Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

Upregulation of estrogen receptor subtypes and vitellogenin mRNA in cinnamon clownfish *Amphiprion melanopus* during the sex change process: Profiles on effects of 17β-estradiol

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ARTICLE INFO

ABSTRACT

In the present study, we investigated the expression pattern of estrogen receptors (*esr*) and vitellogenin (*vtg*) mRNA in the gonads and liver during sex change in cinnamon clownfish by using quantitative polymerase chain reaction. We divided gonadal development during the sex change from male to female into 3 stages (mature male, male at 90 days after removing female, and mature female) and investigated *esr* and *vtg* mRNA expressions during the sex change. With female, the *esr* and *vtg* mRNA expressions increased. In western blot analysis, Esr1 protein was detected only in the ovaries of female cinnamon clownfish. Also, to understand the effect of 17β-estradiol (E_2), we investigated the *esr* and *vtg* mRNA expression patterns in the gonads and liver, and the changes in plasma E_2 level after E_2 injection. E_2 treatment increased both mRNA expression levels of *esr* and *vtg* and plasma E_2 levels. The present study describes the molecular characterization of *esr* subtypes and the interactions between *esr* and *vtg* after E_2 treatment in cinnamon clownfish.

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1. Introduction

Estrogen is an essential steroid hormone in reproduction and plays important roles in sexual maturation and differentiation, which include oogenesis, vitellogenesis, and testicular development. In addition, estrogen is a sex steroid hormone that influences growth, gonad sex differentiation, the reproductive cycle and lipid and bone metabolisms (Auchus and Fuqua, 1994; Pakdel et al., 2000). Estrogen action is primarily mediated by nuclear estrogen receptors (*esr1* and *esr2*), which function as ligand-dependent transcription factors that regulate transcription of target genes containing the consensus estrogen response element (ERE) in their promoter regions. Also, estrogen exerts its action through a membrane *esr*, and various signaling pathways (Ca^{2+}, cyclic AMP, protein kinase cascades) are rapidly activated and ultimately influence downstream transcription factors (Zhang and Trudeau, 2006).

Members of this superfamily share several common features that can be divided into 6 distinct domains (Krust et al., 1986; Kumar et al., 1987), including the highly conserved C (DNA-binding domain; DBD) and E (ligand-binding domain; LBD) domains, as well as many variable regions at the N and C termini between the DBD and LBD (the A/B, F and D domains) (Krust et al., 1986). In fish, 3 *esr* have been identified and characterized: *esr1* and *esr2a1* (Chang et al., 1999; Tchoudakova et al., 1999; Xia et al., 1999; Hawkins et al., 2000; Ma et al., 2000; Pakdel et al., 2000; Patiño et al., 2000; Rogers et al., 2000; Socorro et al., 2000; Huang and Chang, 2002; Choi and Habibi, 2003; Halm et al., 2004), *esr2a2* (Tchoudakova et al., 1999; Ma et al., 2000; Menuet et al., 2002; An et al., 2008), and *esr3* (Hawkins et al., 2000; Halm et al., 2004), which has been cloned and is genetically distinct from the other two.

Induction of vitellogenin (*vtg*), a precursor yolk protein, in response to estrogens by an *esr*-mediated pathway is well documented in several oviparous (egg-laying) fish species (Ryffel, 1978; Pakdel et al., 1991), and *vtg* in males is widely accepted as a biomarker of exposure to environmental estrogens (Hutchinson and Pickford, 2002; Filby et al., 2006). To date, more than two *vtg* transcripts have been discovered in at least 17 teleost species (Hiramatsu et al., 2006).

In this study, cDNAs encoding one type of *vtg* have been cloned and appear to belong to the *vtgA* group (Finn and Kristoffersen, 2007). In largemouth bass (*Micropterus salmoides*) and Atlantic salmon (*Salmo salar*), the *esr1* subtype is highly correlated with *vtg* mRNA levels in the liver (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006). However, in cinnamon clownfish, the role of *esr* subtypes in the regulation of genes such as *vtg in vivo* and *in vitro* is unknown.

