

Simultaneous exposure to estrogen and androgen resulted in feminization and endocrine disruption

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Abstract

Estrogen, which is synthesized earlier in females than androgen in males, is critical for sex determination in non-mammalian vertebrates. However, it remains unknown that what would happen to the gonadal phenotype if estrogen and androgen were administered simultaneously. In this study, XY and XX tilapia fry were treated with the same dose of 17 α -methyltestosterone (MT) and 17 β -estradiol (E₂) alone and in combination from 0 to 30 days after hatching. Treatment of XY fish with E₂ resulted in male to female sex reversal, while treatment of XX fish with MT resulted in female to male sex reversal. In contrast, simultaneous treatment of XX and XY fish with MT and E₂ resulted in female, but with *cyp11b2* and *cyp19a1a* co-expressed in the ovary. Serum 11-ketotestosterone level of the MT and E₂ simultaneously treated XX and XY female was similar to that of the XY control, while serum E₂ level of these two groups was similar to that of the XX control. Transcriptomic cluster analysis revealed that the MT and E₂ treated XX and XY gonads clustered into the same branch with the XX control. However a small fraction of genes, which showed disordered expression, may be associated with stress response. These results demonstrated that estrogen could maintain the female phenotype of XX fish and feminize XY fish even in the presence of androgen. Simultaneous treatment with estrogen and androgen up-regulated the endogenous estrogen and androgen synthesis, and resulted in disordered gene expression and endocrine disruption in tilapia.

Key Words

- ▶ 17 α -methyl testosterone
- ▶ 17 β -estradiol
- ▶ simultaneous administration
- ▶ disordered gene expression

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Introduction

In vertebrates, sex is determined either genetically (genetic sex determination, GSD) or environmentally (environmental sex determination, ESD), or a combination of both. In mammal, sex is completely determined by GSD. In contrast, in non-eutherian vertebrates, environmental factors, especially steroid hormones, play a critical role in the sex determination.

In teleost, endogenous estrogens act as the natural inducer of ovarian differentiation, while androgen is not synthesized in the gonad during the critical period of sex determination (Nagahama 2000). In tilapia, several essential steroidogenic enzymes, Cyp11a1, 3 β -HSD, Cyp17 α 1 and Cyp19a1a, were detected in the gonads of XX fry at 10 days after hatching (dah) by immunohistochemistry

(IHC). In contrast, *Cyp11a1*, 3β -HSD, *Cyp17 α 1* and *Cyp11b2* were not observed in the testis of XY fish until 30 dah (Kobayashi *et al.* 1996, Morrey *et al.* 1998). In a recent study of gonadal transcriptomes from the Nile tilapia, we further confirmed that almost all steroidogenic enzyme genes, including *cyp19a1a* but not including *cyp11b2*, were up-regulated in the XX gonads at 5 dah, while these enzyme genes, including *cyp11b2* but not including *cyp19a1a*, were detected at 30 dah and significantly up-regulated from 90 dah onwards in the XY gonads (Takatsu *et al.* 2013). The pivotal role of estrogens in sex determination and differentiation has been demonstrated in various teleosts (Piferrer *et al.* 1994, D'Cotta *et al.* 2001, Kobayashi *et al.* 2003, Vizziano *et al.* 2008) and other non-eutherian vertebrates (Wibbels & Crews 1994, Merchant-Larios *et al.* 1997, Hudson *et al.* 2005).

Endocrine disruptor chemicals are referred to those exogenous substances that can interfere with the endocrine system and then lead to a range of developmental, reproductive, immune, neurological or metabolic diseases in human and animals (Diamanti-Kandarakis *et al.* 2009). Up to now, steroid hormones have been widely used for sex control in fish for higher commercial value in aquaculture. Environmental sex steroid can mimic the natural sex steroid and disrupt the endocrine system of aquatic animals, which results in disorders and epigenetic modification in sex determination and differentiation. Endogenous hormones such as estrogens and androgens could activate nuclear receptors and then regulate the expression of a large number of genes which would have profound effects on the organisms (Li *et al.* 2015a,b).

Epigenetic mechanisms provide organisms with the ability to modify the activity of their genes in response to changes in the internal or external environment (Piferrer 2013). Genes in the sex determination pathways are the major targets of substantial methylation modification (Navarro-Martin *et al.* 2011, Zhang *et al.* 2013, Parrott *et al.* 2014, Shao *et al.* 2014). The different expression patterns of DNA methyltransferases (DNMTs) in gonads are probably essential for the acquisition of a sex-specific methylation pattern (La Salle *et al.* 2004).

Actually, fishes and other vertebrates live in an environment with both androgenic and estrogenic substances, and therefore, it would be interesting to know i) what would happen to the gonadal phenotype if XX and XY fish were exposed simultaneously to estrogen and androgen during the critical period of sex differentiation? ii) Is their sex still controlled by the genetic sex or otherwise by estrogen irrespective to their genetic sex? iii) Is the gonadal gene expression profile of the treated fish

testis-like, ovary-like or disrupted? iv) Is epigenetic modification (methyltransferase *dnmt* genes expression) involved in the process? To answer these questions, we treated all-XX and all-XY tilapia fry with 17β -estradiol (E_2) and 17α -methyltestosterone (MT) alone or simultaneously from 0 to 30 dah, and analyzed the gonadal phenotype and gene expression profile. Our results indicated that MT and E_2 treated fish developed into females irrespective of their genetic sex. The global gene expression profiles of the gonads were in conformity with their phenotype, while a small fraction of genes showed disordered expression profiles.

Materials and methods

Animals

The Nile tilapia (*Oreochromis niloticus*) was reared in recirculating aerated freshwater tanks at 26 °C under a natural photoperiod. All-XX progenies were obtained by crossing a XX pseudomale with a normal XX female. All-XY progenies were obtained by crossing a YY supermale with a normal female. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Hormone treatment

XX fry were divided into MT and E_2 treatment (MT+ E_2 -XX), MT treatment (MT-XX) and XX control (Ctrl-XX) groups. While XY fry were divided into MT and E_2 treatment (MT+ E_2 -XY), E_2 treatment (E_2 -XY) and control (Ctrl-XY) groups. The control fish were raised in steroid-free water and fed with normal commercial diet. In treatment group, the newly hatched XX and XY fry were first exposed to water containing 50 μ g/l of MT and E_2 (Sigma–Aldrich) alone or in combination from 0 to 5 dah, and then fed with commercial diet sprayed with 95% ethanol containing the same dose (50 μ g/g feed) of MT and E_2 alone or in combination until 30 dah. Later on, all fish were raised in steroid-free water and fed with normal commercial diet. The treatments were repeated three times.

