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Regulation of reproduction- and biomarker-related gene expression by sex steroids in the livers and ovaries of adult female western mosquitofish (*Gambusia affinis*)

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ABSTRACT

To assess the adverse toxicological effects of steroid hormones on western mosquitofish (*Gambusia affinis*), 180 adult females were exposed to individual or binary combinations of progesterone (1 μg/L), testosterone (1 μg/L) and 17β-estradiol (1 μg/L) for eight days. The expression patterns of vitellogenin, estrogen receptor, androgen receptor, metallothionein, and cytochrome P450 1A genes in mosquitofish varied according to tissue as well as the specificity of steroids. Treatment by progesterone or testosterone alone inhibited target gene expression in the livers. The expression levels of both vitellogenin A and vitellogenin B mRNAs were up-regulated by17β-estradiol, and a parallel induction of estrogen receptor α mRNA expression was also observed in the livers. In addition, 17β-estradiol treatment alone suppressed androgen receptor α, metallothionein and cytochrome P450 1A mRNA expression in the livers. In general, multiple hormone treatments had different effects on target gene expression compared with corresponding hormone alone. The results demonstrate that steroid hormones cause multiple biological responses including the expression of vitellogenin, estrogen receptor and androgen receptor mRNA in the hormone signaling pathways and the expression of metallothionein and cytochrome P450 1A mRNA in the xenobiotic signaling pathway.

1. Introduction

During the past decade, concerns have been raised about endocrine disrupting chemicals (EDCs) in the environment since these compounds can interfere with normal hormone signaling pathways through disturbance of necessary gene expression for normal development and reproduction, or activation of normally quiescent genes (Miracle et al., 2006; Kim et al., 2008). As the aquatic environment represents the ultimate sink for most anthropogenic contaminants, fish are directly exposed to a wide variety of EDCs that include natural and synthetic hormones as well as a variety of industrial and agricultural compounds (Sun et al., 2011). Steroidal hormones originating from the excrements of human and animals enter the aquatic environment in their active forms (Lange et al., 2002). The primary steroid hormones are β-estradiol (E2), progesterone (P), testosterone (T), and cortisol. Although those hormones are naturally produced, they can lead to disorders of the endocrine system in aquatic organisms if they exceed a certain concentration in the aquatic environment.

The most commonly observed impacts of steroids on fish are the induction of the female specific vitellogenins (Vtgs) mainly by estrogens (Lange et al., 2002; Leusch et al., 2005; Kim et al., 2010). Feminization of male fish has been reported in rivers of many regions (Matthiessen et al., 2002; Kolok et al., 2007; Xie et al., 2010). Vtgs are egg yolk precursor proteins in the serum of oviparous teleosts. They are synthesized in the liver of females under the control of E2, secreted into the bloodstream and migrated to the ovaries where they are processed into egg yolk proteins (Miracle et al., 2006). Fish Vtgs have been divided into three types based on primary structure. The two Vtg types that possess two lipovitellin domains and one phosvitin domain are classified as VtgA and VtgB, and the third type is classified as VtgC, which contains no polyserine phosvitin domain (Henry et al., 2009; Kim et al., 2010). Generally, Vtgs are undetectable in male and immature female fish unless exposure to exogenous estrogenic compounds has occurred. Due to this, Vtgs have been extensively used as a biomarker of reproductive disruption (Henry et al., 2009). However, other classes of steroid hormones are able to regulate Vtg synthesis in uncertain modes. In primary hepatocyte cultures from male immature rainbow trout, Vtg mRNA expression was induced by progesterone and by both low and high concentrations of androgens including T (Mori et al., 1998). Recent studies have shown that Vtg gene expression is decreased in the liver of female fathead minnows following exposure to a synthetic androgen 17β-trenbolone used as the beef cattle growth promoter (Miracle et al., 2006).

One route of action of steroid hormones is mediated through nuclear hormone receptors, which are activated by steroid ligands and regulate gene expression by interacting with specific DNA sequences upstream of their target genes (Kim et al., 2010; Smolinsky et al., 2010). As a consequence, nuclear receptors play key roles in reproduction and sexual differentiation (Seo et al., 2006). Both estrogen receptors (ERs) and androgen receptors (ARs) are important members

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of nuclear hormone receptor family. ERs were localized in both sexes in numerous tissues and are able to modify transcriptional rates of estrogen response genes through binding as dimers to estrogen response elements (EREs) in the promoter regions of those target genes (Janošek et al., 2006; Pinto et al., 2006; Kim et al., 2010). Until now, two distinct types of ERs (called ERα and ERβ) have been isolated in several fish species including largemouth bass (Sabo-Attwood et al., 2004), mosquitofish (Katsu et al., 2007), tilapia (Esterhuyse et al., 2010), and in cinnamon clownfish (Kim et al., 2010). Role of the androgen receptor (AR) in male organism is very similar to that of estrogen receptor in females (Janošek et al., 2006; Smolinsky et al., 2010). According to structure, two distinct ARs, termed ARα and ARβ, have been identified and characterized in a number of teleosts such as stickleback (Olsson et al., 2005), mosquitofish (Sone et al., 2005), and Japanese eel (Tosaka et al., 2010).