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Cinnamon clownfish (*Amphiprion melanopus*) groups consist of a mated adult pair and an immature individual, and sex is controlled by social rank in the group. The female is largest and is dominant in the group. If the dominant female dies or is absent, the male undergoes a sex change to female (Godwin and Thomas, 1993; Godwin, 1994). Hence, cinnamon clownfish are a good model to study the mechanisms of male to female sex change in protandrous hermaphrodite fish. So far, only studies investigating changes in steroid hormones (Godwin and Thomas, 1993) and gonad histological analyses (Godwin, 1994) have been reported with regard to sex change processes in cinnamon clownfish; neither expression of *esr* subtypes and *vtg* involved in sex change progression nor expression of these genes by steroid hormone treatment has yet been examined. Thus, the aims of this study were to determine the changes in the mRNA expression of the 3 *esr* subtypes and *vtg* during sex change from male to female, to determine the role of E_2 in the changes in *esr* and *vtg* gene expressions and in plasma 17β-estradiol (E_2) levels, and to characterize the interaction between E_2 and these genes in cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

The cinnamon clownfish (*Amphiprion melanopus* Perciformes, Pomacentridae; fishbase.org name: fire clownfish), [immature ($n = 12$, 7.1 ± 0.8 g), mature male ($n = 12$, 10.5 ± 1.2 g), and mature female ($n = 12$, 22.2 ± 2.1 g)] were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea). Groups consisting of a mated pair (dominate female and male) were made in 1 of 12 50-L circulation filter tanks prior to the experiments. The temperature was maintained at 28 ± 0.5 °C, and the photoperiod was a 12:12-h light–dark cycle. Fish were fed a commercial marine aquarium fish feed (Jeilfeed Company, Kyoungnam, Korea) twice a day.

In the present study, the experimental design for sex reversal was a modified version of methods described by Godwin and Thomas (1993). Sex change was induced in male fish by removing their female mate from each group and adding one immature clownfish in its place. Ninety days after the female was removed, we observed the male fish undergo the sex change process from male to female. The fish were anesthetized with tricaine methane sulfonate (200 mg/L, MS-222; Sigma-Aldrich, St. Louis, MO, USA) prior to blood and tissues collection. Blood was taken from the caudal vasculature using a 1 mL heparinized syringe. After centrifugation (1500 g, 4 °C, 5 min), the plasma was stored at −80 °C before analysis, and fish were killed by spinal transection for collection of the pituitary glands and gonads. Pituitary glands and gonads from the fish of the sex change process (mature male; male at 90 days after removal of the female; and mature female) were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until total RNA was extracted for analysis.

2.2. 17β-estradiol treatment

17β-estradiol (E_2, Sigma, USA) was dissolved and diluted in 0.9% physiological saline. After anesthesia, immature fish were injected intraperitoneally of E_2 (0.1 and 1 μg/g, body mass, BM) at a volume of 1 μL/g BM. After injection, the gonad, liver and blood were sampled from the 5 fish at each of the following time periods: 0, 6, 12, 24, and 48 h. Water temperature was maintained at 28 ± 0.5 °C during the injection periods. All fish survived the experimental periods.

2.3. Primary hepatic culture

Livers were removed from 5 cinnamon clownfish, and cut by scalpel into 1–3 mm³ pieces were weighed, placed in a 24-well culture plate. Under a sterile hood, the pieces were washed several times with culture medium solution (M199, Invitrogen, USA). The liver pieces were added

in equal amounts (approximately 50 mg) to each well of a 24-well plate and a total of 2 mL of fresh culture media was added. The liver pieces were allowed to acclimatize at room temperature with access to ambient air for 2 h, after which the indicted concentrations of E_2 (10^{-6} and 10^{-5} M) were added, with equal volume of distilled water (dH_2O) being added to the control group. The liver pieces were cultured for 0, 6, 12, 24, and 48 h in an incubator at 28 °C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20 °C, 10,000 g, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at −80 °C.

2.4. Total RNA extraction and reverse transcription (RT)

Total RNA from the gonads and liver at each maturity stage during the sex change process was extracted using the Trizol method according to the manufacturer's instructions (Gibco/BRL, USA). Concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (3 μg) was reverse transcribed in a total volume of 20 μL using an oligo-d(T)$_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for use in polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.5. Quantitative polymerase chain reaction

QPCR was conducted to determine the relative expression of the *esr* subtypes (*esr1*, *esr2a1*, and *esr2a2*) and *vtg* mRNA using total RNA extracted from the gonads and livers of cinnamon clownfish ($n = 5$). Primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows (GenBank accession nos.: *esr1*, HM185179; *esr2a1*, HM185180; *esr2a2*, HM185178; *vtg*, HM185181): *esr1* forward primer (5′-CTG GGC TGG AGG TGC TGA TG-3′), *esr1* reverse primer (5′-TCA ACA CAG TCG CCT TCG TTC C-3′), *esr2a1* forward primer (5′-GCT TCA GGC TAC CAC TAT-3′), *esr2a1* reverse primer (5′-CGG CGG TTC TTG TCT ATA-3′), *esr2a2* forward primer (5′-GTC TCG GTT CCG TGA GCT GAA G-3′), *esr2a2* reverse primer (5′-GCA CAG ATT GGA GTT GAG GAG GAT C-3′), *vtg* forward primer (5′-CGA GAT TCT GAA ACA CCT G-3′), *vtg* reverse primer (5′-GCT GAA ATA ATT CCA CAA ACT T-3′), β-actin forward primer (5′-GCG ACC TCA CAG ACT ACC TCA TG-3′), and β-actin reverse primer (5′-AAG TCC AGG GCA CAA TAG CAG AG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold-cycle (Ct) level. All analyses were based on the Ct values of the PCR products. Ct level was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve.