Gonads were sampled at 5, 10, 30 and 90 dah and fixed in Bouin's solution for subsequent histological observations and IHC analyses. Gonads were also sampled at 90 dah for real-time PCR and transcriptome analyses. Six fish from the Ctrl-XX, MT-XX, MT+ E_2 -XX, Ctrl-XY,

sex reversed E₂-XY and sex reversed MT+E₂-XY fish were sampled for real-time PCR. The gonad phenotype was determined by histological examination. The sex reversal rate and survival rate were calculated at 90 dah.

IHC and western blot

Gonads were dissected, fixed in Bouin's solution for 12 h at room temperature and embedded in paraffin. All tissue blocks were sectioned at 5 µm for IHC analyses. Antibodies against Cyp19a1a, the key enzyme for E₂ synthesis, and Cyp11b2, the key enzyme for 11-ketotestosterone (11-KT) synthesis, were produced by our laboratory, and were diluted by 1:1000 and 1:500 respectively in use. IHC analyses were performed as described previously (Sun *et al.* 2014). Photographs were taken under light microscope (Olympus BX 51). Total protein was extracted from Ctrl-XX, MT+E₂-XX, MT+E₂-XY and Ctrl-XY gonads of 90 dah tilapia and western blot analyses were performed as reported previously (Li *et al.* 2012).

Measurement of serum 11-KT and E₂ levels by EIA

Blood samples were collected from the caudal veins of the treatment and control fish at 90 dah, and then, kept at 4 °C overnight. Serums were collected after centrifugation and stored at -20 °C until use. Serum 11-KT and E₂ levels were measured using the enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's instructions.

Transcriptome analysis

The Ctrl-XX and Ctrl-XY gonad transcriptomes were sequenced previously by our group (Tao *et al.* 2013). One ovary from each of the MT+E₂-XX fish and the sex reversed MT+E₂-XY fish was used for transcriptome analyses. Total RNAs were extracted from ovaries using RNeasy Mini Kit (50) (Qiagen) according to the manufacturer's instructions. The extracted RNAs were further treated with deoxyribonuclease 1 (ribonuclease free) to eliminate genomic DNA contamination. The oligo (dT) beads enriched mRNA was disrupted into short fragments (200–700 nt) using fragmentation buffer. These short fragments were used as templates for first- and second-strand cDNA synthesis using a DNA synthesis kit (Invitrogen). A QiaQuick PCR purification kit (Qiagen) was used to purify these fragments, and the elution buffer was used for end repair and addition of the poly (A) tail. Then, these short fragments were ligated with sequencing

adapters. After agarose gel electrophoresis, fragments between 320 and 370 nt were cut from the gel for PCR amplification. Two cDNA libraries were constructed from the two samples and sequenced on an Illumina HiSeq 2000 instrument. Clean reads with a quality score \geq Q20 from each library were aligned to the reference genome (Orenil1.0, http://www.ensembl.org/Oreochromis_niloticus/Info/Index) using Tophat with default parameters, and the reads per kb per million reads (RPKM) method was used to calculate gene expression level (Mortazavi *et al.* 2008). The assembled transcripts were merged with the reference annotation (Oreochromis_niloticus.Orenil1.0.78.gtf, downloaded from Ensembl) using cuffmerge, and differential expression analysis was performed using cuffdiff.

Four transcriptomes of Ctrl-XX, Ctrl-XY, MT+E₂-XX and MT+E₂-XY were used to analyze the genes expression profiles. The threshold for the *P* value was determined using false discovery rate (FDR), and was widely set at 10^{-2} (Tao *et al.* 2013). In this study, genes with RPKM < 1 in the transcriptome were considered as background expression. They were excluded from analysis. Gonadal expressed genes were divided into non-differentially expressed genes between Ctrl-XX and Ctrl-XY (F/M-NDGs), Ctrl-XX up-regulated genes (F-UPGs) and Ctrl-XY (M-UPGs) up-regulated genes. (M-UPGs) ' $FDR > 10^{-2}$ ' and ' $-1 < \log_2(\text{Ctrl-XX_RPKM}/\text{Ctrl-XY_RPKM}) < 1$ ' were used to identify F/M-NDGs, ' $FDR \leq 10^{-2}$ ' and ' $|\log_2(\text{Ctrl-XX_RPKM}/\text{Ctrl-XY_RPKM})| \geq 1$ ' were used to identify F-UPGs and M-UPGs. These standards were also used for identification of non-differentially expressed, up- and down-regulated genes in MT+E₂-XX and MT+E₂-XY. Cluster analyses of gene expression patterns were performed with cluster and Java Treeview Software (<http://jtreeview.sourceforge.net/>; <http://rana.lbl.gov/EisenSoftware.htm>) (Eisen *et al.* 1998).

Real-time PCR analysis

Real-time PCR was carried out on an ABI-7500 real-time PCR machine according to the protocol of SYBR Premix Ex Taq II (Takara, Tokyo, Japan) using β -actin, *gapdh* and *eEF1A1a* as internal control. Gonads from Ctrl-XX, MT-XX, MT+E₂-XX, Ctrl-XY, E₂-XY and MT+E₂-XY fish were analyzed. Six samples were used for each group. Total RNAs were extracted from each sample and reverse transcribed using PrimeScript RT Master Mix Perfect Real Time Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. *igf3*, *cyp19a1b*, *wt1b*, *dnmt1*, *dnmt3* and *dnmt7* were selected to validate the transcriptome data by real-time PCR using gene-specific primers (Table S1, see

section on [supplementary data](#) given at the end of this article). The relative abundance of mRNA transcripts was evaluated using the formula: $R = 2^{-\Delta\Delta C_t}$, as described previously (Tao *et al.* 2013).

Results

MT and E₂ treatment on gonad phenotype

The XX and XY fry were treated by MT and E₂ alone or simultaneously from 0 to 30 dah. The gonad phenotypes were determined histologically at 90 dah. All Ctrl-XX and MT + E₂-XX fish developed as female with normal ovary. All MT-XX fish displayed female to male sex reversal with typical testis. These sex reversed fish were fertile as they produced normal sperms which could fertilize the eggs to produce viable offspring. In contrast, all Ctrl-XY fish developed as male with normal testis, while 63% E₂-XY fish and 52% MT + E₂-XY fish displayed male to female sex reversal with typical ovaries. These sex reversed fish were fertile as they produced normal eggs which could be fertilized by the sperms to produce viable offspring. Survival and sex reversal rates of the control and treatment groups were shown in [Table 1](#).