Metallothioneins (MTs) are a family of low-molecular-mass and cysteine-rich proteins that bind many different metals through their sulfydryl groups (Huang and Wang, 2010). They are involved in heavy metal detoxification and homeostasis in a wide variety of organisms including animals, plants, eukaryotic microorganisms and prokaryotes (Rhee et al., 2009; Huang and Wang, 2010). Because induction of MT levels has frequently been associated with metal exposure, analysis of MT levels has been used as a biomarker of metal pollution in the aquatic environment. However, MT gene expression was affected by metals (Rhee et al., 2009), chemicals stressors (Gerpe et al., 2000; Werner et al., 2003) and other factors including age, sex and tissue type (Rhee et al., 2009). Although MT genes have been cloned and their expression has been studied in a number of fish species in response to heavy metals, relatively little is known about how those genes are affected by natural or synthetic hormones (Gerpe et al., 2000; Werner et al., 2008; Rhee et al., 2009).

Cytochrome P4501A (CYP1A) is a member of the cytochrome P450 superfamily and has attracted particular attention because of its inducible biochemical response in fish exposed to numerous exogenous organic compounds including polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins (Navas and Segner, 2001). Induction of CYP1 gene expression is triggered by xenobiotic compounds (such as PAHs, PCBs and dioxins) binding to an arylhydrocarbon receptor (AhR), which then binds to xenobiotic responsive elements (XREs) in the promoter of the CYP1A gene to induce transcription (Jinno et al., 2006). CYP1A has been studied extensively due to its importance in the metabolism and toxicity of certain xenobiotics. Indicators of induction of CYP1A in fish have been used routinely to assess dioxin-like xenobiotic compounds in the aquatic environment. In addition, CYP1A mRNA or protein was also induced by hormones (Jinno et al., 2006). For example, equilenin (an estrogen) was able to dose-dependently increase both ethoxyresorufin *O*-deethylase activity (EROD) in mice, and it also induced CYP1A mRNA in treated HepG2 cell lines (Jinno et al., 2006). However, on the contrary, some previous studies showed that E2 could inhibit CYP1A mRNA expression in fish (Navas and Segner, 2001; Cionna et al., 2006; Kim et al., 2008). Thus, it is interesting to study CYP1A mRNA expression in fish in response to steroid hormones.

Mosquitofish (*Gambusia affinis*) have attractive features including a relatively small size, short generation time, a prominent masculine sexual character for internal fertilization (intromission) (Sone et al., 2005), and survival in highly contaminated environments (Xie et al., 2010). With those advantages, mosquitofish have become an interesting biological model for assessing the reproduction and development in laboratory or field studies (Sone et al., 2005; Xie et al., 2010). Chemical regulation of gene expression is a potential application to risk assessment and identification of EDCs (Kolok et al., 2007; Sellin et al., 2010). Although some genes implicated in reproduction (Vtg isoforms, ER isoforms and AR isoforms) as well as some genes related to detoxification (MT and CYP1A) in mosquitofish have been cloned and studied (Leusch et al., 2005; Sone et al., 2005; Katsu et al., 2007), the mechanisms of those genes' expression in response to EDCs have not been well elucidated.

The aim of the present study was, therefore, to use real-time quantitative RT-PCR to characterize Vtg isoform, ER isoform, AR isoform, MT and CYP1A mRNA expression patterns in the livers and ovaries in mosquitofish exposed to three types of steroid hormones. The compounds chosen in the experiments were progesterone (P), testosterone (T) and 17β-estradiol (E2). This study will help understand better the molecular pathways that control physiological processes in western mosquitofish. Moreover, the results generated from this study are also of critical importance in clarifying how organisms respond to stressors.

2. Materials and methods

2.1. Experimental fish

Mosquitofish (*G. affinis*) were captured from the Liuxi River, which is located at nearby Conghua town in Guangzhou city (southern China) and protected from pollution (Xie et al., 2010). They were transported back to the laboratory and then kept in tanks of circulating freshwater at 24 ± 1 °C. The fish were fed daily with commercially available flake red worms (Binhai, Tianjin, China). The light regime followed a 12 h:12 h light:dark cycle. Fish were acclimated in the laboratory for three weeks prior to experimentation.

2.2. Experimental chemicals

Three natural steroids progesterone (P), testosterone (T) and 17β-estradiol (E2) were obtained from Sigma–Aldrich (USA) with a purity of 99%, 99% and 98%, respectively. Stock solutions of 1 mg/mL each were prepared in ethanol for the three compounds.

2.3. Exposure of fish to steroid hormones

180 healthy adult female mosquitofish were chosen at random and transferred to 12-liter glass aquaria containing 5 L of water. After three weeks acclimatization period, 10 adult female mosquitofish were exposed continuously for eight days to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) in individual tanks. A stock solution of each chemical (1 mg/mL) was diluted appropriately to provide the nominal doses. Only ethanol was added to the control group. The final ethanol concentration in each aquarium was 0.002% (v/v). The exposure concentrations of hormones and exposure durations were chosen based on the previous studies on mosquitofish (Leusch et al., 2005). Populations of 10 individuals lived in 5 L of medium. Three replicate tanks were included for each treatment. The experiment was performed under the static condition and eighty percent of the experimental water was renewed daily during the experimental period. Mosquitofish were maintained at approximately 24 ± 1 °C with a natural dark/light cycle of 12/12 h. Fish were fed once daily during the exposure. Residual bait and feces in the glass aquaria were removed daily. At the conclusion of the exposure, mosquitofish were anesthetized using 0.01% tricaine methanesulfonate (TMS). The livers and ovaries were excised from four fish in each treatment and preserved in RNAlater (Sigma) at −20 °C until RNA was extracted.

