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Molecular Cloning and Characterization of the Expression Profiles of Vitellogenin Transcripts in the Dojo Loach (*Misgurnus anguillicaudatus*) in Response to 17 α -ethinylestradiol and 17 β -estradiol Administration

Meiqin Wu¹, Osamu Nishimiya¹, Misato Nakamori², Kiyoshi Soyano²,
Takashi Todo³, Akihiko Hara³, and Naoshi Hiramatsu^{3*}

¹Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

²Institute for East China Sea Research, Nagasaki University, Nagasaki, Nagasaki 851-2213, Japan

³Division of Marine Life Sciences, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

The gene, *vitellogenin* (*vtg*) was cloned and characterized in the dojo loach (*Misgurnus anguillicaudatus*), an indigenous freshwater species in East Asia, in order to develop tools for detecting the effects of estrogenic endocrine-disrupting chemicals (EEDCs). Full-length cDNAs encoding seven distinct *vtg* transcripts (*vtg1–7*) were obtained. The corresponding deduced amino acid sequences (*Vtg1–7*) were divided into two types; type I (*Vtg1–6*; 89–99% identical), which contained both lipovitellin (Lv) and phosvitin (Pv), and type II (*Vtg7*), which contained Lv alone. Phylogenetic analysis revealed that the type I and type II *Vtgs* in the loach could be classified as *VtgAo1* and *VtgC* types, respectively. Immuno-biochemical analyses using type-specific *Vtg* antisera revealed that *VtgAo1* proteins appeared to be the major *Vtg* type in this species. Males were administered (aqueous exposure) either 17 β -estradiol (E2) or 17 α -ethinylestradiol (EE2), the results from which were used to determine that hepatic *vtgAo1* expression was estrogen-sensitive. The precise classification of the loach *vtg/Vtg* products, as well as their induction profiles following the estrogenic stimulation, provide a basis for their use as sensitive biomarkers when EEDC activities are evaluated in the freshwater environments in East Asia.

Key words: 17 α -ethinylestradiol, 17 β -estradiol, estrogenic endocrine-disrupting chemicals, *Misgurnus anguillicaudatus*, quantitative realtime PCR, vitellogenin

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are increasingly found in the effluents of industrial, agricultural, and domestic sewage treatment plants (Sumpter and Johnson, 2005; Porte et al., 2006; Oh et al., 2009). Some EDCs are known to induce an estrogenic response by mimicking the action of endogenous estrogens (Kolpin et al., 2002). One such xenoestrogen, 17 α -ethinylestradiol (EE2), which has been widely used in oral contraceptives and hormone substitute treatments, has been consistently detected during investigations of the effects of EDCs on aquatic environments (Williams et al., 2003; Johnson et al., 2005; Muller et al., 2008).

Vitellogenin (*Vtg*), a major precursor of egg yolk proteins, is normally synthesized in the liver of reproductive female oviparous vertebrates in response to the endogenous estrogen, 17 β -estradiol (E2) (Wahli et al., 1981). It is then released into the blood, sequestered into oocytes by

receptor-mediated endocytosis, and deposited as yolk proteins following limited enzymatic processing. While *Vtg*-derived yolk proteins typically consist of lipovitellin (Lv), phosvitin (Pv), and β' -component (β' -c) in teleosts (Hiramatsu et al., 2005, 2006), an additional yolk protein class encoding the C-terminal end of *Vtg* (termed as C-terminal peptide) has been detected in the barfin flounder (*Verasper moseri*) (Matsubara et al., 1999).

Vtg is also induced following exposure to estrogenic EDCs (EEDCs) in males and juveniles (Sumpter and Jobling, 1995; Hiramatsu et al., 2005, 2006; Matozzo et al., 2008; Robinson and Scott, 2012). Therefore, the appearance of *Vtg* in males and juveniles has been utilized as an effective, sensitive biomarker for detecting estrogenic activity in the aquatic environment.

Various subtypes of the *Vtg* transcript have been classified in teleosts (Finn and Kristoffersen, 2007). However current methods for the detection and quantification of fish *Vtg* have often been developed on the basis of a single unclassified type of *Vtg* (Hiramatsu et al., 2005, 2006). It is therefore important that the various *Vtg* subtypes are characterized and quantified in model animals before utilizing them as bio-

* Corresponding author. Tel. : +81-138-40-8878;
Fax : +81-138-40-8878;
E-mail: naoshi@fish.hokudai.ac.jp

markers for studying EEDC exposure.

The impact of EDCs on fish has been evaluated and studied extensively (De Vlaming et al., 2007). The dojo loach (*Misgurnus anguillicaudatus*) is a widespread freshwater fish in East Asia. Due to its moderate size (10–20 g for adult fish), easy identification of the sexes (by the shape of pectoral fin), and the close association of its diet with (polluted) sediments, this species is considered to be a candidate organism for field and laboratory toxicological studies (Shao et al., 2005). In addition, this species inhabits rice fields and rivers that are likely contaminated with high levels of pesticides and other anthropogenic chemicals, and is thus exposed to pollution-associated risk. A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of Vtg in the plasma of *M. anguillicaudatus* by Shao et al. (2005), which was subsequently used to assess the effects of E2 in the male loach (Lv et al., 2006, 2007). However, the presence of multiple Vtg subtypes needs to be verified prior to the development of precise quantification methods for the hepatic *vtg* gene expression and circulating Vtg levels in loach species.

The goal of the present study was to survey the impact of EEDC contamination in freshwater environments in East Asia by establishing a research model that utilizes the Vtg of the dojo loach as an estrogen-sensitive biomarker. To this end, the focus in this present study was to (1) clone and characterize full-length cDNAs encoding Vtg in the dojo loach and (2) investigate the profiles of *vtg* gene expression in the male loach following the exposure to the model estrogenic compounds, natural estrogen (E2) and synthetic xenoestrogen (EE2). Additionally, some biological parameters are provided in order to complement this information and assess the influence of exposure to aqueous EDCs.

MATERIALS AND METHODS

Experimental fish and sample collection

Adult female dojo loach [$n = 10$; 25.3 ± 0.9 g in body weight; 16.4 ± 0.3 cm in total length; $10.69 \pm 1.94\%$ in gonadosomatic index (GSI)] were bought from Gondo fish farm (Fukuoka, Japan) and raised in glass tanks with aerated water at $26 \pm 1^\circ\text{C}$ in the institute for East China Sea Research of Nagasaki University. The photoperiod was set at 14 h:10 h (light:dark). The fish were anesthetized with 2-phenoxyethanol (Kanto Chemical Co., Tokyo, Japan) before sampling. Liver samples were collected and incubated in RNAlater (Ambion, Austin, TX, USA) overnight at 4°C , and kept at -30°C until use for preparing an inter-assay control RNA for quantitative realtime PCR (qRT-PCR; pooled RNA from $n = 10$ individuals, see later section for more details). A part of this liver samples ($n = 4$) were also used in molecular cloning procedure.

One male loach ($n = 1$; 10.1 g; 13.1 cm; 0.50% GSI), which was induced to produce Vtg by estrogen injection, was injected twice with 5 mg/kg body weight of E2 (prepared as 2 mg E2/ml in propylene glycol) into the abdominal cavity at 3-day intervals. A blood sample was collected two days after the second treatment. Serum was separated by centrifugation at $10,000 \times g$ for 10 min and stored at -30°C until use (for Vtg detection).