2.6. Western blot analysis

Western blots were performed based on the protocols described previously (An et al., 2010a). Briefly, the total protein isolated from the gonads of cinnamon clownfish during salinity changes was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin, pH 7.4), sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A protein ladder (PageRuler™ Prestained Protein Ladder, SM0671, Fermentas, USA) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye

front ran off of the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min followed by washing in TBS. Membranes were incubated with polyclonal rabbit antibody to Esr1 (dilution 1:1000, E1528, Sigma) followed by horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (dilution 1:2000, Bio-Rad) for 60 min. Bands were detected using standard ECL as well as more sensitive ECL systems (ECL Advance; GE Life Sciences, Sweden) and exposure to autoradiography-sensitive film for 3 min.

2.7. Plasma analysis

Plasma E_2 levels were analyzed by radioimmunoassay (RIA) using an E_2 RIA kit (Adaltis, Bologna, Italy).

2.8. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Newman Keuls multiple range test) was used to compare differences in the data at a significance level of $P<0.05$. Results are presented as mean ± SD. The inter- and intra-assay CV were calculated as the SD divided by the mean.

3. Results

3.1. Quantification of esr1, esr2a1, esr2a2, and vtg mRNA expressions during sex change

The *esr1*, *esr2a1*, *esr2a2*, and *vtg* mRNA expression changes in the gonads during sex change from male to female by QPCR are shown in Fig. 1. Expressions of *esr1*, *esr2a1*, *esr2a2*, and *vtg* mRNA in the gonads were increased as the fish progressed toward the mature female state.

Expression of these genes in the livers was similar to that in the gonads (Fig. 2).

3.2. Western blot analysis

The Esr1 protein corresponding to the predicted size (approximately 66 kDa) for cinnamon clownfish was detected only in the female ovary, and not in males or transitioning fish (Fig. 3).

3.3. Plasma E_2 concentration during sex change

The plasma E_2 level was 182.1 + 30.2 pg/mL in male with a female present, elevated to 893.1 + 55.3 pg/mL in the male after removal of the female, and was higher once the male changed to a female (1192.4 + 40.4 pg/mL) (Fig. 4).

3.4. Quantification of esr1, esr2a1, esr2a2, and vtg mRNA expressions by E_2 in vivo

The *esr1*, *esr2a1*, *esr2a2*, and *vtg* mRNA expression changes in the gonads by E_2 injection are shown in Fig. 5. The *esr* mRNA expression was highest at 12 h after injection (*esr1*: approximately 20-fold; *esr2a1*: 12.1-fold, and *esr2a2*: 15.4-fold, respectively, at 1 μg/g E_2) and then decreased, and *vtg* mRNA continuously increased during the experimental period. The *esr1* mRNA expression was highest in the liver at 6 h after injection (approximately 6-fold at 1 μg/g E_2) and then decreased, and the *esr2a1* mRNA expression increased up to 24 h and then decreased at 48 h. The *esr2a2* was highest at 12 h (approximately 2.5-fold, at 1 μg/g E_2), and *vtg* mRNA increased at 12 h (7.5-fold, at 1 μg/g E_2) and then decreased (Fig. 6).