2.4. Chemical analysis

Water samples were collected from each tank into flasks and the actual concentrations of P, T or E2 were determined in three parallel samples from each treatment using RRLC–MS/MS with electrospray ionization (ESI) according to the protocols by Liu et al. (2011). For measurement of P or T, the water samples were filtered through a

Table 1
The nominal and actual concentrations (μg/L) of test chemicals in each treatment.

Treatments	P nominal concentration	T nominal concentration	E2 nominal concentration	P actual concentration	T actual concentration	E2 actual concentration
Control	0	0	0	0	0	0
P	1	0	0	1.11 ± 0.13	–	–
T	0	1	0	–	1.01 ± 0.02	–
E2	0	0	1	–	–	1.03 ± 0.08
P + E2	1	0	1	0.91 ± 0.03	–	0.93 ± 0.09
T + E2	0	1	1	–	0.94 ± 0.06	1.01 ± 0.09

– No detection.

0.45 μm membrane filter into a 2 mL amber glass and directly analyzed by RRLC–MS/MS. For measurement of E2, 100 mL of water samples were extracted using solid phase extraction (SPE) as previously described in Liu et al. (2011) and then analyzed by RRLC–MS/MS. Mean measured concentrations of the test chemicals in each treatment are given in Table 1. Data are presented as means ± standard error (S.E.) from three parallel samples in each treatment.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from tissues of four individual fish from each of the three replicate tanks per treatment using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Following resuspension of total RNA in water treated with diethyl pyrocarbonate (DEPC), each total RNA sample was then treated with RQ1 RNase-free DNase (Promega) at 37 °C for 30 min to eliminate contamination of genomic DNA. Total RNA was quantified by measuring the spectrophotometric absorbance at 260 nm and the purity was assessed by measuring the optical density at 260/280 nm. 1 μg of DNase-treated total RNA was then reverse transcribed into cDNA in a volume of 20 μL using SuperScript® First-Strand Synthesis System (Invitrogen) as described by the manufacturer. Oligo(dT) primers were used for reverse transcription. The reaction was incubated at 42 °C for 60 min. The first strand cDNA was diluted to a final volume of 100 μL by adding 80 μL nuclease-free water and stored at −20 °C for subsequent real-time PCR.

2.6. Quantification of gene expression

All real-time quantitative PCR analyses were carried out using ABI 7500 (ABI) with THUNDERBIRD SYBR® qPCR Mix (Toyobo) following procedures recommended by the manufacturer. The amplification was set at 20 μL, each reaction containing 10 μL of Real-time PCR Master Mix, 0.6 μL of each the forward and reverse primer (10 μM), 4 μL of cDNA and 4.8 μL of nuclease free water. Nuclease-free water for the replacement of cDNA template was used as negative control. The specific primers of each gene for real-time quantitative PCR are shown in Table 2. Primer Premier 5.0 (Premier) was used for primer design. The cycling profile used consisted of 95 °C for 1 min and 45 cycles at 95 °C for 15 s and 60 °C for 40 s. To determine if non-specific products were being amplified, the fluorescence signal was captured at the end of each cycle and a melting curve analysis was performed from 60 to 95 °C. The dissociation curves of all ten genes showed a single peak at the expected melting temperature. β-actin was used as a housekeeping reference to normalize gene expression levels in each sample. Each gene relative expression for each sample was calculated according to the equation ($2^{-\Delta\Delta Ct}$) described by Huang and Wang (2010).

2.7. Statistical analysis

Data are presented as mean ± SE (n = 3) from three separate experiments in each treatment. Data analysis was carried out by one-way analysis of variance using SPSS 13.0. Duncan's multiple range tests were performed to determine the significant differences between experimental groups. Statistical significance was set at $P<0.05$.

3. Results

3.1. Modulation of Vtg gene expression in female mosquitofish exposed to steroid hormones

The mRNA expression levels of the three Vtg isoforms varied in response to steroid hormone exposure (Fig. 1). The mRNA expression

Table 2
Nucleotide sequences of primer pairs used for SYBR Green real-time RT-PCR.

Gene	Primer sequence (5′→3′)	Annealing temperature (°C)	Amplicon length (bp)	GenBank accession no.
β-Actin	Forward: GATCTGGCATCACACCTTCTACAA	60.9	149	AB182330
	Reverse: CGTACATGGCAGGAGTGTTGAA	61.0		
VtgA	Forward: CCTCCAACCCAGTCAAATCATC	60.8	206	AB181835
	Reverse: GATGTAGGCACCGTTTTCACTG	59.8		
VtgB	Forward: TCCACCAGCATTCCATCTCAG	60.6	135	AB181836
	Reverse: TAATGGCACGGACAAGGACTG	60.9		
VtgC	Forward: TGAGCGACAACACTTCAGTGC	59.4	214	AB181837
	Reverse: AGCCTTTGGTCCTGGGTTATC	59.8		
ERα	Forward: CTTGCCGACTCAGGAAGTGTTAC	60.8	102	AB295655
	Reverse: TGACGCCAGTCTGTCGTTTGT	61.2		
ERβ	Forward: TTACTGACAGCCCATCATCCAT	59.4	133	AB295657
	Reverse: GGTGGGTTTGGTTCATTGTAGAC	60.3		
ARα	Forward: GCTTCAGGCACGAGGATTTC	59.7	104	AB182328
	Reverse: GGTGACCGCTCCGTAATGAC	60.1		
ARβ	Forward: CGATGCCCAGACCCAGATTAC	61.7	126	AB182329
	Reverse: GAGGCGAGGTGATGAAAATGC	62.0		
MT	Forward: CTGCTCTTGCTGCCCATCA	60.2	77	AB455145
	Reverse: AGCCCTTGTCGCAGGTCTTC	61.6		
CYP1A	Forward: GTCTGTCGTGGGCAGTGATGTA	60.7	171	AB371607
	Reverse: GGTATGAGGAATGACGGAAGAGC	61.9		