Other live male loach ($n = 105$) were bought from a local store (Hakodate, Japan), transferred into the three 60 L recirculating tanks, and acclimated for about two weeks under the conditions described above at Hokkaido University. This group was used in the E2 and EE2 exposure experiments (see details in section 'Aqueous exposure to 17α -ethinylestradiol and 17β -estradiol'). Body weight and total length, as well as liver and gonad weight, were recorded at each sampling to provide data regarding the biology and health of the experimental individuals (Table 1).

All fish used in these experiments were fed a commercial carp diet (Marubeni Nisshin Feed Co., Tokyo, Japan) during the acclimation period, but starved during experimental periods.

Chemicals

Solutions (1 mg/ml) of EE2 and E2 (Nacalai Tesque Co., Kyoto, Japan) were initially prepared in 99.5% ethanol (Kanto Chemical Co.). These high stock solutions were serially diluted to working concentrations (10 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$) and stored at 4°C until they were further diluted to the final experimental concentrations (i.e., concentrations in the experimental rearing water).

Aqueous exposure to 17α -ethinylestradiol and 17β -estradiol

Following two weeks of acclimation as described above, five males were sampled as the initial control group and the remaining males (the experimental group) were randomly assigned into groups of 10 individuals and kept in 8 L glass tanks for three days prior to the onset of the exposure. After three days of acclimation, the experimental group was exposed to either EE2 or E2 at nominal concentrations of 1, 10, 100, 1000 ng/L, with continuous aeration to maintain adequate dissolved oxygen, but without recirculation. The fish in the solvent control group were exposed to the solvent carrier only. The final concentration of solvent carrier (ethanol) in all aquaria was 0.01%. The experimental water containing the above concentrations of hormones was freshly made every day using the

Table 1. Biological parameters of male loach used in estrogen exposure experiments.

Group (ng/L) ^a	Duration (d)	Total length (cm)	Body weight (g)	GSI (%) ^b	HSI (%) ^c
Initial		9.7 ± 0.1	3.7 ± 0.2	0.66 ± 0.08	0.60 ± 0.07
control		8.6 ± 0.3	2.6 ± 0.3	0.36 ± 0.12	0.59 ± 0.10
1		9.4 ± 0.3	3.4 ± 0.5	0.46 ± 0.03	0.59 ± 0.10
10	2	9.2 ± 0.2	2.8 ± 0.3	0.58 ± 0.11	0.70 ± 0.17
100		9.8 ± 0.1	3.5 ± 0.3	0.62 ± 0.14	0.70 ± 0.09
1000		10.1 ± 0.4	3.7 ± 0.6	0.45 ± 0.07	1.03 ± 0.21
EE2		9.3 ± 0.4	3.0 ± 0.5	0.46 ± 0.08	0.64 ± 0.10
control		10.0 ± 0.2	3.4 ± 0.4	0.46 ± 0.07	0.71 ± 0.03
1	7	9.8 ± 0.4	3.6 ± 0.4	0.38 ± 0.07	0.52 ± 0.03
10		9.1 ± 0.3	3.1 ± 0.3	0.56 ± 0.07	0.94 ± 0.15
100		9.8 ± 0.3	3.6 ± 0.3	0.65 ± 0.08	$2.32 \pm 0.34^*$
control		8.8 ± 0.3	2.5 ± 0.2	0.50 ± 0.03	0.59 ± 0.06
1		9.2 ± 0.2	2.6 ± 0.1	0.37 ± 0.04	0.61 ± 0.07
10	2	8.8 ± 0.3	2.5 ± 0.3	0.44 ± 0.07	0.66 ± 0.07
100		9.1 ± 0.6	2.7 ± 0.3	0.47 ± 0.14	0.49 ± 0.04
1000		9.2 ± 0.3	2.7 ± 0.3	0.93 ± 0.35	0.78 ± 0.04
E2		9.2 ± 0.4	3.0 ± 0.5	0.56 ± 0.08	0.71 ± 0.07
control		8.9 ± 0.4	2.5 ± 0.4	0.59 ± 0.09	0.64 ± 0.08
1	7	9.8 ± 0.4	3.3 ± 0.2	0.52 ± 0.05	0.64 ± 0.02
10		9.0 ± 0.3	2.7 ± 0.3	0.58 ± 0.09	0.64 ± 0.07
100		9.3 ± 0.3	2.8 ± 0.2	0.72 ± 0.07	$1.08 \pm 0.09^*$

Results are shown as mean $\pm$ standard error. ^aEach group consists of five individuals.

^bGSI = gonad weight / body weight $\times 100$. ^cHSI = liver weight / body weight $\times 100$.

*Significant difference ($P < 0.05$) as determined by Tukey's honestly significant difference (HSD) test.

stock hormone solutions; the corresponding aquarium's water was exchanged during the experimental period everyday with this freshly made water in order to avoid possible degradation and to keep the actual concentration as close as possible to the desired nominal concentrations. At two and seven days following the initial exposure, five fish were sampled randomly from each tank. The gonad and liver tissues were dissected, weighed, and incubated in RNAlater solution as described above, and then stored at -30°C until quantification of *vtg* transcripts. The GSI and hepatosomatic index (HSI) were calculated as gonad and liver weight / body weight $\times 100$, respectively.

RNA extraction and reverse transcription

Total RNA was extracted from the liver tissues according to the method described in our previous report (Wu et al., 2011). Loach cDNA was synthesized from 1 μg of hepatic total RNA using SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) in a 10 μl reaction volume according to the manufacturer's instructions, unless otherwise stated (see below). In addition to the normal reverse transcription (RT) reaction, a reaction omitting the reverse transcriptase (no RT control: NRT) was also performed in parallel. The following RT conditions were used: 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min. The resulting first-strand cDNA were used as templates for either cDNA cloning or qRT-PCR analyses.

Molecular cloning of the partial cDNAs

The sampled liver tissues from four female individuals (23.89 ± 0.86 g; 16.53 ± 0.46 cm; $10.09 \pm 3.06\%$ GSI) were pooled prior to total RNA extraction for molecular cloning. Degenerate oligonucleotide primers were designed for the isolation of partial cDNA for loach *vtgC* based on the *vtgC* sequences of different teleost species available in GenBank: zebrafish (*Danio rerio*) *vtgC* (GenBank accession number AF254638), yellowfin goby (*Acanthogobius flavimanus*) *vtg-320* (GenBank accession number AB088474), western mosquitofish (*Gambusia affinis*) *vtgC* (GenBank accession number AB181837), grey mullet (*Mugil cephalus*) *vtgC* (GenBank accession number AB288934), and red seabream (*Pagrus major*) *vtgC* (GenBank accession number AB181840). Primers were also designed for cDNA amplification of A-type loach *vtg* (hereby referred to as *vtgAo* according to Finn and Kristoffersen, 2007) based on the conserved coding regions of zebrafish *vtg1* (GenBank accession number NM_001044897), *vtg2* (GenBank accession number NM_001044913), *vtg4* (GenBank accession number NM_001045294), *vtg5* (GenBank accession number BC097081), *vtg6* (GenBank accession number NM_001122610), *vtg7* (GenBank accession number NM_001102671). Polymerase chain reaction (PCR) was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster city, CA, USA) in a reaction volume of 10 μl containing each degenerate primer (1 μM in the final condition) listed in Table 2 and the first-strand cDNA template according to the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 90 s, ending with 10 min of elongation at 72°C . The amplified

products were analyzed by a 1.5% agarose gel electrophoresis and purified using GENECLAN Turbo Kit (MP Biomedicals Europe, Illkirch, France). The isolated cDNA was inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA) and positive clones were sequenced according to the methods described in Morita et al. (2011).