3.5. Plasma E_2 concentration by E_2 injection

The plasma E_2 level was 35.9 + 5.2 pg/mL at the start of the experiment, increased to 375.5 + 15.3 pg/mL at 24 h (0.1 μg/g E_2) and 560.4 + 40.1 pg/mL at 24 h (1 μg/g E_2), and then decreased (Fig. 7).
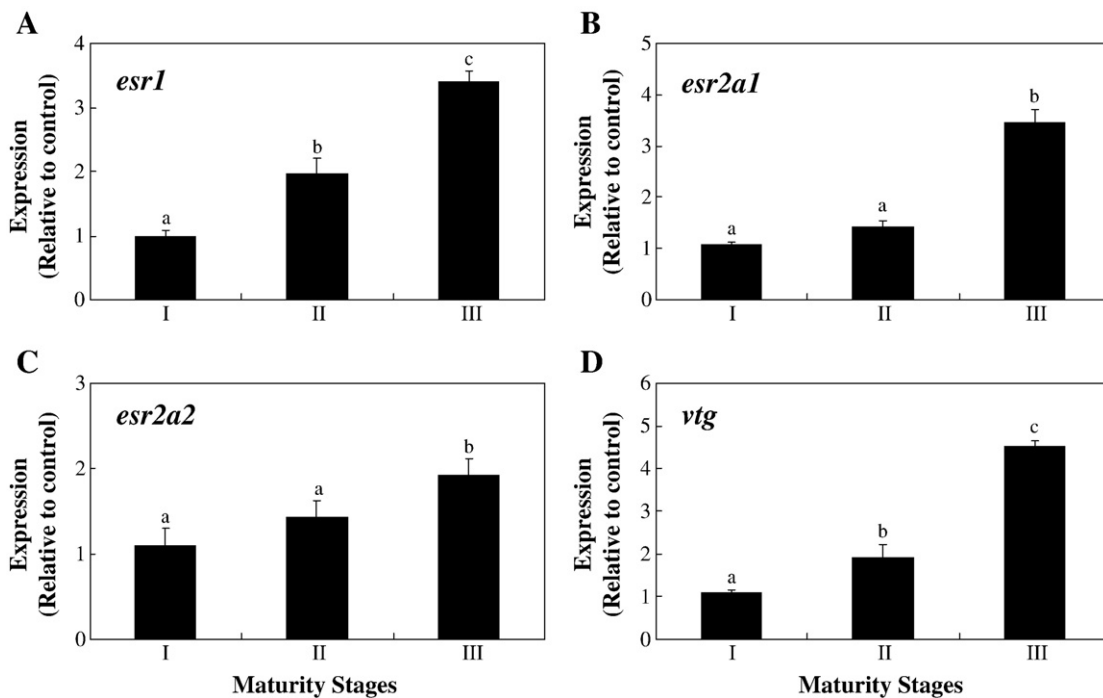

Fig. 1. Expressions of *esr1* (A), *esr2a1* (B), *esr2a2* (C), and *vtg* (D) mRNA in the gonads of cinnamon clownfish using quantitative PCR. I: mature male; II: male at 90 days after removal of the female; III: mature female. Total RNA (3 μg) prepared from the gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values indicate means + SD ($n=5$).

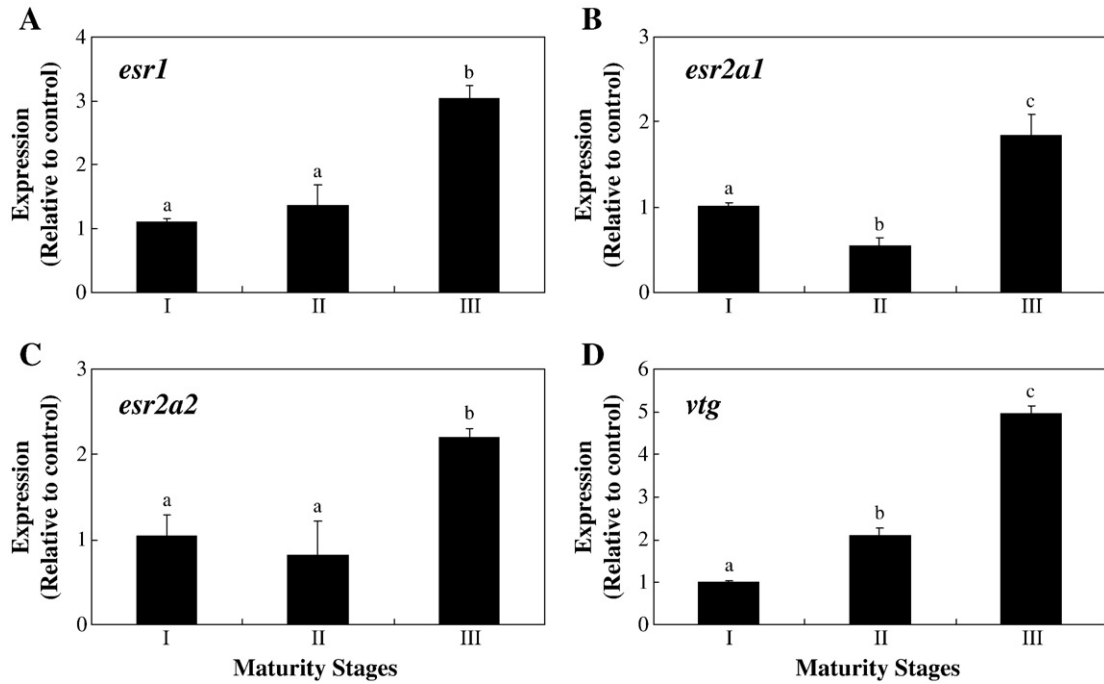**Fig. 2.** Expressions of *esr1* (A), *esr2a1* (B), *esr2a2* (C), and *vtg* (D) mRNA in the livers of cinnamon clownfish using quantitative PCR. I: mature male; II: male at 90 days after removal of the female; III: mature female. Total RNA (3 μg) prepared from the livers was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values indicate means + SD ($n=5$).