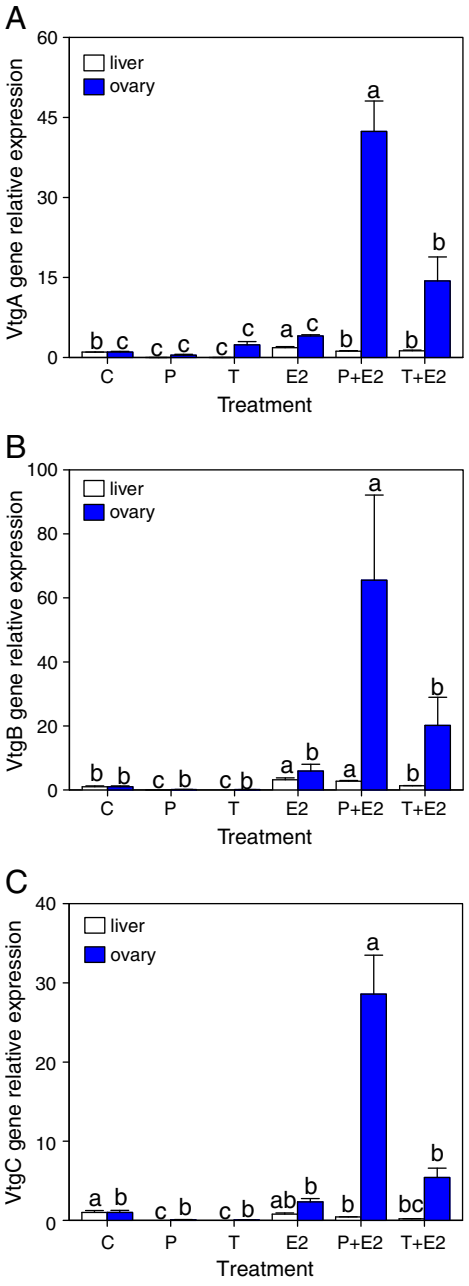

Fig. 1. Expression patterns of VtgA (A), VtgB (B) and VtgC (C) mRNAs in the livers and ovaries of adult female western mosquitofish (*G. affinis*) after exposure to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) for eight days (P: progesterone, T: testosterone, E2: 17β-estradiol). The relative mRNA expression levels of Vtg isoform gene as expressed by $2^{-\Delta\Delta Ct}$ were determined for each group and vertical bars represented the mean ± SE (n = 3). Different letters indicate significant difference among treatment groups at a particular tissue ($P<0.05$).

levels of each Vtg isoform were significantly repressed in the livers in P-treated and T-treated groups in comparison with the controls. E2 treatment induced Vtg isoform mRNA expression in the livers in comparison with the controls except VtgC. When E2 was combined with P or T, VtgA mRNA expression in the livers had no significant change, whereas VtgC mRNA expression in the livers was significantly decreased in comparison with the control. Moreover, in the livers, when E2 was combined with P, VtgB mRNA expression was significantly induced. In the ovaries, the transcription levels of each Vtg isoform gene were significantly induced in P + E2 treatment in comparison with the controls, and VtgA mRNA expression also had significant induction in T + E2 treatment in comparison the control.

3.2. Modulation of ER gene expression in female mosquitofish exposed to steroid hormones

Fig. 2 shows the effect of hormone treatments on the mRNA expression levels of ERα (A) and ERβ (B) in the livers and ovaries in mosquitofish. In the livers, treatment with P or T alone significantly reduced the mRNA leve1s of ERα and ERβ in comparison with their respective controls. In the livers, although E2 treatment significantly increased the expression of ERα mRNA, it significantly decreased the expression of ERβ mRNA in comparison with the control. A significant decrease in ERβ mRNA expression in the livers was noticed in the P + E2-treated group compared to the control. In the T + E2-treated group, both ERα and ERβ mRNA levels in the livers showed a significant decrease in comparison with the control group.

3.3. Modulation of AR gene expression in female mosquitofish exposed to steroid hormones

Fig. 3 shows AR isoform mRNA expression levels assessed using real-time quantitative PCR. P treatment significantly inhibited the expression of ARα and ARβ mRNAs in the livers, but it significantly induced the expression of ARα and ARβ mRNAs in the ovaries in comparison with the controls. T treatment of mosquitofish significantly suppressed the levels of ARα mRNA in the livers. On the contrary, T treatment significantly induced the levels of ARβ mRNA in the ovaries. E2 treatment of mosquitofish had significant inhibition