A cDNA encoding a fragment of elongation factor 1- α (*ef1- α*) was amplified by PCR using primers (Table 2) designed based on known *ef1- α* sequences of zebrafish (GenBank accession number BC064291), common carp (GenBank accession number AF485331), goldfish (GenBank accession number AB056104), and fathead minnow (GenBank accession number AY643400); this was used as a reference gene normalizing *vtg* mRNA expression levels in a qRT-PCR analysis (see section 'Quantitative realtime reverse transcription PCR for *vtg* mRNA expression').

Rapid amplification of cDNA ends (RACE) and final cloning of full-length cDNA sequence

Templates of 5'- and 3'-RACE were synthesized from female liver total RNA using the SMARTer[™] RACE cDNA Amplification Kit (Clontech, Takara Bio Inc., Shiga, Japan) according to manufacturer's protocols. These were done using a universal primer mix (UPM) supplied by the kit, in conjunction with the gene-specific primers (GSPs, Table 2) designed based on the partial sequences of loach *vtgAo/vtgC*.

Following the cloning of the 5' and 3' ends of *vtg* sequences by RACE, gene primers (Table 2) were newly designed to obtain consecutive full-length *vtg* sequences. The resulting PCR products were sub-cloned into the pGEM-T Easy vector as described above for sequencing.

Alignment and phylogenetic analysis

Homology search of the deduced amino acid (AA) sequences of putative loach Vtgs was done using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis of vertebrate Vtg AA sequences was performed using Neighbor Joining (NJ) method of MEGA version 5.05 software (Tamura et al., 2011). Statistical sig-

Table 2. Primers used in cDNA clonings of two vitellogenin subtypes (*vtgAo* and *vtgC*) and elongation factor 1- α (*ef1- α*), as well as in quantification of the corresponding mRNA expression in the dojo loach.

Primer name	Primer sequences	Purpose
vtgC-degenerate-F1	F: 5'-TWMRGCCVYTGSTGRAHATGG-3'	Partial <i>vtgC</i> cloning
vtgC-degenerate-R1	R: 5'-AKYCHTCHGSRGRAYACC-3'	Partial <i>vtgC</i> cloning
vtgAo-degenerate-F1	F: 5'-AGYTCAAGCTCHTBCWTGTCCA-3'	Partial <i>vtgAo</i> cloning
vtgAo-degenerate-R1	R: 5'-CATTGTSATCTCTGGAAAYCCTARCA-3'	Partial <i>vtgAo</i> cloning
vtgC-5'RACE-GSP1	R: 5'-CGGACACCAAACTCAAGCCATTG-3'	5'RACE for <i>vtgC</i>
vtgAo-5'RACE-GSP1	R: 5'-GGTCAATCTCTTTATCGCTGTTCTGGGC-3'	5'RACE for <i>vtgAo</i>
vtgC-3'RACE-GSP2	F: 5'-GCTCTGGTCTCAGTAATAAGCGAGGTTTC-3'	3'RACE for <i>vtgC</i>
vtgAo-3'RACE-GSP2	F: 5'-CAAAGGCAAGAAGCAGTGGAAGC-3'	3'RACE for <i>vtgAo</i>
vtgAo-3'RACE-NGSP2	F: 5'-GCCGTCCTTTGCCCGTGCTGTGAG-3'	3'RACE for <i>vtgAo</i> (Nest)
vtgC-full primer-F1	F: 5'-ACCATGTGGCAGCTGTATCTTTGTCTC-3'	Full-length <i>vtgC</i> cloning
vtgC-full primer-R1	F: 5'-GAAGCAAGCCATGTTACATTCTCCACTAC-3'	Full-length <i>vtgC</i> cloning
vtgAo-full primer-F1	F: 5'-ACACAACCACATGAGCATGAGAGC-3'	Full-length <i>vtgAo</i> cloning
vtgAo-full primer-R1	R: 5'-TTGTGGTACATTTGATACATTACGTTCAAA-3'	Full-length <i>vtgAo</i> cloning
vtgAo-full primer-R2	R: 5'-TGCTTGCTGAAATTTATTAGGATCACATT-3'	Full-length <i>vtgAo</i> cloning
vtgC-rVRBP-F1	F: 5'-CACGTGAATTCGCTCTATGAAGTCTTGAGTTGGGA-3'	In-fusion cloning of <i>vtgC</i>
vtgC-rVRBP-R1	R: 5'-AATATCATCGATCTCACTGGTTGCACCATCCAC-3'	In-fusion cloning of <i>vtgC</i>
vtgAo-rVRBP-F1	F: 5'-CACGTGAATTCGCTCTATGAAGTCAAGAGGCTG-3'	In-fusion cloning of <i>vtgAo</i>
vtgAo-rVRBP-R1	R: 5'-AATATCATCGATCTCAAGAGCATCATGGACC-3'	In-fusion cloning of <i>vtgAo</i>
ef1- α -F1	F: 5'-TGGTATCTCCAAGAACGGAC-3'	Partial <i>ef1-α</i> cloning
ef1- α -R1	R: 5'-GTGGTTCAGGATGATGACC-3'	Partial <i>ef1-α</i> cloning
ef1- α qPCR-F1	F: 5'-TCAGCGCCTACATCAAGAAG-3'	qRT-PCR for <i>ef1-α</i>
ef1- α qPCR-R1	R: 5'-TTACGCTCAACCTTCCATCC-3'	qRT-PCR for <i>ef1-α</i>
vtgAo qPCR-F1	F: 5'-GGTCACTGCTGATTGGAAAC-3'	qRT-PCR for <i>vtgAo</i>
vtgAo qPCR-R1	R: 5'-TTCACGTACTGCTGGGATTG	qRT-PCR for <i>vtgAo</i>

B = C/G/T, H = A/T/C, K = G/T, M = A/C, R = A/G, S = C/G, V = A/C/G, W = A/T, Y = C/T.

nificance was evaluated by a bootstrap analysis (1000 replicates).

Synthesis of recombinant Vtg proteins and production of polyclonal antisera

Based on full-length *vtg* sequences, two pairs of specific primer (Table 2) were designed to amplify cDNA fragments from full-length cDNA templates of *vtgAo* (*vtg1*–6) and *vtgC* (*vtg7*); namely, one pair was designed to amplify a common sequence encoded by *vtg1*–6 (i.e., amino acid positions 151–330 in the deduced Vtg1 sequence; *vtg1* cDNA was used as the representative template of *vtg1*–6), while the other was designed to amplify a part of *vtgC* (i.e., amino acid positions 151–330 in the deduced VtgC sequence).

Recombinant partial Vtgs were produced using the following kits according to the manufacturer's protocols. The PCR products obtained above were isolated and ligated into pET302/NT-His (Invitrogen) vector using In-Fusion Advantage PCR Cloning Kit (Clontech) as fusion proteins with a C-terminal 6 × His tag to facilitate purification. Expression of recombinant proteins was induced in Rosetta-gami B strain of *E. coli* cells (Merck, Darmstadt, Germany). Purification of recombinant Vtg-His fusion proteins was carried out under denaturing conditions using His-Bind Resins (Novagen, San Diego, CA, USA).