3.6. Quantification of the esr1, esr2a1, esr2a2, and vtg mRNA expressions by E_2 in vitro

The expression changes of *esr1*, *esr2a1*, *esr2a2*, and *vtg* mRNA by QPCR are shown in Fig. 8. The expression of *esr* mRNA was highest at 12 h (*esr1*: approximately 58.4 fold, *esr2a1*: 13.7 fold, and *esr2a2*: 17.6 fold, respectively at 10^{-5} M E_2) and then decreased, and *vtg* mRNA was highest at 12 h (approximately 25.2 fold, at 10^{-5} M E_2) and then decreased.
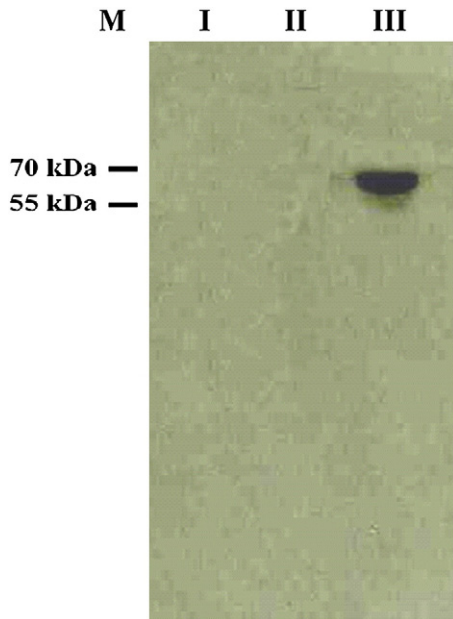

Fig. 3. Western blot of Esr1 protein (approximately 66 kDa) expression in cinnamon clownfish gonads during sex change. M: protein marker; I: mature male; II: male at 90 days after removing the female; III: mature female.

4. Discussion

In the present study, we examined *esr* subtype and *vtg* mRNA expression and plasma E_2 levels during a gonadal sex change from male to female in cinnamon clownfish. Also, to understand the role of *esr* and *vtg* genes in the gonads and livers, we examined the expression of *esr* subtypes and *vtg* mRNA and plasma E_2 levels after E_2 treatment (*in vivo* and *in vitro* experiments) in the gonads and livers of cinnamon clownfish.

During the sex change process of cinnamon clownfish, we detected that *esr* subtypes and *vtg* mRNA expressions were increased in the gonads and livers of fish that changed to female (Figs. 1 and 2). In addition, Esr1 protein in western blot analysis was only detected in the female ovary (Fig. 3), and plasma E_2 was highest in females during the sex change (Fig. 4); these data supported the expression data. Generally, *esr* and *vtg* genes are involved in development and maturity of oocytes in fish, as well as E_2 (Nagahama et al., 1995; Tyler and Sumpter, 1996). It has been reported that *esr* and *vtg* are
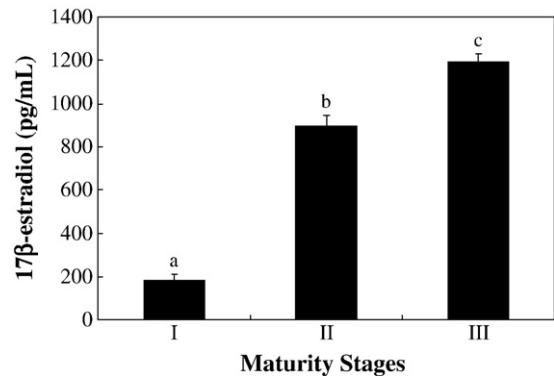