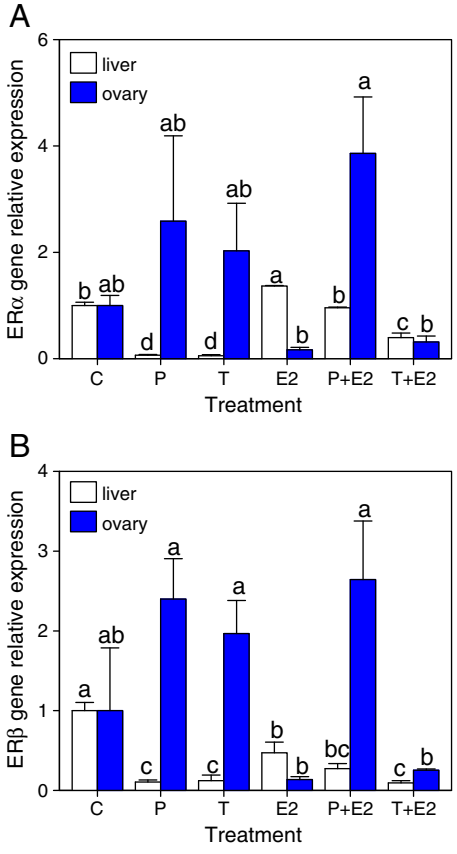

Fig. 2. Expression patterns of ERα (A) and ERβ (B) mRNAs in the livers and ovaries of adult female western mosquitofish (*G. affinis*) after exposure to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) for eight days (P: progesterone, T: testosterone, E2: 17β-estradiol). The relative mRNA expression levels of ER isoform gene as expressed by $2^{-\Delta\Delta Ct}$ were determined for each group and vertical bars represented the mean ± SE (n = 3). Different letters indicate significant difference among treatment groups at a particular tissue ($P<0.05$).

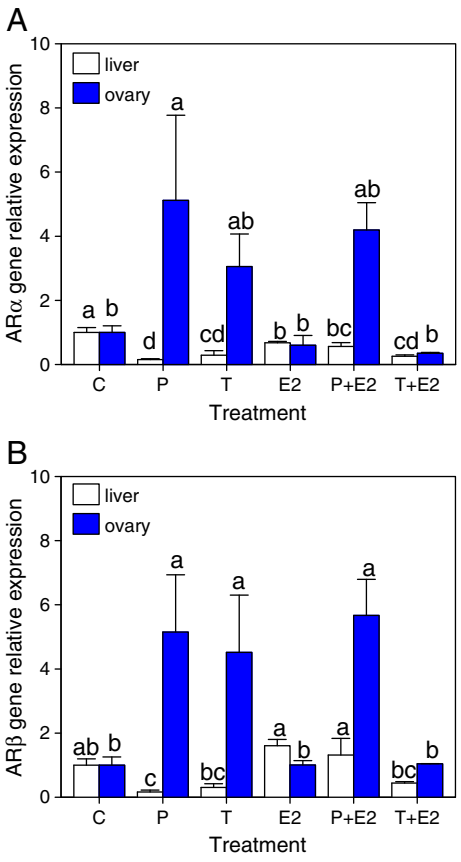

Fig. 3. Expression patterns of ARα (A) and ARβ (B) mRNAs in the livers and ovaries of adult female western mosquitofish (*G. affinis*) after exposure to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) for eight days (P: progesterone, T: testosterone, E2: 17β-estradiol). The relative mRNA expression levels of AR isoform gene as expressed by $2^{-\Delta\Delta Ct}$ were determined for each group and vertical bars represented the mean ± SE (n = 3). Different letters indicate significant difference among treatment groups at a particular tissue (P<0.05).

on ARα mRNA expression in the livers in comparison with the control. The expression levels of ARα and ARβ mRNAs in the livers in the P + E2-treated group was significantly induced compared with the P-treated group. A significant decrease in ARα and ARβ mRNA expression in the livers was noticed in the T + E2-treated group compared with the E2-treated group.

3.4. Modulation of MT gene expression in female mosquitofish exposed to steroid hormones

As depicted in Fig. 4, hormone treatments significantly reduced the expression of MT mRNA in the livers as compared to the control. In the ovaries, E2 treatment alone significantly reduced MT mRNA expression as compared to the control. Combination treatment with T and E2 significantly inhibited the level of expression of MT mRNA in the ovaries in comparison to the control.

3.5. Modulation of CYP1A gene expression in female mosquitofish exposed to steroid hormones

The expression pattern of CYP1A mRNA in mosquitofish is showed in Fig. 5. In the livers, steroid-treated groups showed significantly lower levels of CYP1A transcripts compared to the control group. In the ovaries, the difference in the expression of CYP1A mRNA is statistically significant in the T group in comparison to the P, E2, P + E2 and T + E2 groups.

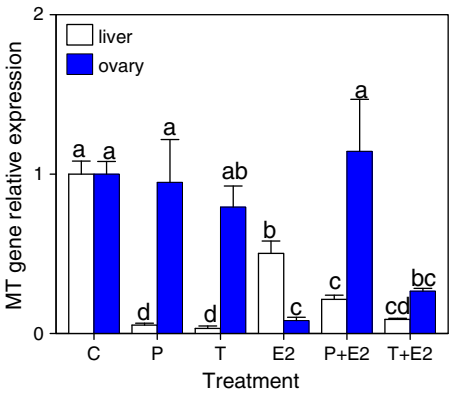

Fig. 4. Expression patterns of MT mRNA in the livers and ovaries of adult female western mosquitofish (*G. affinis*) after exposure to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) for eight days (P: progesterone, T: testosterone, E2: 17β-estradiol). The relative mRNA expression levels of MT gene as expressed by $2^{-\Delta\Delta Ct}$ were determined for each group and vertical bars represented the mean ± SE (n = 3). Different letters indicate significant difference among treatment groups at a particular tissue (P<0.05).