Two New Zealand white rabbits were individually immunized by subcutaneous injection of each of the purified recombinant proteins (1 mg in total) emulsified in Freund's complete adjuvant (Merck) according to the method described in Amano et al. (2007).

Electrophoresis and immunobiochemical procedures

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). The sera of male and E2-treated fish were electrophoresed on 7.5% polyacrylamide gels. Gels were stained with 0.1% Coomassie Brilliant Blue R250 (CBB; Bio-Rad, Hercules, CA, USA). Western blotting was performed according to the method of Kwon et al. (1990) using the polyclonal Vtg antisera (1:2000 dilution) described above.

Quantitative realtime reverse transcription PCR for *vtg* mRNA expression

Primers used for qRT-PCR were designed based on the cloned *vtgAo* sequences using the Primer3Plus primer pick tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>), and are listed in Table 2. The primers for *vtgAo* were designed to target a common region found among all *vtgAo* sequences (i.e., the primer set amplifies total *vtgAo* expression).

qRT-PCR was done using Stepone Plus (Applied Biosystems) under the following conditions: 5 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl cDNA template in a reaction volume of 10 µl with primer concentration of 50 nM. Experimental liver samples were reverse transcribed as described above and applied to the assay. Beside these RT samples, total RNA preparations from the liver of reproductive female fish ($n = 10$) were pooled, reverse transcribed as described above, and used as an inter-assay control (IAC). A no-reverse transcript (NRT) control was also prepared through the reverse transcription reaction conducted without the addition of reverse transcriptase enzyme.

Each reaction plate also contained duplicate standard samples generated by a serial dilution of plasmid *vtgAo* and *ef1-α* cDNAs (10^1 – 10^6 copies) in order to provide copy numbers of the target gene (*vtgAo*) and the reference gene (*ef1-α*), respectively. Copy numbers of the target gene (*vtgAo*) were normalized to those of the reference gene (*ef1-α*). Results were then reported as a fold change in abundance relative to IAC values obtained in each reaction.

Statistical analysis

Data from the aqueous hormone exposure experiments were statistically analyzed using one-way ANOVA followed by Tukey's

honestly significant difference (HSD) test. Differences between two sampling periods (two days and seven days of exposure) within each treatment group were tested for statistical significance using the Student's *t*-test. Analyses were performed using the JMP program (SAS Institute Inc., Cary, NC, USA). Differences among dose groups were considered to be significant at $P < 0.05$. All samples were analyzed in duplicate and the results are expressed as mean $\pm$ standard error (SEM). Correlation analyses among biological parameters and *vtg* expression levels were performed using the Excel software package (Office 2010, Microsoft, Redmond, WA, USA).

RESULTS

Cloning and molecular characterization of *vtg* full-length cDNAs

In the present study, 32 random clones, which were assumed to contain full-length dojo loach *vtg* cDNAs, were selected and sequenced following the ligation and transformation of PCR products obtained using *vtgAo* primer sets (*vtgAo*-full primer-F1, *vtgAo*-full primer-R1, and *vtgAo*-full primer-R2). Twenty six clones could be grouped into six distinct *vtgs*. The most abundant clones ($n = 16$), herein temporarily named *vtg1* cDNA clone (4235 bp, GenBank accession number KF733650), consisted of a complete open reading frame (ORF: 4089 bp) encoding 1363 AA residues, with a 16 bp 5'-untranslated region (UTR) and a 130 bp 3' UTR. Other *vtg* clones (*vtg2*–6) also exhibited similar structural features as *vtg1*. In addition to these *vtg* clones, 16 random clones were selected and sequenced following the ligation and transformation of PCR products obtained using *vtgC* primer sets (*vtgC*-full primer-F1 and *vtgC*-full primer-R1), with the result that all of them appeared to encode a single *vtg* product (designated *vtg7*). The *vtg7* cDNA (to be renamed later as *vtgC*, 4448 bp, GenBank accession number KF733656) consisted of a complete ORF (3771 bp) encoding 1257 AA, with a 3 bp 5' UTR and a 674 bp 3' UTR. The deduced AA sequences of loach Vtg1 and Vtg7 exhibited theoretical molecular weights of 149 and 140 kDa, respectively. The overall primary structures of these two clearly distinct types of dojo loach Vtg (Vtg1 and Vtg7) are schematically represented in Fig. 1.

When the deduced AA sequences of loach Vtg were aligned, six clones (Vtg1–6) were 89–99% identical (Table

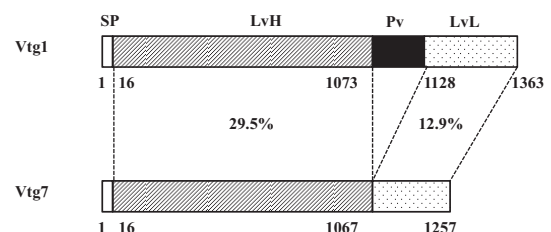


Fig. 1. Schematic alignment of deduced amino acid (AA) sequences of loach vitellogenin (Vtg). Two discrete Vtg representatives, Vtg1 and Vtg7, belong to VtgAo and VtgC, respectively (see more details in Discussion). The signal peptide (SP), lipovitellin heavy chain (LvH), phosvitin (Pv) and lipovitellin light chain (LvL) are represented by blank, lined, black and stippled rectangles, respectively. The numbers beneath the rectangles indicate the positions of AA residues at the boundary of each domain. The percent identity between the two sequences for two domains is also given.

3); these Vtgs were designated as type I Vtgs, while Vtg7 was designated as a type II Vtg. When the loach Vtg1 and Vtg7 were aligned with Vtgs of other teleosts, loach Vtg1 shared the closest identity with the Vtg1 of common carp (82%), while the loach Vtg7 most closely resembled zebrafish Vtg3 (77%). When the deduced AA sequences were characterized for type I Vtgs, they contained two major yolk protein domains (lipovitellin: Lv and phosvitin: Pv) but largely lacked the C-terminal domains (designated as C-terminal half) consisting of a portion of Lv light chain (LvL) and

the downstream sequences, β' -component and C-terminal peptide. In contrast, the type II Vtg lacked both Pv and the C-terminal half, consisting of Lv alone.

Phylogenetic analysis

In order to classify the loach Vtgs, a phylogenetic tree consisting of loach and vertebrate Vtg sequences was constructed using the Neighbor Joining method (Saitou and Nei, 1987). The phylogenetic analysis showed that loach Vtgs were clearly divided into two clusters, VtgAo and VtgC, according to the nomenclature of Finn and Kristoffersen (2007); six type I loach Vtgs (Vtg1–6) were closely related to a branch of VtgAo of Ostariophysi fish but formed an independent loach cluster, while the type II Vtg (Vtg7) was included in the teleost VtgC cluster, which also contained the zebrafish VtgC (Fig. 2). Based on the phylogenetic analysis, the loach Vtgs were hereby categorized as follows: type I Vtgs (Vtg1–6) appeared to be loach VtgAo paralogs, while the type II Vtg (Vtg7) appeared to be loach VtgC. The loach VtgAo paralogs were further characterized in their domain structures as such paralogous VtgAo in zebrafish were additionally classified into two types by Finn and Kristoffersen (2007). Because all loach VtgAo paralogs were found to lack a part of yolk protein domains locating at the C-terminal side, they were hereby classified into VtgAo1 type, but not VtgAo2 type, paralog.