Fig. 4. Plasma 17β-estradiol (E_2) levels during sex change in cinnamon clownfish. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values indicate means + SD ($n=5$).
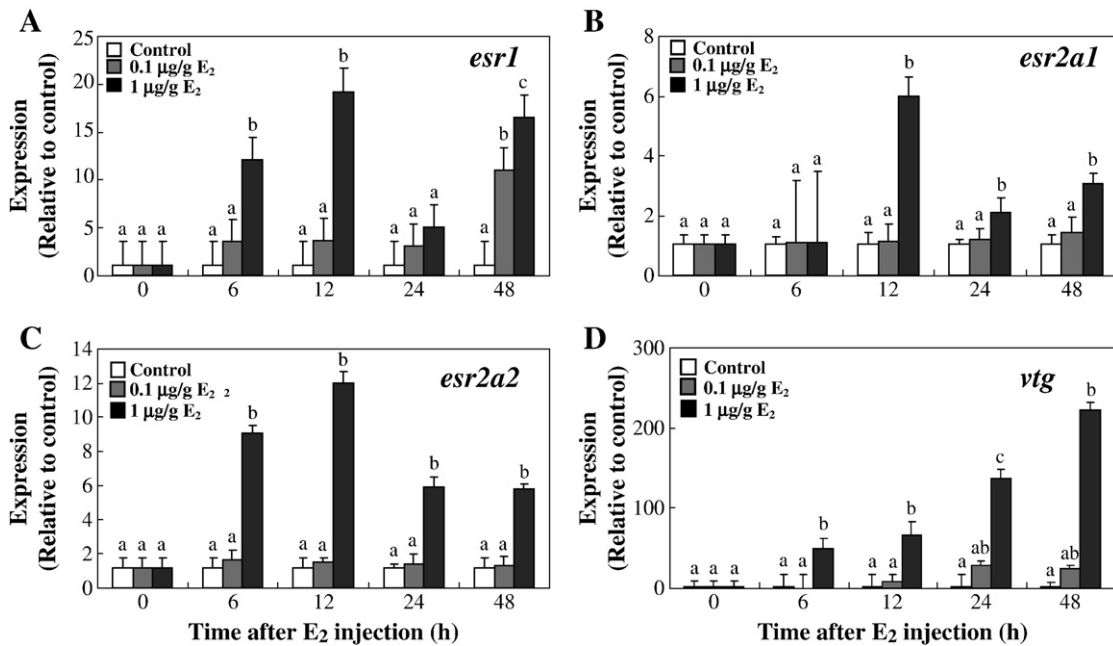
Fig. 5. Expressions of *esr1* (A), *esr2a1* (B), *esr2a2* (C), and *vtg* (D) mRNA in the gonads of cinnamon clownfish using quantitative PCR after E_2 injection. Total RNA (3 μg) prepared from the gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). Values indicate means + SD ($n = 5$).

involved in maturity of oocytes after synthesis of *vtg* by binding E_2 and *esr* in the liver (Flouriot et al., 1996; Bowman et al., 2002; Davis et al., 2009), so we judged that oocytes developed as a result of increasing *esr* and *vtg* mRNA levels during sex change to female in cinnamon clownfish. The *esr* were detected in the males, which agreed with the studies that *esr* were detected in the testes and sperm of rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*), implying that E_2 and *esr* are important in the regulation of gametogenesis in both sexes (Bouma and Nagler, 2001; Wu et al., 2001).

To study the role of E_2, we investigated *esr* and *vtg* mRNA expressions and the levels of plasma E_2 after intraperitoneal injection of E_2 in cinnamon clownfish. The *esr* and *vtg* mRNA expressions in the gonads tended to increase (Fig. 5). This result concurred with studies finding that expression of *esr* mRNA was increased by E_2 injection in goldfish (*Carassius auratus*) gonads (Nelson et al., 2007; Marlatt et al., 2008). Among *esr* subtypes, *esr1* had the highest levels after E_2 injection, and previous studies demonstrated that *esr1* was important for the synthesis of *vtg* after E_2 injection (Sabo-Attwood et al., 2004; Marlatt et al., 2008). In the present study, *esr1* mRNA was highest, and
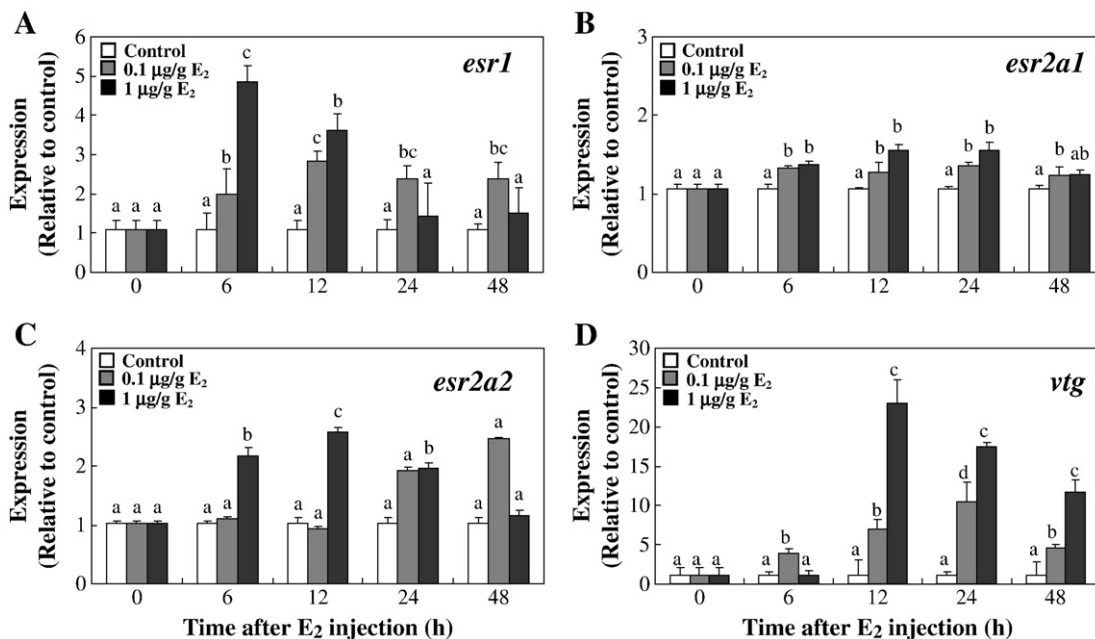