4. Discussion

4.1. Modulation of Vtg gene expression

The assessment of different Vtg isoform mRNA expression patterns in response to steroid hormones provides a rare opportunity to study the reproductive physiology of fish and environmental endocrine disruptors (Henry et al., 2009). Although the mRNA expression levels of VtgA, VtgB and VtgC in two tested tissues have some differences in response to steroid hormones in this study, it should be noted that their expression patterns in general are very similar. The present results showed a significant increase of both VtgA and VtgB mRNA expression by E2 treatment in the livers of mosquitofish compared with the controls. This is in agreement with previous studies which have demonstrated the induction of Vtg mRNA expression in response to E2 in several fish species (Tong et al., 2004; Leusch et al., 2005). Vtg genes are a very sensitive target for E2. Mori et al. (1998) thought that the effects of E2 on Vtg mRNA expression levels in the livers of fish could be interpreted by at least two mechanisms: 1) a stimulation of Vtg gene transcription through activation of estrogen receptors by E2; 2) a stabilization of Vtg mRNAs in the presence

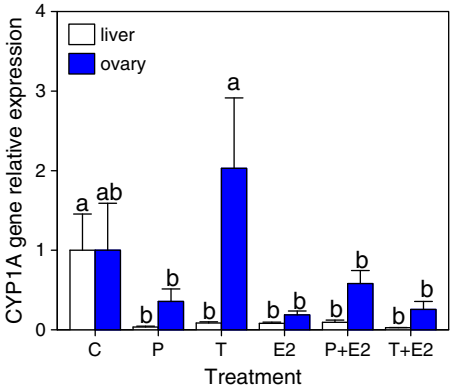

Fig. 5. Expression patterns of CYP1A mRNA in the livers and ovaries of adult female western mosquitofish (*G. affinis*) after exposure to P (1 μg/L), T (1 μg/L), E2 (1 μg/L), P (1 μg/L) + E2 (1 μg/L) or T (1 μg/L) + E2 (1 μg/L) for eight days (P: progesterone, T: testosterone, E2: 17β-estradiol). The relative mRNA expression levels of CYP1A gene as expressed by $2^{-\Delta\Delta Ct}$ were determined for each group and vertical bars represented the mean ± SE (n = 3). Different letters indicate significant difference among treatment groups at a particular tissue (P<0.05).

of E2. The present study also confirmed that E2 is able to significantly increase the expression of ERα mRNA in the livers of mosquitofish. However, VtgC mRNA expression in the livers of mosquitofish had slight decrease in the E2-treated group compared to the control. The differential patterns of Vtg isoforms in the livers modulated by E2 are interesting, which may be due to their different structures. Although VtgA and VtgB possess two lipovitellin domains and one phosvitin domain, VtgC contains no polyserine phosvitin domain (Henry et al., 2009). Both P and T exhibited important inhibition in the expression levels of three Vtg isoform mRNA in mosquitofish. In addition, treatment of E2 in combination with either P or T showed that the presence of P or T reduces the mRNA levels of Vtg isoforms in the livers of mosquitofish induced by E2. These results demonstrated that both P and T have a physiological role in inhibiting the Vtg gene expression in the livers of mosquitofish. To date, there are no reports of progesterone receptor (PR) genes in mosquitofish; it is not known whether P participates in regulating Vtg mRNA expression in the present study through its own receptor. In a previous study, exposure of female fathead minnow to the synthetic androgen 17β-trenbolone could also significantly depress Vtg mRNA levels (Miracle et al., 2006). In addition, in the male turtle (*Chrysemys picta*), both P and T have been shown to inhibit E2-induced Vtg mRNA expression levels, T being the most potent inhibitor (Custodia-Lora et al., 2004a). However, surprisingly, P or T increased each Vtg isoform mRNA expression induced by E2 in the ovaries of mosquitofish. A similar phenomenon was observed in the study of Custodia-Lora et al. (2004b), where P treatment alone did not significantly influence hepatic Vtg mRNA levels in female turtles, but P slightly increased E2-induced Vtg mRNA in the livers of female turtles (*C. picta*). Moreover, Mori et al. (1998) also showed that several hormones including progesterone and androgens induced Vtg mRNA in primary cultures of male immature rainbow trout hepatocytes. Although the mechanism of action of P and T on Vtg gene expression in mosquitofish is not understood, both P and T significantly suppressed hepatic Vtg isoform mRNA expression with an accompanying decrease in ER and AR isoform mRNA expression in the livers. A possible key role of both P and T is in controlling the formation of yolk. Marina et al. (2008) reported that P regulates the synthesis of Vtg proteins in *Torpedo* in a biphasic manner, upregulating when its titer is moderate, downregulating when it is high. Induction of gene expression by steroid hormones is related to steroid receptor regulation and a previous study has established that Vtg gene expression is closely related to ER regulation (Mori et al., 1998). The results from the present study also showed a direct transcriptional relationship between hepatic ER and Vtg genes in mosquitofish exposed to P and T alone. However, hormones can also have effects on organisms via non-receptor mechanisms, such as the cytochrome P450 family of enzymes that are participated in biosynthesis or catabolism of E2 (Kim et al., 2008). In accordance with that finding, the results from the present study showed a direct transcriptional relationship between hepatic ER and Vtg genes in mosquitofish exposed to P and T alone.