MT and E₂ treatment on gonadal *cyp19a1a*, *dmrt1* and *cyp11b2* expression

IHC analyses were performed for *cyp19a1a*, *dmrt1* and *cyp11b2* ([Fig. 1](#)). *cyp19a1a* was found to be expressed in the gonad of the Ctrl-XX and MT + E₂-XX at 5, 10 and 30 dah, in the gonad of the MT-XX at 5 and 10 dah and in the gonad of both MT + E₂-XY and E₂-XY at 10 and 30 dah. *dmrt1* was expressed in the gonad of MT-XX at 10 and 30 dah, in the gonad of both MT + E₂-XY and E₂-XY at 5 and 10 dah and in the gonad of Ctrl-XY at 5, 10 and 30 dah. *cyp11b2* was expressed in the gonad of the MT-XX, MT + E₂-XX, MT + E₂-XY and Ctrl-XY at 30 dah. At 90 dah, *Cyp19a1a* was expressed in the interstitial cells of the

Ctrl-XX and E₂-XY ovaries, while *cyp11b2* was expressed in the Leydig cells of the MT-XX and Ctrl-XY testes. However, *cyp19a1a* and *cyp11b2* were co-expressed in the interstitial cells of the sex reversed ovaries of MT + E₂-XX and MT + E₂-XY at 90 dah ([Fig. 1](#)). Like Ctrl-XY, only *Cyp11b2* was detected in the Leydig cells of the testis of the MT + E₂-XY and E₂-XY fish which experienced no sex reversal ([Fig. S1](#), see section on [supplementary data](#) given at the end of this article). Consistently, by western blotting strong band of *Cyp19a1a* was detected in the ovaries of the Ctrl-XX fish, and strong band of *Cyp11b2* was detected in the testes of the Ctrl-XY fish, while strong band of *Cyp19a1a* and weak band of *Cyp11b2* were detected in the ovaries of both MT + E₂-XX and MT + E₂-XY fish ([Fig. 2](#)).

MT and E₂ treatment on serum 11-KT and E₂ levels

At 90 dah, the serum E₂ and 11-KT levels of the MT-XX were similar to those of the Ctrl-XY. Serum E₂ level of E₂-XY was significant lower than while serum 11-KT level similar to that of the Ctrl-XX. Interestingly, serum E₂ level of both MT + E₂-XX and MT + E₂-XY showed no significant difference with that of the Ctrl-XX. Serum 11-KT level of both MT + E₂-XX and MT + E₂-XY was similar to that of the Ctrl-XY ([Fig. 3A and B](#)).

MT and E₂ treatment on gonad global gene expression

Sequencing of gonadal transcriptomes from MT + E₂-XX and MT + E₂-XY yielded a total of 123897206 and 118566266 reads respectively. The total gene counts of both MT + E₂-XX (19979) and MT + E₂-XY (19034) were larger than that of Ctrl-XX (18100), but the total gene counts of MT + E₂-XY was less than that of Ctrl-XY (19790) ([Fig. 4A](#)).

Cluster analysis of gene expression patterns of gonadal transcriptomes from MT + E₂-XX, MT + E₂-XY, Ctrl-XX and Ctrl-XY at 90 dah was performed. The resulting

Table 1 Survival and sex reversal rate after MT and E₂ treatment in tilapia

Genetic sex	Treatment	Fish number treatment	Fish number after treatment	Survival rate (%)	Sex reversal rate (%)
XX	Ctrl-XX	120	114	95	0
	MT-XX	120	90	75	100
	MT + E ₂ -XX	120	75	63	0
XY	Ctrl-XY	120	117	98	0
	E ₂ -XY	120	106	88	63
	MT + E ₂ -XY	120	64	54	52

The fish were treated with E₂ and MT along or in combination, 50 µg/l water bath from 0 to 5 dah and then fed with 50 µg/g diet to 30 dah. Survival rate and sex reversal rate were examined and calculated at 90 dah.

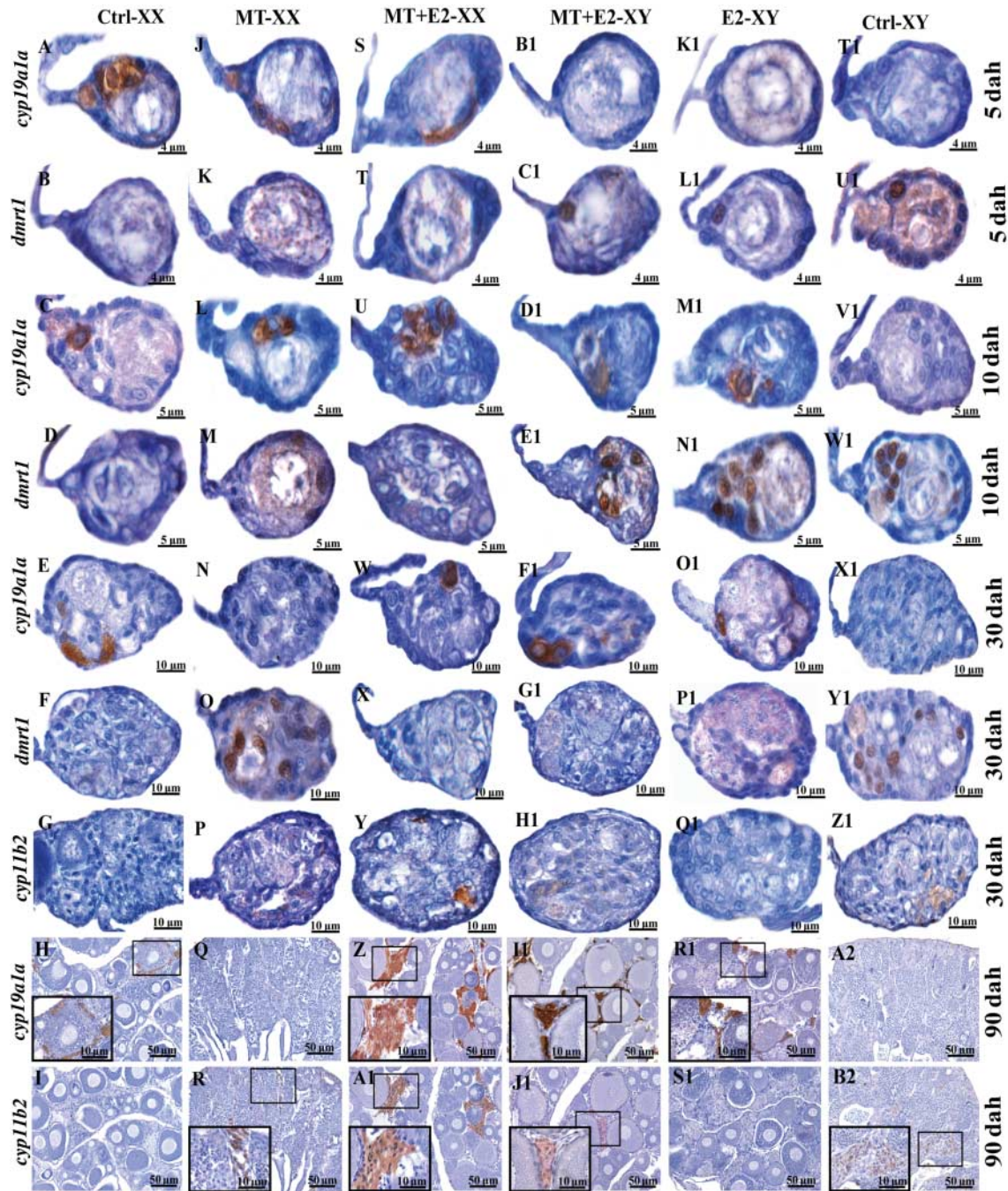
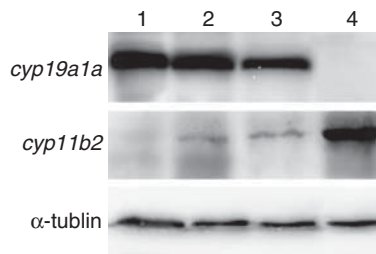