Fig. 6. Expressions of *esr1* (A), *esr2a1* (B), *esr2a2* (C), and *vtg* (D) mRNA in the livers of cinnamon clownfish using quantitative PCR after E_2 injection. Total RNA (3 μg) prepared from the livers was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). Values indicate means + SD ($n = 5$).
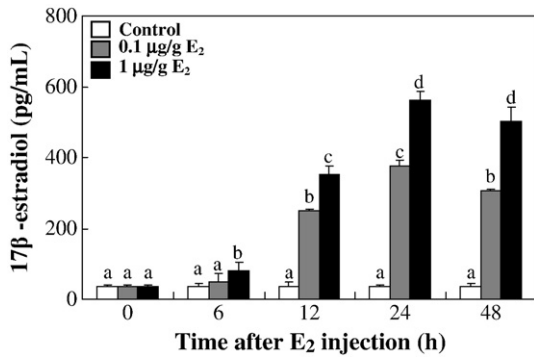
Fig. 7. Plasma 17β-estradiol (E$_2$) levels by E$_2$ injection in cinnamon clownfish. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values indicate means + SD ($n=5$).

we thought *esr1* affected *vtg* synthesis in cinnamon clownfish. Generally, although *vtg* was mainly synthesized in the liver, *vtg* was detected in the testes of various fish including tilapia (*Oreochromis mossambicus*), sea bream (*Sparus auratus*), and zebrafish (*Danio rerio*) (Wang et al., 2005; Pinto et al., 2006; Davis et al., 2008). In previous studies, *esr1*, *esr2*, and *vtg* mRNA increased in the testes of male tilapia by injection of E$_2$ (Davis et al., 2008), and the increases of *vtg* mRNA seen in the testes of sea bream after E$_2$ injection (Pinto et al., 2006) were similar to ours. Also, Chang et al. (1995) and An et al. (2009) mentioned that using protandrous black porgy (*Acanthopagrus schlegeli*), the plasma E$_2$ level was 9.4 + 0.7 pg/mL at the start of the experiment, increased to a maximum of 260.1 + 23.54 pg/mL after 3 days, and then decreased to 96.0 + 13.2 pg/mL after 9 days by 17β-estradiol injection (1.5 μg/g) in black porgy. An et al. (2008) found that the maximum plasma E$_2$ level (3 days; 260.1 + 23.54 pg/mL) following E$_2$ injection was lower than that of mature female black porgy (705.6 + 70 pg/mL) during sex change determined in our previous study. These data suggest that the plasma E$_2$ concentration attained following the exogenous administration of E$_2$ did not affect sex change but only temporarily increased plasma levels in immature

black porgy. Therefore, these results combined with these previous reports suggest that E$_2$ is involved in ovarian maturation as well as the male to female sex change in cinnamon clownfish.

As in the gonads, expressions of the 3 *esr* and *vtg* mRNA in the livers of immature cinnamon clownfish increased (Fig. 6). Marlatt et al. (2008) reported increased *esr1* mRNA expression in the livers of male goldfish (*C. auratus*) after E$_2$ injection, and Soverchia et al. (2005) also found increased *esr2a1* mRNA expression in the livers of immature goldfish by E$_2$, results that are both similar to ours. Expression of *vtg* mRNA in the livers of cinnamon clownfish increased in the present study, which is in agreement with previous studies that found significantly increased expression of *vtg* mRNA in the livers by E$_2$ in goldfish (*C. auratus*) (Marlatt et al., 2008) and tilapia (*O. mossambicus*) (Davis et al., 2008; Esterhuyse et al., 2009). E$_2$ may first increase the plasma levels of E$_2$ and the expression of *esr* mRNA in the gonads and then induce *esr* and *vtg* expressions in the liver (Flouriot et al., 1996; Bowman et al., 2002). We observed that expression of *esr* mRNA was higher after treatment with high-dose E$_2$ at an early hour (1 μg/g BM) than after treatment with low-dose E$_2$ (0.1 μg/g BM), indicating that E$_2$ has a dose-dependent effect. This result agreed with the previous study in which liver *esr1* was induced in a dose-dependent manner with a single E$_2$ injection (from 0.5 to 2.5 mg/kg) in male largemouth bass (*M. salmoides*) (Sabo-Attwood et al., 2004). In addition, we examined gonadal histology during sex change in cinnamon clownfish in our previous study (An et al., 2010b). As a results, we found that the oocytes had developed and the testicular tissue were regressed from male to female.