Detection of multiple Vtgs in serum

Partial sequences encoding loach VtgAo1 and VtgC were subcloned into the expression vector and their corre-

Table 3. Amino acid sequence identities (%) of dojo loach vitellogenins (VtgAo paralogs).

	Vtg1	Vtg2	Vtg3	Vtg4	Vtg5	Vtg6
Vtg1	100					
Vtg2	89	100				
Vtg3	98	96	100			
Vtg4	91	93	95	100		
Vtg5	91	93	95	99	100	
Vtg6	91	92	95	99	98	100

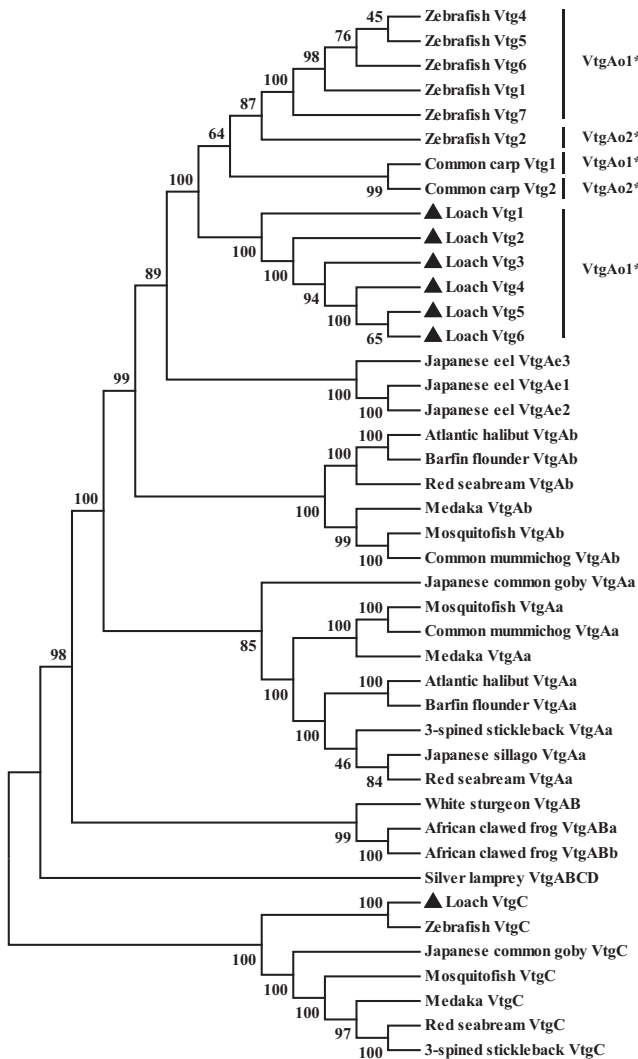


Fig. 2. Phylogenetic inference of the deduced amino acid sequences of vitellogenin (Vtg) in loach and various vertebrate species. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.05. The GenBank accession numbers used in the analysis are listed below: zebrafish (Vtg1, NP_001038362; Vtg2, NP_001038378; VtgC, AAG30407; Vtg4, NP_001038759; Vtg5, AAH97081; Vtg6, NP_001116082; Vtg7, NP_001096141), common carp (Vtg1, AAL07472; Vtg2, BAD51933), Japanese eel (VtgAe1, AAV48826; VtgAe2, AAR82899; VtgAe3, AAR82898), Atlantic halibut (VtgAa, ABQ58113; VtgAb, ABQ58114), barfin flounder (VtgAa, BAD93695; VtgAb, BAD93696), red seabream (VtgAa, BAE43870; VtgAb, BAE43871; VtgC, BAE43872), medaka (VtgAa, BAB79696; VtgAb, BAB79591; VtgC, ENSORLP00000008173), mosquitofish (VtgAa, BAD93697; VtgAb, BAD93698; VtgC, BAE43872), common mummichog (VtgAa, AAA93123; VtgAb, AAB17152), 3-spined stickleback (VtgAa, ENSGACP000000012923; VtgC, ENSGACP000000012536), Japanese silago (VtgAa, BAC20186), white sturgeon (VtgAB, Q90243), African clawed frog (VtgABa, NP_001152753; VtgABb, NP_001094404), silver lamprey (VtgABCD, AAA49327), and Japanese common goby (VtgAa, BAC06190; VtgC, BAC06191). Numbers next to the nodes represent bootstrap values (%). The asterisks indicate additional classification of VtgAo paralogs, which are referred to as VtgAo1 and VtgAo2 according to the nomenclature of Finn and Kristoffersen (2007).

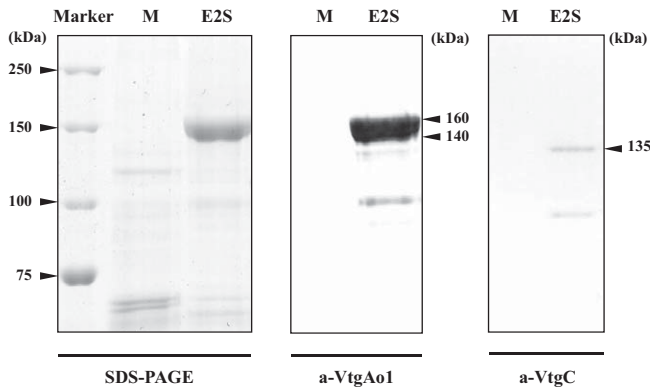


Fig. 3. Results of 7.5% SDS-PAGE and corresponding Western blotting of sera of loach using antisera obtained from immunized rabbits against recombinant loach VtgAo1 (a-VtgAo1) and VtgC (a-VtgC). The sera were obtained from male loach (M) and 17 β -estradiol-treated fish (E2S). Numbers at the right side of the blot represent the relative molecular mass (kDa) of the corresponding immunoreactive bands (i.e., putative loach Vtgs).

sponding recombinant Vtg proteins were generated and purified (data not shown). Subsequently, VtgAo1 and VtgC were independently immunized into rabbit to make specific antisera (designated here as a-VtgAo1 and a-VtgC, respectively). These antisera were used in Western blotting. Multiple Vtg proteins were identified by Western blotting in the serum of E2-treated loach using a-VtgAo1 and a-VtgC (Fig. 3). Each antiserum appeared to detect each target Vtg in the serum of E2-treated fish, but did not react with male serum proteins; a broad band of size ~140 kDa to ~160 kDa reacted with a-VtgAo1, while a minor band of ~135 kDa reacted with a-VtgC. Both antisera reacted with a few smaller minor bands that perhaps were degraded products of Vtg monomers.

Effects of aqueous exposure to 17 α -ethinylestradiol and 17 β -estradiol

Induction rates of *vtgAo1* mRNA

No *vtgAo1* mRNA was found in the initial group and control group up to two days post initiation of the exposure experiment (2 dpi), while 80% and 40% of the solvent control fish exhibited a trace level of *vtgAo1* expression when E2 and EE2 exposure experiments were separately conducted for seven days (i.e., at 7 dpi), respectively (Fig. 4).

When male loach were exposed to EE2 for two days, fish of 1 ng/L group and 10 ng/L group showed *vtgAo1* induction at rates of 20% and 80%, respectively (Fig. 4A). The period of exposure appeared to affect the lowest-observed-effect concentration (LOEC, i.e., the lowest concentration at which all tested individuals in the group exhibited positive *vtgAo1* induction); in this study, the LOEC for EE2 in loach *vtgAo1* expression was 100 ng/L at 2 dpi; whereas it was 10 ng/L at 7 dpi.