Figure 1

cyp19a1a, *dmrt1* and *cyp11b2* expression in gonads of Ctrl-XX (A-I), MT-XX (J-R), MT + E₂-XX (S-A1), MT + E₂-XY (B1-J1), E₂-XY (K1-S1) and Ctrl-XY (T1-B2) fish from 5 to 90 dah by immunohistochemistry. XY and XX fish were treated by 17 β -estradiol (E₂) and 17 α -methyltestosterone (MT) respectively for 30 days by exposed to 50 μ g/l of MT and E₂ alone and in combination from 0 to 5 dah, fed with commercial diet with 50 μ g/g feed of MT and E₂ alone or in combination until 30 dah, and then fed with control diet to 90 dah. The control fish were raised in steroid-free water and fed with normal commercial diet. The treatment was repeated three times. *cyp19a1a* was expressed in the gonad of the Ctrl-XX and MT + E₂-XX at 5, 10 and 30 dah (A, C, E and S, U, W), in the gonad of the MT-XX at 5 and 10 dah

(J and L) and in the gonad of both MT + E₂-XY and E₂-XY at 10 and 30 dah (D1, F1 and M1, Q1). *dmrt1* was expressed in the gonad of MT-XX at 10 and 30 dah (M and O), in the gonad of both MT + E₂-XY and E₂-XY at 5 and 10 dah (C1, E1 and L1, N1) and in the gonad of Ctrl-XY at 5, 10 and 30 dah (U1, W1 and Y1). *cyp11b2* was expressed in the gonad of Ctrl-XY (Z1) and in MT-XX, MT + E₂-XX and MT + E₂-XY at 30 dah (P, Y, H1). *cyp19a1a* was expressed in the Ctrl-XX ovary at 90 dah (H), while *cyp11b2* was expressed in the Leydig cells of the Ctrl-XY (B2). However, *cyp19a1a* and *cyp11b2* were co-expressed in the interstitial cells of the MT + E₂-XX and MT + E₂-XY (Z, A1 and I1, J1) ovaries. dah, day after hatching. The positive signal corresponds to the brownish color.

**Figure 2**

Expression of *cyp11b2* and *cyp19a1a* in tilapia gonads at 90 dah by western blotting. Lanes 1–4, proteins extracted from the gonads of Ctrl-XX, MT+E₂-XX, MT+E₂-XY and Ctrl-XY fish at 90 dah respectively. Cyp19a1a was detected in the gonads of Ctrl-XX, MT+E₂-XX and MT+E₂-XY fish, while Cyp11b2 was detected in the gonads of MT+E₂-XX, MT+E₂-XY and Ctrl-XY fish. α -tubulin was used to validate equal loading for western blot analysis and as reference protein for densitometry analysis.

dendrogram contained two main branches (I and II). Gonadal transcriptome from Ctrl-XY group clustered into branch II and those from the other three groups, which displayed extremely similar expression profile, clustered into branch I (Fig. 4B).

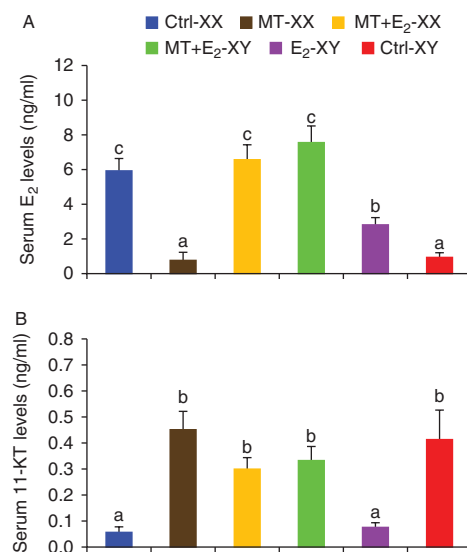
Of the gonadal expressed genes, 5170 genes were F-UPGs, account for 33%; 5662 genes were F/M-NDGs, account for 36%; 4864 genes were M-UPGs, account for 31%. Of the F-UPGs 14% in MT+E₂-XX, 13% in MT+E₂-XY and 8% in both were up-regulated; 56% in MT+E₂-XX, 77% in MT+E₂-XY and 49% in both displayed no change, which were consistent with the ovarian phenotype; however, 30% in MT+E₂-XX, 10% in MT+E₂-XY (of which 159 genes, 3%, represented with shadow, were up-regulated compared with Ctrl-XY) and 7% in both were down-regulated compared with Ctrl-XX, which were inconsistent with the ovarian phenotype (Fig. 4C). Of the F/M-NDGs 17% in MT+E₂-XX, 12% in MT+E₂-XY and 8% in both were up-regulated; 56% in MT+E₂-XX, 73% in MT+E₂-XY and 48% in both displayed no change; 27% in MT+E₂-XX, 15% in MT+E₂-XY and 9% in both were down-regulated (Fig. 4D). Of the M-UPGs 3% in MT+E₂-XX, 1% in MT+E₂-XY and 0.5% in both were up-regulated compared with Ctrl-XY; and 21% in MT+E₂-XX, 11% in MT+E₂-XY and 7% in both displayed no change; in contrast, consistent with the ovarian phenotype, 76% in MT+E₂-XX, 88% in MT+E₂-XY and 73% in both were down-regulated (Fig. 4E).

In general, the expression profiles of most genes, especially *foxl2*, *cyp19a1a*, *nanos3*, *dnd*, *dmrt1* and *gsdf*, were consistent with their ovarian phenotype in both MT+E₂-XX and MT+E₂-XY (Fig. S2, see section on supplementary data given at the end of this article).