4.2. Modulation of ER gene expression

It is well known that Vtg mRNA expression is induced in oviparous fish species in response to estrogens by an ER-mediated pathway (Kim et al., 2010). It is not known however, whether each ER isoform contributes equally to the transcriptional regulation of Vtg genes in many species. It should be noted that two distinct ER isoforms have been cloned in western mosquitofish and termed as ERα and ERβ (Katsu et al., 2007). However, the ER isoform expression patterns and their role in the regulation of Vtg genes in mosquitofish in response to hormones are unknown. In previous transient transfection study, the three mosquitofish ER isoform proteins displayed estrogen-dependent activation of transcription (Katsu et al., 2007). In this study, the transcript levels of ERα (but not ERβ) in the livers of mosquitofish were significantly up-regulated by E2, and a parallel induction of the mRNA expression of both VtgA and VtgB in the livers was also observed. Many previous studies in teleosts showed that the expression of hepatic ERα mRNA is significantly induced by E2 treatment (Sabo-Attwood et al., 2004; Esterhuyse et al., 2010; Marlatt et al., 2010). In contrast to ERα, ERβ transcriptional regulation by estrogen seems to be more variable depending on the species and ERβ subtypes (Sabo-Attwood et al., 2004; Pinto et al., 2006; Esterhuyse et al., 2010). ERα auto-regulation in the livers is a common feature of teleosts that has been attributed to be involved in Vtg gene transcription in the livers of mature females in response to E2 (Pinto et al., 2006). Although the results in the present study do not show a direct mechanistic contribution of ER isoforms to the transcriptional regulation of Vtg genes in the livers of mosquitofish, they suggest that ER isoforms are not regulated in the same manner by E2 and do not contribute equally to those gene regulation. In addition, the results in the present study also indicate that the role of ERα associated with reproduction in the livers of fish may be more important than that of ERβ. In contrast to the livers of mosquitofish, a slight down-regulation by E2 of both ERα and ERβ is found in the ovaries, suggesting the regulation of ER subtypes varies among tissues. Mosquitofish treated with P or T alone showed a significant reduction in the mRNA expression of two ER isoforms preceding significant inhibition of three Vtg isoform mRNA levels in the livers. Although the reasons for tissue-specific inhibition of ER isoforms by P and T, seen only in the liver but not ovaries, are unclear, it does point to a targeted disruption of liver steroid signaling in female mosquitofish. In addition, treatment of E2 in combination with either P or T showed that either P or T had a negative effect on the E2 up-regulation of ERα and strengthened the E2 down-regulation of ERβ in the livers. In the present study, P or T may act as an antagonist of ER gene transcripts in the livers of mosquitofish. However, this does not seem to be the case in the present study where ovarian ER isoform transcription levels were increased in P or T treatment compared with the controls, though not to significant levels. Moreover, it should be noted that P blocked the E2 down-regulation of both ERα and ERβ mRNA expression in the ovaries. The results indicate an organ specific auto-regulation of ER isoforms by hormones and suggest ER isoforms in the livers are a more sensitive automatic adjusting loop than those in the ovaries. Although the rainbow trout ER gene promoter contains EREs that regulate its own transcription (Le Dréan, et al., 1995), it is not clear in fish how ER genes are regulated by P and T, whether they can be regulated through the promoters of ER genes. Given the unique roles of ER isoforms in gonadal differentiation, reproduction, metabolism and behavior, the effects reported in the present study show harmful consequences for mosquitofish in response to steroid hormones.

4.3. Modulation of AR gene expression

The role of ARs in male organisms is very similar to that of ERs in females (Janošek et al., 2006; Smolinsky et al., 2010). Androgens regulate genes by binding to androgen receptors (ARs), which usually play a pivotal role in sex differentiation, sex change and maturation in fish (Seo et al., 2006; Smolinsky et al., 2010). In previous studies, two subtypes of ARs have been cloned and characterized in western mosquitofish (Sone et al., 2005). Furthermore, the investigations of the binding affinities of mosquitofish ARs for various steroids including E2, T, P, corticosterone, diethylstilbestrol (DES), and 5α-dihydrotestosterone (DHT) showed that only androgens, T and DHT were effective in inducing luciferase activity by ARα and ARβ (Katsu et al., 2007), Which indicated that the cloned ARα and ARβ cDNAs encode functional mosquitofish ARs (Katsu et al., 2007). Larsson et al. (2002) reported that gonadal factors, including both androgens and estrogens, are involved in the physiological

regulation of brain androgen receptors in a teleost species during the reproductive cycle. However, compared with ERs, relatively little is known about how ARs are affected by external compounds. To begin to elucidate the physiological roles of ARα and ARβ, it is necessary to assess how hormones and other factors regulate their expression. Although E2 primarily acts by binding to estrogen receptors, it may have other mechanisms of action. E2 has been shown to be able to regulate AR mRNA levels (Larsson et al., 2002). In this study, E2 decreased ARα mRNA expression in the livers, which is agreement with previous observations about the AR mRNA and protein levels of stickleback kidney in response to E2 (Olsson et al., 2005). Olsson et al. (2005) found that down-regulation of AR by estrogens is a common feature of AR. However, in mosquitofish, two AR isoforms in the ovaries and ARβ in the ARβ in the livers have no significant change compared with the controls, suggesting that the regulation of AR depend on the species, organs and AR subtypes. In the present study, P could regulate AR mRNA expression: up-regulation in the livers and down regulation in the ovaries, indicating that the role of P varies according to tissue type (Seo et al., 2006). In addition, the results about each AR isoform mRNA expression in mosquitofish treated with P in combination with E2 indicate that E2 play a main role in regulating AR isoforms in the livers, but P does key function in the ovaries. In contrast to ERs that are auto-regulated by estrogens, AR is not usually up-regulated by androgens and no androgen-responsive elements (AREs) have been found in the promoter of cloned ARs (Olsson et al., 2005). Androgens induce or inhibit AR mRNA expression levels depending on the species and tissues (Larsson et al., 2002). Previously, synthetic androgens, 17β-trenbolone (TB) and methyltestosterone could induce masculinization of the anal fin accompanied by a transient up-regulation of ARα and ARβ in adult female western mosquitofish (Sone et al., 2005). However, when three-spined sticklebacks were exposed to DHT, 11-ketoandrostenedione (KA) or T, none of the three tested androgens regulated AR mRNA or AR protein (Olsson et al., 2005). The results of AR isoform mRNA expression in mosquitofish exposed to T alone are similar to those in mosquitofish exposed to P alone, probably because P is the precursor of T. However, AR isoform transcription in ovaries of mosquitofish treated by T + E2 was found distinctly different from that in P + E2 treated group. The regulation of AR isoform mRNA expression in multiple hormone treatments in mosquitofish appears to be under complex transcriptional regulation.