At 2 dpi of E2 exposure (Fig. 4B), 20–40% of the 1–100 ng/L group exhibited *vtgAo1* mRNA expression. At 7 dpi of E2 exposure, the induction rates were within 0–40% in 1 ng/L and 10 ng/L groups. The exposure time appeared to affect the LOECs for E2 exposure in the loach *vtgAo1* expression; LOECs were 1000 and 100 ng/L at 2 and 7 dpi, respectively.

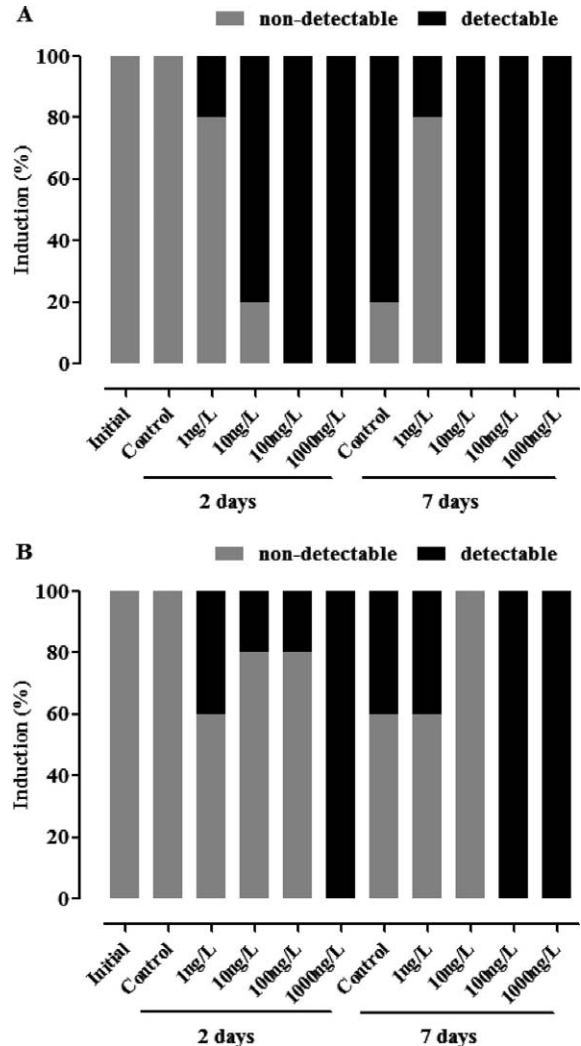


Fig. 4. Induction rates of male loach (percent *vtg* mRNA positive individuals) expressing vitellogenin mRNA during aqueous estrogen exposure experiments. Male loach were sampled before (Initial) or after exposure (two and seven days post initiation of the exposure) to rearing water supplemented with different concentrations (1–1000 ng/L) of 17 α -ethinylestradiol (A) or 17 β -estradiol (B), or without hormone (Control). The induction rates in individuals of *vtgAo1* transcripts were detectable and non-detectable are marked by grey and black boxes, respectively.

Expression levels of *vtgAo1* mRNA

At 2 dpi of EE2 exposure (Fig. 5A, grey columns), *vtgAo1* mRNA expressions significantly increased between 100 and 1000 ng/L groups. The highest expression of *vtgAo1* mRNA was found at 1000 ng/L group; the average expression level of 1000 ng/L groups was 6.4-fold higher than that of the 100 ng/L group.

At 7 dpi of EE2 exposure (Fig. 5A, black solid columns), significant and dose-dependent increases of *vtgAo1* mRNA expression were found among the 10, 100, and 1000 ng/L groups (Fig. 5B). In comparison with the average expression level of the 10 ng/L group, *vtgAo1* expression was 53.2-fold and 108.6-fold higher in the 100 and 1000 ng/L groups, respectively.

Time-dependent increases (between 2 dpi and 7 dpi) in

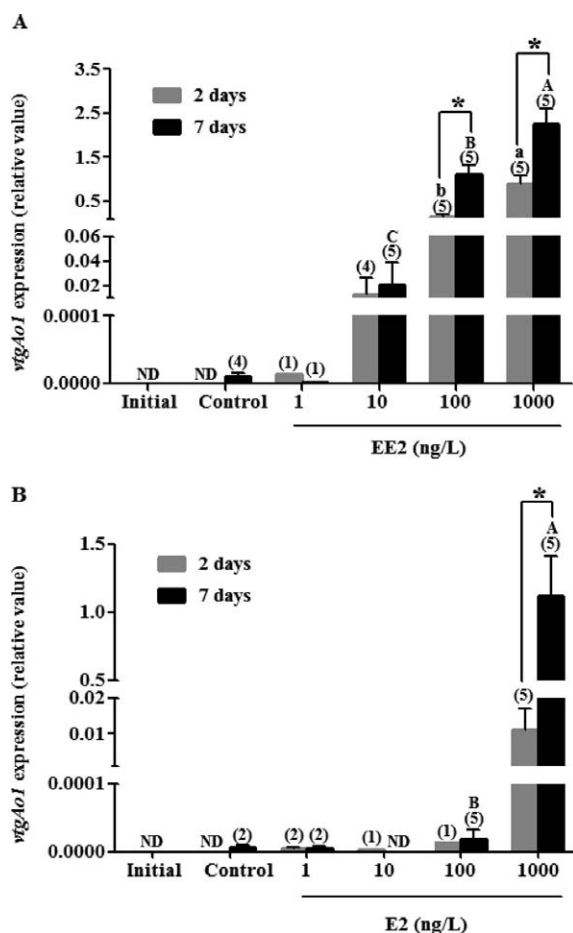


Fig. 5. Effects of aqueous exposure of 17 α -ethinylestradiol (EE2, panel A) and 17 β -estradiol (E2, panel B) on the hepatic expression of vitellogenin (*vtg*) transcripts in male loach. The experimental fish were sampled as described in Fig. 4. Expression levels of total *vtgAo1* transcripts were quantified and expressed as relative values (see Materials and Methods for details). Error bars indicate standard error ($n = 5$). ND indicates that all test fish sampled at the corresponding sampling points were negative (non-detectable) for the induction of *vtgAo1* transcripts. Number(s) of fish that exhibited detectable levels of *vtgAo1* transcripts are indicated in parentheses. Mean values bearing different letter superscripts are significantly different ($P < 0.05$) between different doses in the same period. Significant differences between the same exposure dose in the different periods are indicated by an asterisk ($P < 0.05$).

the expression levels of *vtgAo1* mRNA were also statistically significant when they were exposed to 100 and 1000 ng/L EE2.

At 7 dpi of E2 exposure, the *vtgAo1* mRNA expressions appeared to be dose-dependent between the 100 and 1000 ng/L groups (Fig. 5B, black solid columns). The average level of *vtgAo1* mRNA of 1000 ng/L group was 60786.9-fold higher than that of the 100 ng/L group. Time-dependent increases (between 2 and 7 dpi) in *vtgAo1* mRNA was also statistically significant in the 1000 ng/L group.