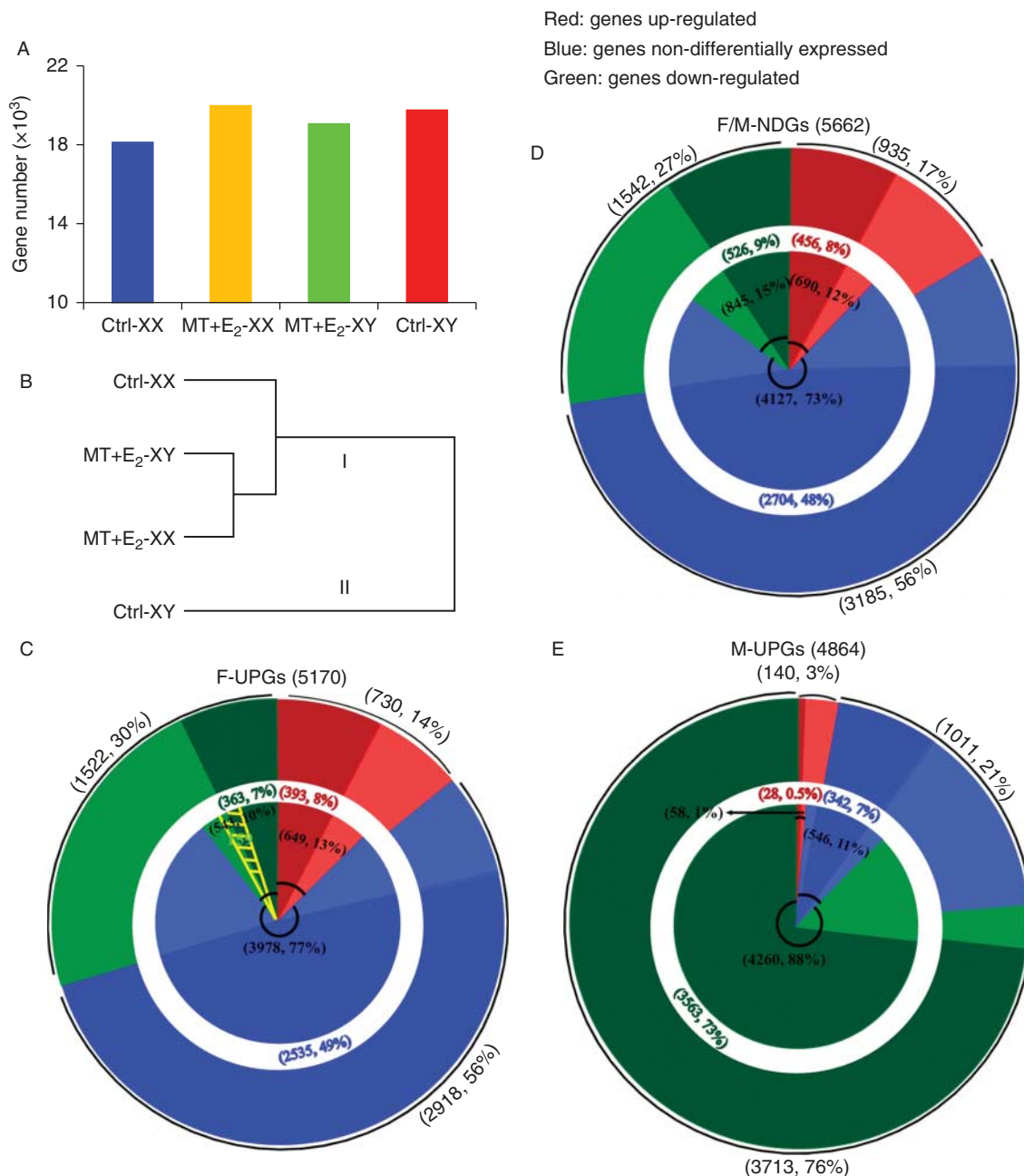
Surprisingly, there were still minority of genes including *igf3*, *cyp19a1b*, *wt1b* and some ribosome genes, displayed disrupted expression profile, were inconsistent with their ovarian phenotype (Fig. S3).

MT and E₂ treatment on expression profiles of DNMT genes in gonads

The expression level of maintenance methyltransferase gene *dnmt1* was significantly up-regulated in both MT+E₂-XX and MT+E₂-XY gonads compared with the Ctrl-XX, Ctrl-XY, MT-XX and E₂-XY gonads which displayed no significant difference among them. The expression levels of *de novo* DNMTs genes *dnmt3* and *dnmt7* were down-regulated in the MT+E₂-XX gonad while up-regulated in the MT+E₂-XY gonad compared with their respective control. However, the expression levels of *de novo* DNMTs genes *dnmt4*, *dnmt6* and *dnmt8* were down-regulated in the MT+E₂-XY gonad compared with the Ctrl-XY and these genes displayed no significant difference in expression level between the MT+E₂-XX and the Ctrl-XX gonad (Fig. 5). Consistently, similar expression profiles of selected DNMTs genes were observed by real-time PCR. Some genes, such as *igf3*, *cyp19a1b*, *wt1b*, *dnmt1*, *dnmt3* and *dnmt7* were selected for validation of

**Figure 3**

Serum E₂ (A) and 11-KT (B) level in MT-XX, E₂-XY, MT+E₂-XX, MT+E₂-XY, Ctrl-XX and Ctrl-XY fish at 90 dah by enzyme immunoassay. The fish were treated from 0 to 30 dah and then fed with control diet to 90 dah. Data were expressed as the mean \pm s.e.m. ($n=6$). Different letters above the error bar indicate significant difference ($P<0.05$) as determined by one-way ANOVA followed by *post-hoc* analysis. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0432>.

**Figure 4**

Transcriptomic analyses of gene expression profiles in Ctrl-XX, Ctrl-XY, MT+E₂-XX and MT+E₂-XY gonads at 90 dah. (A) Gene number expressed in gonadal transcriptomes. (B) Classification of gonadal transcriptomes using an unsupervised average linkage clustering of the gene expression profiles. (C, D, E) Gonad expressed genes were divided into three parts: (C) Ctrl-XX up-regulated genes (F-UPGs, 5170, 33%); (D) non-differentially expressed genes between Ctrl-XX and Ctrl-XY (F/M-NDGs, 5662, 36%); (E) Ctrl-XY up-regulated genes (M-UPGs, 4864, 31%). Of the F-UPGs 14% in MT+E₂-XX, 13% in MT+E₂-XY and 8% in both were up-regulated; 56% in MT+E₂-XX, 77% in MT+E₂-XY and 49% in both still displayed no change; 30% in MT+E₂-XX, 10% in MT+E₂-XY (of which 159 genes, 3%, represented with shadow, were up-regulated compared with Ctrl-XY) and

7% in both were down-regulated. Of the F/M-NDGs 17% in MT+E₂-XX, 12% in MT+E₂-XY and 8% in both were up-regulated; 56% in MT+E₂-XX, 73% in MT+E₂-XY and 48% in both still displayed no change; 27% in MT+E₂-XX, 15% in MT+E₂-XY and 9% in both were down-regulated. Of the M-UPGs 3% in MT+E₂-XX, 1% in MT+E₂-XY and 0.5% in both were up-regulated; 21% in MT+E₂-XX, 11% in MT+E₂-XY and 7% in both still displayed no change; 76% in MT+E₂-XX, 88% in MT+E₂-XY and 73% in both were down-regulated. Outside and inside pie represent gene expression profile of MT+E₂-XX and MT+E₂-XY respectively. Light and dark colors represent different and shared genes between MT+E₂-XX and MT+E₂-XY respectively.