4.4. Modulation of MT gene expression

MT has been shown to be regulated during the period of sexual maturation as well as during early embryonic development (Olsson et al., 1995). Tissue-specific regulation of MT mRNA in mosquitofish has been observed following steroid hormone exposure. Although the different mechanism of hormone regulation of MT mRNA levels in different tissues of mosquitofish is not known from the present study, a similar phenomenon was also observed in a previous study (Rhee et al., 2009). E2 treatment resulted in a significant down-regulation of MT mRNA expression in two tested tissues of female mosquitofish in the present study. In a previous study, Gerpe et al. (2000) reported that MT mRNA expression was not affected in the liver when arctic char (*Salvelinus alpinus*) were exposed to E2 or mimic estrogens alone, but those compounds could inhibit Cd-induced MT mRNA expression in that tissue. Werner et al. (2003) also reported that MT concentrations were significantly reduced in the livers of lake trout exposed to the high and medium concentrations of ethynylestradiol. Moreover, Costa et al. (2010) showed that the levels of MT decreased in the liver of mature male fish (*Rhamdia quelen*) exposed to the highest E2 dose with a higher E2 level in plasma. Previous studies showed that E2 or other estrogenic compounds might inhibit MT expression by reducing free intracellular Zn being available to bind to the metal responsive elements in the promoters of MTs during Vtg synthesis (Olsson et al., 1995; Gerpe et al., 2000; Werner et al., 2008). In the present study, MT mRNA expression was also significantly inhibited in the livers of mosquitofish exposed to P or T alone. In a previous study, Sogawa et al. (2001) also found that both P and T could reduce the Cd-induced MT-I mRNA expression in ovariectomized mice. However, there were inconsistent results in adult tilapia (*Oreochromis mossambicus*) showing that medium and high doses of P treatments resulted in an increase in MT levels in the livers (Wu et al., 2002). Chan and Chan (2008) showed that the promoter of MT genes in tilapia contains metal-regulatory elements (MRE) and glucocorticoid-responsive elements (GRE). Furthermore, P is a precursor for the major glucocorticoids. Thus, it is easy to understand the up-regulation of MT in tilapia by P in the experiments performed by Wu et al. (2002). Whether P regulates MT mRNA expression through GRE or other mechanisms in the present study requires further study. To date, there is no available information regarding the MT expression in response to T, so it is difficult to elucidate the down-regulation MT mRNA by T.

4.5. Modulation of CYP1A gene expression

CYP1A is a typical biomarker for the biotransformation and detoxification of numerous exogenous organic compounds, including polycyclic aromatic hydrocarbons (PAHs) (Kim et al., 2008). Steroid hormone treatments caused a significant decrease of CYP1A mRNA expression in the livers of female mosquitofish, although they had no profound effect on ovarian CYP1A mRNA levels compared to the controls. Inhibition of CYP1A mRNA expression levels implies that hormones caused some form of cellular toxicity in female mosquitofish since its corresponding protection against xenobiotic compounds would also be reduced (Kim et al., 2008). Navas and Segner (2001) have reported that hepatic CYP1A expression in fish can be modulated by the female sex hormone. Although data concerning the responses of CYP1A to P and T in fish are unavailable from previous studies, some studies about the effects of E2 or xenoestrogen on CYP1A mRNA expression are in agreement with our results. For example, E2 and xenoestrogen (nonylphenol, NP) could inhibited the expression levels of CYP1A mRNA in juvenile and adult gray mullet (Cionna et al., 2006). Kim et al. (2008) also found CYP1A expression tended to decrease in the liver of male medaka after exposure to E2. Moreover, E2 reduced the expression levels of CYP1A mRNA of cultured trout hepatocytes, but inhibitory action by E2 did not overcome xenobiotic-induction of CYP1A mRNA (Navas and Segner, 2001). Navas and Segner (2001) hypothesized that the inhibitory effect of E2 on CYP1A mRNA expression could be due to the E2–ER complex interfering with the CYP1A gene directly, or interacting with the AhR to regulate CYP1A gene expression. Moreover, it is worth noting that the mRNA expression of CYP1A in the present study was similar to that of ERβ in the livers in corresponding hormone treatments.

5. Conclusions

The results from this study have showed that steroid hormones can cause multiple biological responses including the expression of Vtg, ER and AR isoform mRNA in the hormone signaling pathways and the expression of MT and CYP1A mRNA in the xenobiotic signaling pathway. Given that Vtgs, MT and CYP1A have been extensively used as biomarkers for estrogens, metals and hydrocarbons in aquatic environment, respectively; it is very important to consider that hormones may have negative effect on those biomarkers. Thus, it is advisable to use multiple biomarkers in assessing biological effects of environmental contaminants.

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