Relationship between *vtgAo1* mRNA and other factors

At 2 and 7 dpi of EE2 exposure, positive, but weak, correlation ($R^2 = 0.3312$ and 0.5547 , respectively) was

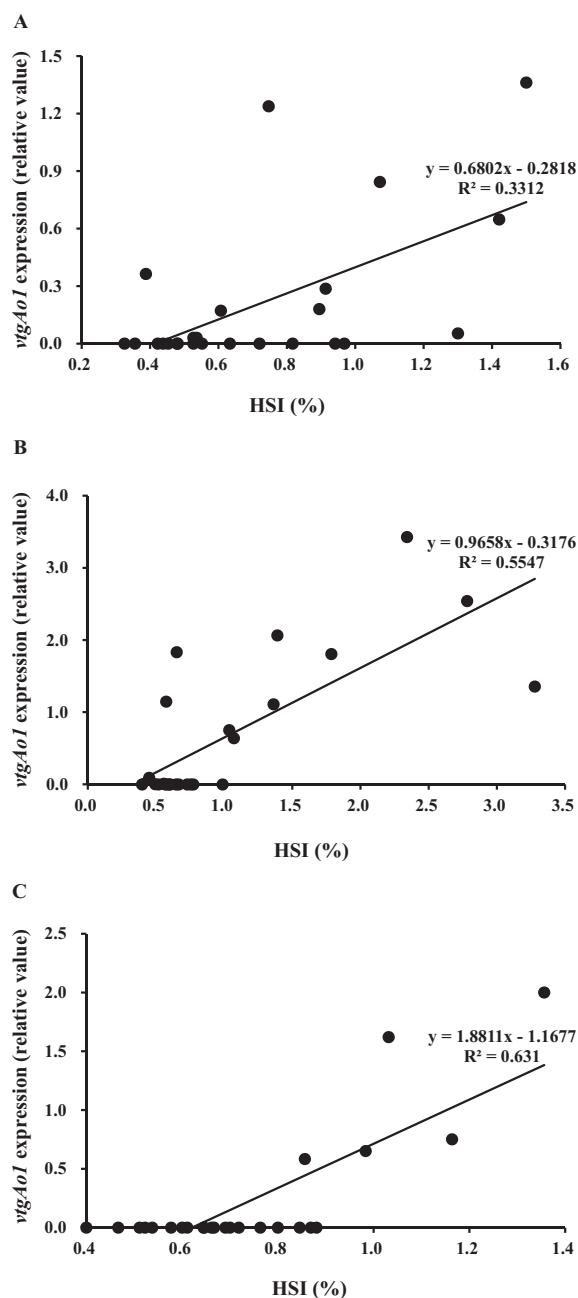


Fig. 6. Relationship between hepatic expression of vitellogenin (*vtg*) transcripts and hepatosomatic index (HSI). (A) 17 α -ethinylestradiol (EE2) exposure for two days; (B) EE2 exposure for 7 days; (C) 17 β -estradiol (E2) exposure for seven days. The data sets of this analysis for total *vtgAo1* levels and HSI were the same as those used for the exposure experiment (Fig. 5 and Table 1).

observed between *vtgAo1* levels and HSI (Fig. 6A, B); while no clear relationship could be observed between *vtgAo1* levels and GSI (data not shown).

At 2 dpi of E2 exposure, no significant correlation between *vtgAo1* levels and HSI was found (data not shown); while a weak but positive linear relationship ($R^2 = 0.631$) was seen at 7 dpi of E2 exposure (Fig. 6C). There was no significant correlation between *vtgAo1* levels and GSI throughout the exposure experiments.

DISCUSSION

EEDCs have recently been detected in aquatic environments and the possible adverse effects of these chemicals on aquatic organisms recognized (Jobling et al., 1998; Hashimoto et al., 2000; Gercken and Sordyl, 2002; Kavanagh et al., 2004). Among the compounds of the EEDCs, E2 is the most potent and biologically active estrogen synthesized in the ovaries. EE2, a synthetic analog of E2 that is widely used in oral contraceptives, enters the aquatic ecosystem through discharges from domestic sewage effluents and disposal of animal waste (Andersson and Skakkeak, 1999; Beardmore et al., 2001). In addition, the consumption of estrogens and progestogens (natural and synthetic) in human medicine and animal farming has increased markedly (Ying et al., 2002; Matozzo et al., 2008). Concentrations of E2 and EE2 reported in the effluents from sewage treatment works range up to tens of ng/L (Routledge et al., 1998; Baronti et al., 2000; Ying et al., 2002; Zhou et al., 2011). Based on this information, EE2 and E2 were chosen as model EEDCs in this study; the nominal exposure concentrations of these chemicals were determined to be in a range of 1–1000 ng/L, which can be expected in actual field surveys.

Vtg mRNA and protein in male fish are frequently used as biomarkers for screening of EEDCs in environmental monitoring programs (Sumpter, 2005; Matozzo et al., 2008). Test organisms commonly include rainbow trout (*Oncorhynchus mykiss*), flounder (*Pleuronectes yokohamae*), and grey mullet, among others (Sumpter and Jobling, 1995; Harries et al., 1997; Hashimoto et al., 2000; Aoki et al., 2010). However, one of the main disadvantages associated with their use as test organisms is the high mobility associated with feeding and reproductive migration and the limited regional distribution of several species. This may explain why biomarker induction has yielded no results in some contaminated areas. The ideal candidate organisms are residents with a wide geographical distribution. In this regard, the dojo loach appears to be a good test species.

As little is known about Vtg in the dojo loach, we cloned and characterized cDNAs encoding seven distinct full-length Vtgs. Multiple Vtg transcripts have been detected in most oviparous vertebrates. Among Cypriniformes, zebrafish possesses at least six forms of VtgAo paralogs other than VtgC; the one (termed VtgAo2 by Finn and Kristoffersen, 2007; GenBank accession number NP_001038378) contains the full suite of YP domains (i.e., designated as complete Vtg), and all the others (termed VtgAo1: GenBank accession number NP_001038362, GenBank accession number NP_001038759, GenBank accession number AAH97081, GenBank accession number NP_001116082, GenBank accession number NP_001096141) lack a portion of LVL and the C-terminal half domain (Wang et al., 2005). Similarly, The Vtg2 (i.e., VtgAo2) of common carp (*Cyprinus carpio*) comprise a full complement of YP domains (GenBank accession number BAD51933), while Vtg1 (i.e., VtgAo1) exhibits the truncated yolk protein domain (GenBank accession number AAL07472) (Kang et al., 2007). These previous studies have indicated that Cypriniformes have the complete VtgAo paralogs (VtgAo2) as well as the truncated VtgAo paralogs (VtgAo1), in addition to VtgC. However, we only

found the truncated VtgAo paralogs (i.e., VtgAo1) in the loach. Our results also confirmed that a-VtgAo1 recognizes the ~160–140 kDa band in a Western blot; the band is clearly the dominant Vtg protein in the E2-treated serum. This indicates that the truncated VtgAo1 is the dominant Vtg in this species, not the complete VtgAo2 or VtgC.

Multiple Vtg proteins were detected by Western blotting in the serum of E2-treated loach using a-VtgAo1 and a-VtgC (Fig. 3). Each antiserum appeared to be specific to the targeted Vtg subtypes, as indicated by a good agreement between the size of immunoreactive bands (~160–140 kDa of VtgAo1, ~135 kDa of VtgC), and the corresponding mass expected from their primary structures (149 kDa of VtgAo1, 140 kDa of VtgC). The truncated VtgAo1 proteins appeared to be highly expressed, whereas VtgC appeared to be the minor Vtg component. Previous reports indicated that the carp Vtg consisted of two polypeptides separated by SDS-PAGE. Western blot analysis using specific Vtg antisera demonstrated that a highly expressed or prominent polypeptide (~160 kDa) and a less expressed, minor polypeptide (~180 kDa) were detected in the sera of females and E2-treated males (Kang et al., 2007). Truncated *vtgAo1* were also detected as the major *vtg* transcripts in other fish of Cyprinidae, including zebrafish and fathead minnow (*Pimephales promelas*) (Wang et al., 2005; Miracle et al., 2006). These results, in conjunction with results obtained in the present study, reveal that truncated VtgAo1 is likely to be the major Vtg type in Cypriniformes. Thus far, this study considered VtgAo1, rather than VtgC, to be a potential candidate as an estrogen-inducible marker in loach since its expression levels are much higher than those of VtgC in this species.