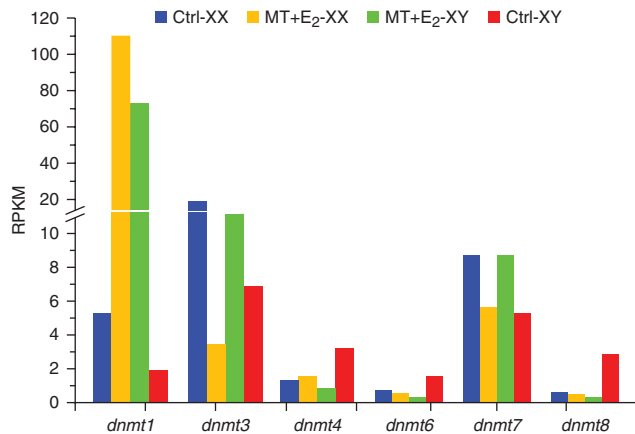


Figure 5

Expression profiles of methyltransferase genes in gonads. The expression levels of the maintenance methyltransferase gene *dnmt1* were up-regulated significantly, while the expression levels of the *de novo* DNA methyltransferases genes *dnmt3* and *dnmt7* were down-regulated in the MT + E₂-XX gonad but up-regulated in the MT + E₂-XY gonad significantly compared with their respective control. However, the expression levels of the *de novo* DNA methyltransferases genes *dnmt4*, *dnmt6* and *dnmt8* were down-regulated in MT + E₂-XY gonad compared with the Ctrl-XY and they displayed no significant change in the MT + E₂-XX gonad compared with the Ctrl-XX. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0432>.

transcriptome data with qPCR. Comparison of the transcriptome data with the qPCR results revealed similar expression profiles (Fig. 6).

Discussion

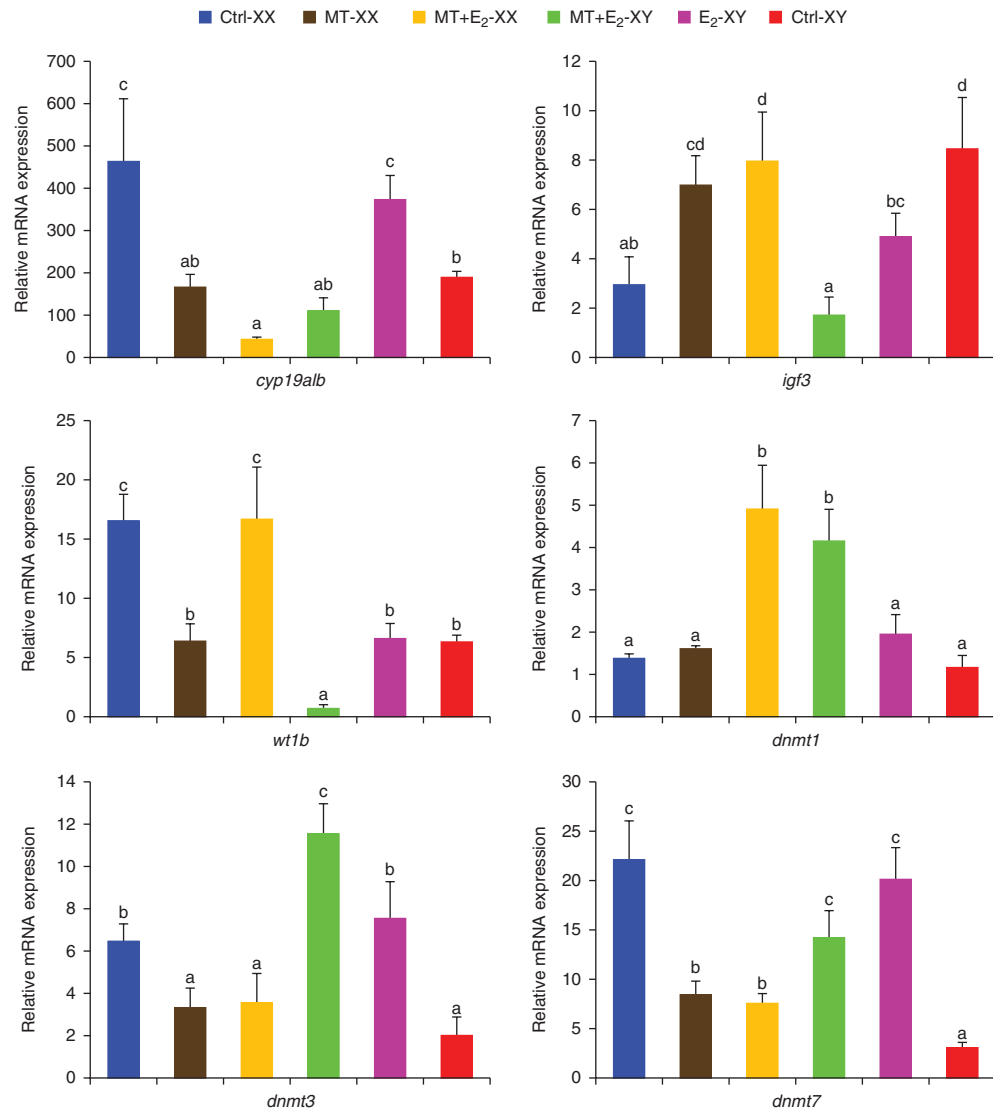
Sex was determined by estrogen even in the presence of androgen

Many studies have been focused on the role of sex hormones during gonadal sex differentiation. Genetic males can be reversed to phenotypic females with estrogens if the treatment is applied before sex differentiation in vertebrates including marsupials (Coveney *et al.* 2001), birds (Scheib 1983), reptiles (Merchant-Larios *et al.* 1997), and teleosts (Kobayashi *et al.* 2003, Baron *et al.* 2004, Gennotte *et al.* 2014). In species with natural sex reversal, increase of serum E₂ levels always associated with male to female sex reversal in protandrous fish (Guiguen *et al.* 1993). In contrast, E₂ levels have been found to decrease during female to male sex reversal in protogynous fish (Yeung & Chan 1987, Nakamura *et al.* 1989). Repression of aromatase expression and decrease of estrogen level were also observed in female to male sex reversal induced by high rearing temperatures (D'Cotta *et al.* 2001). Long-term treatment of aromatase inhibitor