Considering the previous studies and the present study, we believe that the expression pattern of *esr* subtypes is dependent upon the maturity stage, hormone treatment, hormone concentration, and duration of hormone exposure.

Moreover, the *esr* and *vtg* mRNA expressions were elevated after primary hepatic culture in cinnamon clownfish (Fig. 8). High levels of *vtg* mRNA were detected in a high concentration of E$_2$ (1 μg/g BM), which agreed with Esterhuyse et al. (2009) who demonstrated that the expression of *vtg* mRNA was high in a dose-dependent manner after treatment with E$_2$ (0.5, 1.0 and 60 μg/L E$_2$) in the livers of tilapia.
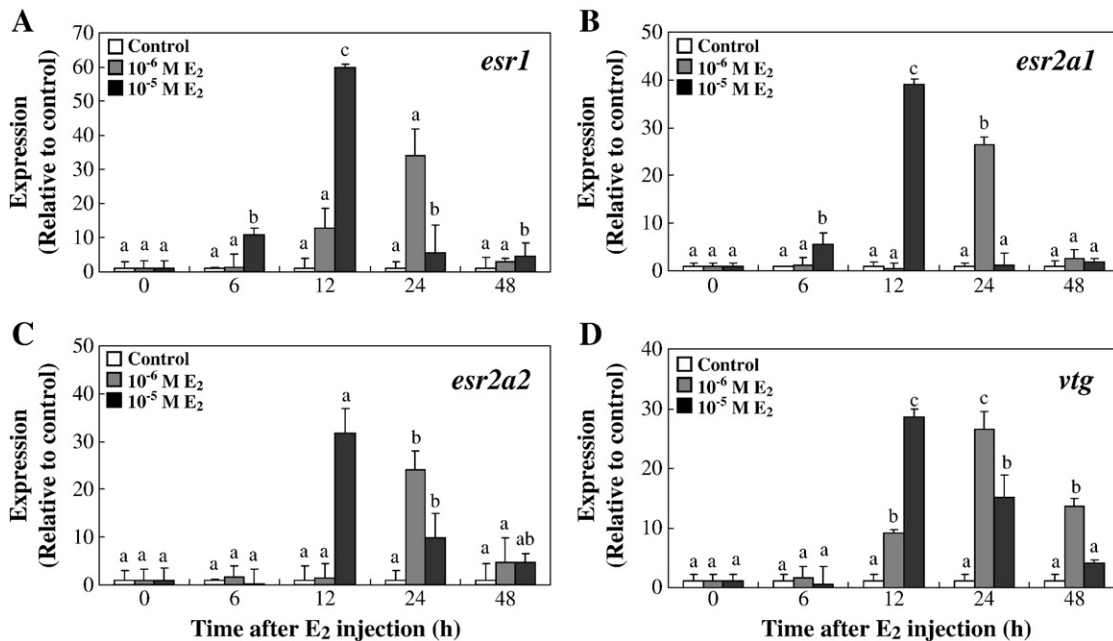

Fig. 8. Expressions of *esr1* (A), *esr2a1* (B), *esr2a2* (C), and *vtg* (D) mRNA in hepatic cultures of cinnamon clownfish using quantitative real-time PCR after E$_2$ injection. Total RNA (3 μg) prepared from hepatic cultures was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values indicate means + SD ($n=5$).

Therefore, we judged that the increased expressions of *esr* and *vtg* in the gonads and livers may be because E$_2$ was a regulator of these genes in cinnamon clownfish.

In brief, we found: 1) increased *esr* and *vtg* mRNA expressions during sex change from male to female in cinnamon clownfish, and 2) increased gene mRNA expression by E$_2$ injection in immature cinnamon clownfish, indicating that E$_2$ induces the increased expressions of *esr* and *vtg* mRNA. As a result, we concluded that E$_2$ is the regulator of *esr* and *vtg* in cinnamon clownfish.

Acknowledgements

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