In the present study, the major *vtg* (*vtgAo1*) mRNAs were inducible within a week in male loach following aqueous exposure to EE2 and E2. This indicates that the loach promises to be a good model to test by means of the bioassay of aqueous EEDC contamination since aqueous exposure is probably the most realistic route to simulate the environmental conditions (Yokota et al., 2001a, b; Seki et al., 2003a, b). Hepatic *vtg* mRNA expression can be more effective and immediate indicator than the circulating Vtg protein to represent current or recent EEDC exposure, due to its rapid disappearance after the removal of an estrogenic stimulus and quick appearance upon presenting the stimulus (Hemmer et al., 2002; Scholz et al., 2004). Previous studies with E2 treatment have shown that a clear elevation of plasma Vtg levels in male loach could be found between 100 and 1000 ng E2/L at 7 dpi of the exposure (Lv et al., 2007); the LOEC was 1000 ng E2/L in this previous study. In the present study, the LOEC of E2 exposure on *vtgAo1* expression was confirmed to be 100 ng/L when the exposure was for seven days. These results suggest that hepatic *vtgAo1* mRNA can be more sensitive than plasma Vtg when they are used in the assay of chemicals with estrogenic activities. In addition, the expression profiles of *vtgAo1* mRNA in the male loach exhibited a dose-dependent and time-dependent increase following exposure to both estrogens used in this study. Thus far, induction profiles described above on loach *vtgAo1* transcripts have been confirmed to be appropriate for the future use in the studies on EEDCs.

In general, EE2 is known to be more potent than E2 in

terms of estrogenic activity (Legler et al., 2002; Muncke et al., 2007). Rose et al. (2002) observed that Vtg induction (LOEC) occurred in whole body homogenates of zebrafish at actual concentrations of 21 ng E2/L and 3.0 ng EE2/L when aqueous exposure experiments were conducted for 8 days. Thomas-Jones et al. (2003) reported that the LOEC on the induction of *vtg* mRNA expression in the juvenile female rainbow trout were 14 ng E2/L and 1 ng EE2/L at 14 dpi of the exposure. Such tendencies were also confirmed in the present study in terms of the appearance rates of *vtg*-positive males, as well as for the LOEC and the induced *vtg* levels. Specifically, the LOECs for EE2 were 100 ng/L at 2 dpi and 10 ng/L at 7 dpi, respectively (Fig. 5A). On the other hand, those for E2 were higher by one order of magnitude, i.e. 1000 ng/L at 2 dpi and 100 ng/L at 7 dpi (Fig. 5B). At the highest exposure (1000 ng/L), EE2 caused hepatic *vtgAo1* mRNA expression to increase 2.0–80.5-fold when compared to E2. At 7 dpi of exposure to a dose of 100 ng/L, the expression level of *vtgAo1* mRNA increased by EE2 was 59393.5-fold the detection of E2. These results represent the effectiveness of the bioassay utilizing loach *vtgAo1*.

The occurrence of *vtgAo1* transcript in EE2-exposed group ($n = 30$ of 40 individuals; 75% total) was more frequently observed than that in E2-exposed group ($n = 21$; 52.5%) in the aggregate (Fig. 4A, B). As expected, the longer exposure periods appeared to lower the threshold concentration that gives rise to 100% in the appearance rate of *vtgAo1* positive males (i.e., LOEC). In both cases, the LOECs of EE2 and E2 were 100 and 1000 ng/L at 2 dpi, respectively, whereas they were 10 and 100 ng/L, respectively, at 7 dpi (Fig. 5A, B). These results suggest that the abnormal induction of *vtgAo1* mRNA in males could be stabilized by the very low concentration of the exogenous estrogenic chemicals during a long exposure period. The LOEC of E2 for the induction of *vtg* mRNA in zebrafish and medaka (*Oryzias latipes*) exposed for two days were 1000 and 100 ng/L, respectively (Tong et al., 2004). The expression of *vtg* in juvenile mud carp (*Cirrhinus molitorella*) significantly increased by exposing E2 at the concentration of 50 and 500 ng/L for 2 days (Liang and Fang, 2012). These results suggested that the sensitivity of dojo loach to the estrogen-responsible *vtg* induction is similar to that of other freshwater fishes.

A possible, albeit weak, effect of hormone solvent (i.e., ethanol, with a final concentration of 0.01% in this study) on hepatic *vtgAo1* mRNA synthesis was observed at 7 dpi on some individuals in this study (Fig. 4A, B). Harris et al. (2001) also indicated that plasma Vtg concentrations were elevated in methanol-exposed (0.002%) rainbow trout. However, Panter et al. (2002) demonstrated that methanol at 100 μ L/L (the same concentration as in the present study) had no effect on *vtg* levels in male fathead minnow after 21 days. It is thus likely that the effect of solvent control (e.g., ethanol) on hepatic *vtg* production is limited in a trace level and varies across and even within species when it is occurred. Other factors than hormone solvent, such as the stress of being reared in a small glass tank, might be a possible cause of the trace level of *vtg* induction (Ding et al., 1994).

The present study revealed that the loach from the EE2 and E2 exposure groups increased the hepatic *vtgAo1*

expression and HSI (as shown in Table 2, Fig. 6). Similarly, studies with male Japanese medaka also reported a significantly greater HSI in response to treatment with 500 ng EE2/L (Zhang et al., 2008). In contrast, no significant relationship between *vtgAo1* mRNA expression and GSI was observed in the present study, although an inverse correlation between Vtg and GSI was reported in adult male roach (*Rutilus rutilus*) (Jobling et al., 1998). In addition, a reduction of GSI in male zebrafish was found at 24 dpi of EE2 exposure (Van den Belt et al., 2002). These altered gonadal features might be obtained by exposure times of longer than seven days in the loach; such effects of estrogenic exposure on the gonads remain to be verified in the present study.

In summary, full length cDNAs encoding seven distinct Vtgs were isolated and characterized in the dojo loach. Phylogenetic analysis classified them into two types, type I (VtgAo1) and type II (VtgC). Immunobiochemical analysis, as well as molecular cloning suggested that *vtgAo1* transcripts and VtgAo1 protein could be found as the major truncated Vtg type in the liver and the circulation, respectively, of the loach. Therefore, VtgAo1 rather than VtgC can be used as an adequate marker in response to estrogenic stimulations. Furthermore, significant expression of loach *vtgAo1* mRNA was induced in time- and dose-dependent manners by exposing male fish to EE2 and E2. Patterns of occurrence and LOECs of *vtgAo1* expression determined for both chemicals appeared to be similar to what have been reported for other model fish species. These results suggest that the dojo loach has the potential to be a test model for monitoring the estrogenic activities in the aquatic fresh water environments across East Asian countries by utilizing Vtg of this species as the marker of estrogenic exposure.

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