Fadrozole even induced secondary sex reversal of the differentiated ovary into functional testis (Paul-Prasanth *et al.* 2013, Sun *et al.* 2014). However, it is unclear whether sex will still be determined by estrogen if the animals were exposed to an environment with both estrogens and androgens. In this study, MT treatment induced 100% sex reversal of all XX fish, and *Cyp19a1a* was detected in gonads at 5 and 10 dah while disappeared from 30 dah onwards. However, MT + E₂ treatment of XX fish resulted in no sex reversal at all, and the expression of *cyp19a1a* were detected in gonads at all time points checked, indicating that estrogen is responsible for the maintenance of female phenotype and androgen cannot induce sex reversal in the presence of estrogen. On the other hand, no significant differences in the percentage of sex reversal were observed between the E₂-XY (63%) and the MT + E₂-XY (52%) fish indicating that estrogen can still induce sex reversal even in the presence of androgen. The remaining 37 and 48% of the E₂-XY and MT + E₂-XY fish developed into male which might be caused by different sensitivity of these fish to steroid treatment. As we all know, the continuity of species is largely dependent on female population. Giving priority to female may be the result of natural selection. Taken together, our results highlight the crucial role of estrogen in sex differentiation and maintenance in non-mammalian vertebrates.

Estrogen is synthesized earlier in females than androgen in males during sex differentiation. Consequently, sex differentiation and meiosis initiate earlier in female than in male. Consistently, in the present study, the expression of *cyp19a1a* started earlier in the Ctrl-XX and MT + E₂-XX (5 dah) female or the sex reversed E₂-XY and MT + E₂-XY (10 dah) female than the expression of *cyp11b2* in the Ctrl-XY male or the sex reversed MT-XX (30 dah) male, and meiosis initiate earlier in the normal and sex reserved female (30 dah) than in the normal and sex reserved male (85 dah) (Kobayashi & Nagahama 2009). One possible explanation might be that oogenesis takes more time than spermatogenesis as the oocytes need to deposit bulk nutrients for the developing embryos which is time consuming.

The earlier occurrence of ovarian differentiation than testicular differentiation in vertebrates gives the impression that it should be easier to induce male to female sex reversal. However, this is opposite to the facts: i) it is easy to induce 100% female to male sex reversal but it is difficult to induce 100% male to female sex reversal and ii) secondary sex reversal of ovary into testis has been reported in medaka, zebrafish and tilapia (Poonlaphdecha *et al.* 2013, Takatsu *et al.* 2013, Sun *et al.* 2014), while there

**Figure 6**

Validation of genes with disrupted expression profile from transcriptome data by real-time PCR. All examined genes displayed similar expression profiles to those from the transcriptome data. Data were expressed as the mean \pm S.E.M. ($n=6$). Different letters above the error bar indicate

significant difference ($P<0.05$) as determined by one-way ANOVA followed by *post-hoc* analysis. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0432>.

has been no report showing the successful sex reversal of a testis into an ovary in teleosts. In fact, high doses of E₂ treatment are needed to induce complete male to female sex reversal in rainbow trout, tilapia and other teleosts (Krisfalusi & Nagler 2000, Bhandari *et al.* 2005, Gennotte *et al.* 2014). This might be explained by the following two possible reasons: i) though the ovarian differentiation occurs earlier than testicular differentiation, the male determining gene, such as *amhy* in tilapia, expresses even earlier than the female pathway genes (Eshel *et al.* 2014, Li *et al.* 2015a,b), and therefore high doses of E₂ are needed

to antagonize the male pathway genes, which is further supported by the differential sensitivity of XY and YY genotypes to embryonic induced-feminization (Gennotte *et al.* 2014); and ii) almost all steroidogenic enzyme genes are expressed in XX gonads but not in XY gonads at 5 dah (Tao *et al.* 2013), and therefore high doses of E₂ are needed to induce those genes, especially *cyp19a1a*, expression for ovarian differentiation.

Signals of Dmrt1 and Cyp19a1a could be simultaneously detected at 10 dah in the MT-XX gonad while Cyp19a1a disappeared later on. Granulosa cell marker

Cyp19a1a was down-regulated while Sertoli cell marker Dmrt1 was up-regulated following androgen treatment, indicating the de-differentiation of the granulosa cells and then trans-differentiation into Sertoli cells as suggested in rainbow trout (Baron *et al.* 2008). Similarly, signals of Dmrt1 and Cyp19a1a could be simultaneously detected at 10 dah in E₂-XY and MT+E₂-XY fish while Dmrt1 disappeared latter on. There would be a process for the de-differentiation of the Sertoli cells and then trans-differentiation into granulosa cells.

There have been many publications showing that ovarian pathway operates by repressing the testicular pathway, e.g. estrogen treatment resulted in down-regulation of P45011 β , 3 β HSD and P450c17, and thus inhibition of the synthesis of testicular androgen. This was suggested as an important step required for the feminization of genetic male fish (Govoroun *et al.* 2001). On the other hand, there have been reports showing that inhibition of estrogen synthesis by aromatase inhibitors or androgen resulted in female to male sex reversal (Baron *et al.* 2007, Poonlaphdecha *et al.* 2013, Sun *et al.* 2014). The two opposite pathways are continuously antagonizing during development as demonstrated by the simultaneous expression of *cyp19a1a* and *dmrt1* during sex reversal in the present study and by the simultaneous expression of *cyp19a1a* and *cyp11b2* during secondary sex reversal (Sun *et al.* 2014). The antagonism is also reflected by sex reversal after mutation of the key genes, such as *foxl2* and *dmrt1*, of the female and male pathway (Li *et al.* 2013, Boulanger *et al.* 2014, Lindeman *et al.* 2015).

The function of androgen in sex differentiation is controversial. It has been long believed that the sex of fish is determined by the dominate steroids. In general, estrogens feminize genetic males and androgens masculinize genetic females (Devlin & Nagahama 2002). This was confirmed by the wide use of steroids for sex reversal in fish. However, lines of evidences demonstrated that the undifferentiated male gonads could synthesize neither estrogens nor androgens (Guiguen *et al.* 1999, Nagahama 2000, Tao *et al.* 2013). Interestingly, in the present study, Cyp11b2 was detected from 30 dah in MT+E₂-XX and MT+E₂-XY gonads which were developed into ovaries, indicating that MT treatment resulted in endogenous androgen synthesis and both exogenous and endogenous androgens were not involved in sex differentiation in the presence of estrogen. The masculinization of female by androgen could be explained by two evidences. One is that androgen receptors are expressed in XX gonads at a time critical for sex determination in tilapia (Tao *et al.* 2013); the other is that androgens probably act as an inhibitor of

female pathway and E₂ synthesis (Bhandari *et al.* 2006, Vizziano *et al.* 2008). The male's main role is the production of sperm, which is critical for improving population viability via sexual selection in the face of genetic stress as demonstrated recently in the flour beetle (Lumley *et al.* 2015). It is well documented that androgen is essential for spermatogenesis (Billard *et al.* 1982, Ruwanpura *et al.* 2010).

