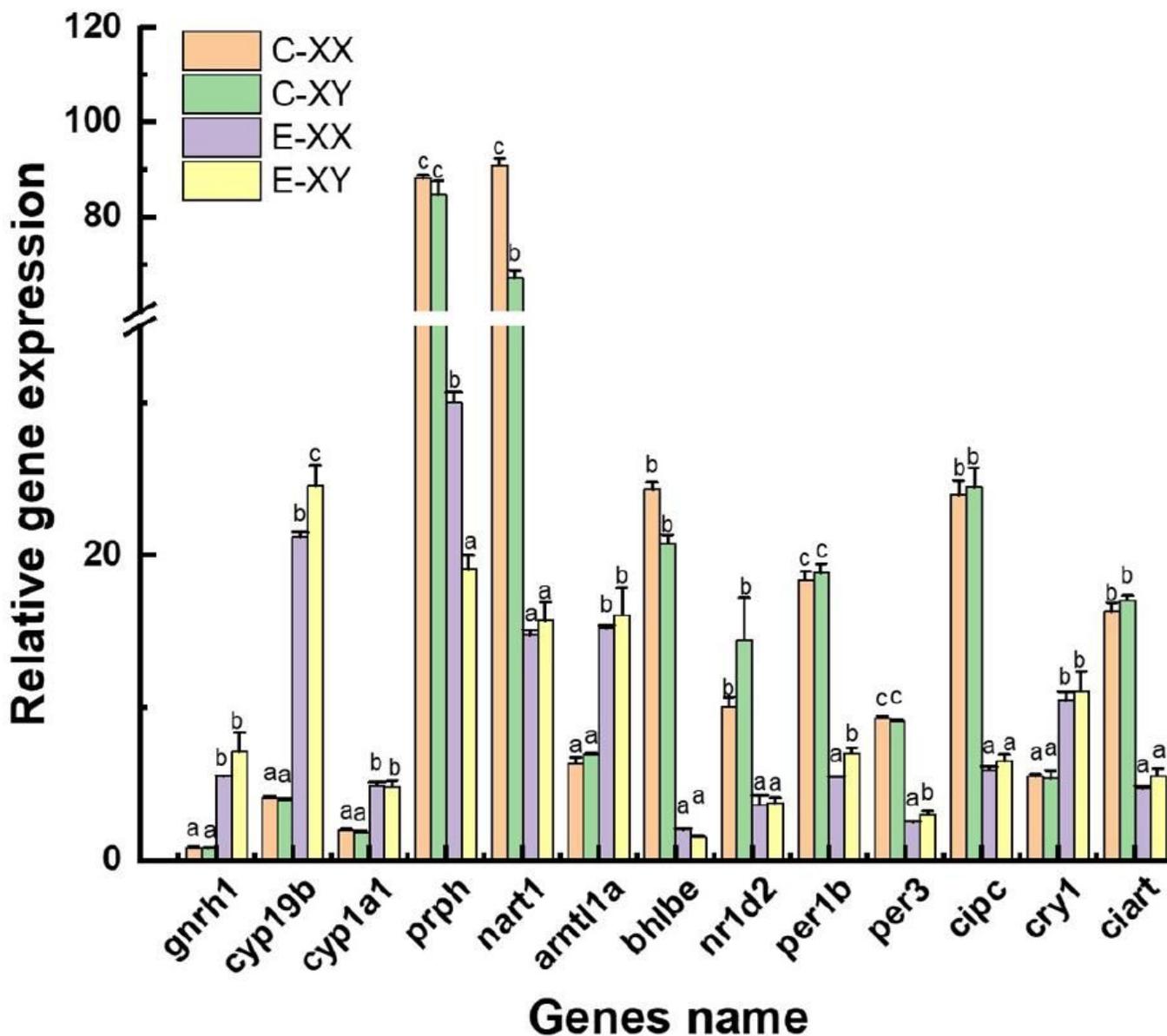


Figure 7

The levels of *gnrh1*, *cyp19a1b*, *cyp1a1*, *prph*, *nart1*, *arntl1a*, *bhlbe*, *nr1d2*, *per1b*, *per3*, *cipc*, *cry1* and *ciart* in the *T. rubripes* brain after E2 treatment. C, Control group; E2, E2-treated group. Each value represents the mean  $\pm$  SD of three measurements. One-way ANOVA ( $p$ -value  $< 0.05$ ) was used for analysis.

## Supplementary Files

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