Simultaneous treatment of MT and E₂ resulted in endocrine disruption and disorder of gene expression

Surprisingly, no differences in the serum 11-KT level were observed among MT+E₂-XX, MT+E₂-XY and Ctrl-XY fish though the former two were female with typical ovaries. Consistently, *cyp11b2* and *cyp19a1a* were co-expressed in the interstitial cells of ovaries in both groups, indicating that steroidogenesis of these fish were disrupted when androgen and estrogen exist simultaneously in the environment. One possible explanation might be that the environmental estrogens and androgens mimic the natural ones and disrupt the endocrine system of aquatic animals as suggested previously by other researchers (Guillette & Gunderson 2001). Endogenous hormones could activate nuclear receptors and then regulate the expression of a sizable proportion of genes would have profound effects on the organisms (Li *et al.* 2015a,b). Our results showed that most of the genes in both MT+E₂-XX and MT+E₂-XY gonads displaying expression profile consistent with ovarian phenotype. Such as high expression of *foxl2*, *cyp19a1a*, *nanos3*, *dnd* and *fig1a* which are key genes involved in ovarian differentiation and development, and low expression of *dmrt1*, *gsdf*, *sf1* and *amh* which are key genes involved in testicular differentiation and development (Baron *et al.* 2008, Ijiri *et al.* 2008, Li *et al.* 2013, Tao *et al.* 2013, Poonlaphdecha *et al.* 2013). Expression profiles of many genes such as *pin1*, *igf2r*, *rspanin1*, *ara* and *erb* displayed no significant difference between MT+E₂-XX, MT+E₂-XY, Ctrl-XX and Ctrl-XY, indicating their essential roles in gonadal development, but not in sex differentiation. There was consistent report showing that *pin1* is involved in the regulation of mammalian primordial germ cells proliferation in both male and female (Atchison *et al.* 2003). However, a small fraction of genes in both MT+E₂-XX and MT+E₂-XY displayed inconsistent expression profile with their ovarian phenotypes, indicating that these genes are not involved in sex differentiation. It is well documented that differences exist between the sexes in their response to environmental stress and females are more influenced

by environmental cues and stress (Senovilla *et al.* 2008, Bertholomey & Torregrossa 2015, Speed *et al.* 2015). Consistently, in the present study, there were more genes in MT+E₂-XX (27%) than in MT+E₂-XY (16%) displayed expression profile inconsistent with their ovarian phenotypes. The expression level of some genes, such as *vasa* and many ribosome genes, were significantly up-regulated while *dazap2* were significantly down-regulated compared with both Ctrl-XX and Ctrl-XY, indicating that they might be involved in stress response in both male and female. *vasa*, the molecular marker of the germ line lineage in many organisms, has been reported to be up-regulated by u.v. radiation stress in *Botryllus schlosseri* (Rosner *et al.* 2009). The up-regulation of ribosome genes has also been reported in rainbow trout by androgen treatment (Baron *et al.* 2007) and in short-lived fish by diapause (Reichwald *et al.* 2015). *dazap2* has been reported to induce stress granules formation in mouse (Kim *et al.* 2008). The expression of some genes, such as some ribosome genes, were significantly changed only in MT+E₂-XX, and some others, such as *sparc*, *igf3* and *wt1b*, only in MT+E₂-XY, which may be involved in genetic female and male stress response respectively. Some of the highly expressed ribosome genes are also reported to be involved in abiotic stress responses (Wang *et al.* 2013). *sparc* has been reported to play a key role in post-synthetic procollagen processing in normal and pressure-overloaded myocardium of mice (Bradshaw *et al.* 2009). The expression of *wt1b*, an epicardial gene in zebrafish, is responsive to heart injury (Itou *et al.* 2012). Estrogen treatments resulted in significant down-regulation of *igf3* mRNA in the testis while ovarian *igf3* mRNA did not respond (Berishvili *et al.* 2010), in contrast, androgens increased *igf3* transcript levels in the ovary (Melo *et al.* 2015). In our study, *igf3* was down-regulated significantly in E₂-XY and MT+E₂-XY compared with Ctrl-XY, while up-regulated significantly in MT-XX and MT+E₂-XX compared with Ctrl-XX. These results demonstrated that simultaneous treatment with estrogen and androgen resulted in severe endocrine disruption.

It has long been proposed that epigenetic regulation may play an important role in ESD species in response to environmental influences, leading to the gonad developmental change (Manolakou *et al.* 2006). Plenty of experiment results suggested that epigenetic regulation plays multiple crucial roles in the gonadal differentiation of teleosts and other vertebrates (Navarro-Martin *et al.* 2011, Zhang *et al.* 2013, Parrott *et al.* 2014, Shao *et al.* 2014). Neonatal exposure to the synthetic estrogen diethylstilbestrol caused abnormal demethylation of the

CpG sites upstream of the estrogen-response element of the lactoferrin promoter (Li *et al.* 1997). In the present study, the *de novo* methyltransferase *dnmt6*, *dnmt8* and *dnmt4*, which are counterparts of mammalian *dnmt3a* and *dnmt3b*, displayed the same expression pattern in the Ctrl-XX, MT+E₂-XX and MT+E₂-XY ovary, while different from that of the Ctrl-XY testis. One possible explanation is estrogen determines sex by influencing the expression of methyltransferase, which in turn, result in the change of gene expression pattern that in favor of the female pathway. Surprisingly, the expression level of maintenance methyltransferase gene *dnmt1* was significantly up-regulated in the gonads of MT+E₂-XX and MT+E₂-XY compared with Ctrl-XX and Ctrl-XY, implying that animals might be able to adapt to the endocrine disruptor polluted environment by change of the gene expression pattern via the up- or down-regulation of methyltransferase gene expression.

In summary, estrogen could feminize XY fish even in the presence of androgen, while androgen could not masculinize XX fish in the presence of estrogen. These results strongly emphasize the critical role of estrogen in sex differentiation and maintenance in teleosts. Simultaneous treatment with estrogen and androgen up-regulated the endogenous estrogen and androgen syntheses, and resulted in disordered gene expression and endocrine disruption. Our study provided a very useful model for the understanding of the molecular mechanism of animals adapt to the estrogen and androgen contaminated environments.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0432>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conceived and designed the experiments: L C and X J; collected sample: L C, X J, H F, Q X and H S; performed the experiments: L C, X J and H F; performed the analyses: L C, W T and L S; wrote the paper: D W, L C and L S.

Data accessibility

The RNA-seq reads reported in this study have been deposited in the National Center for Biotechnology Information Short Reads Archive, www.ncbi.nlm.nih.gov/sra (study accession ID: SRP063372).

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