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Genomic organization and transcriptional modulation in response to endocrine disrupting chemicals of three vitellogenin genes in the self-fertilizing fish *Kryptolebias marmoratus*

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ABSTRACT

Vitellogenin (Vtg) is the precursor of egg yolk proteins, and its expression has been used as a reliable biomarker for estrogenic contamination in the aquatic environment. To examine the biomarker potential of the self-fertilizing killifish *Kryptolebias marmoratus Vtgs* (Km-Vtgs), full genomic DNAs of *Km-Vtgs-Aa*, *Km-Vtgs-Ab*, and *Km-Vtgs-C* were cloned, sequenced, and characterized. Three *Vtg* genes in *K. marmoratus* are tandemly placed in a 550 kb section of the same chromosome. *In silico* analysis of promoter regions revealed that both the *Km-Vtgs-Aa* and *Km-Vtgs-Ab* genes had an estrogen response element (ERE), but the *Km-Vtgs-C* gene did not. However, all three *Km-Vtgs* genes had several ERE-half sites in their promoter regions. Phylogenetic analysis demonstrated that the three deduced amino acid residues were highly conserved with conventional Vtgs protein, forming distinctive clades within teleost Vtgs. Liver tissue showed the highest expression of *Km-Vtg* transcripts in all tested tissues (brain/pituitary, eye, gonad, intestine, skin, and muscle) in response to endocrine disrupting chemical (EDC)-exposed conditions. *Km-Vtg* transcripts were significantly increased in response to 17β-estradiol (E2), tamoxifen (TMX), 4-n-nonylphenol (NP), bisphenol A (BPA), and octylphenol (OP) over 24 hr exposure. The *Km-Vtg-A* gene was highly expressed compared to the control in response to NP and OP. EDC-induced modulatory patterns of *Km-Vtg* gene expression were different depending on tissue, gender, and isoforms.

Introduction

In oviparous oogenesis, incorporation of yolk materials (*e.g.*, vitellogenin) into oocytes and mobilization of these substances during embryogenesis are crucial processes for oocyte development and successful reproduction (Arukwe and Goksøyr, 2003). Vitellogenin (Vtg), the precursor of egg yolk protein, is synthesized by endogenous estrogen regulation in the hepatocytes of oviparous vertebrates (Mommsen and Walsh, 1988). Vtg synthesis is mainly controlled by estrogen, but it can also be regulated by other hormones (Lubzens et al., 2010). In teleosts, the liver tissue may not be the only source for circulating plasma Vtgs, as extra-hepatic expression of *Vtg* genes has been detected in several tissues (Islinger et al., 2003;

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Wang et al., 2005, 2010; Ma et al., 2009; Tingaud-Sequeira et al., 2012; Zhong et al., 2014). Vtg is secreted into the bloodstream and incorporated into the growing oocytes to serve as a nutritional source for developing embryos and larvae (Lubzens et al., 2010). Previous complementary deoxyribonucleic acid (cDNA) cloning and phylogenetic analyses revealed that teleost Vtgs have undergone lineage-specific gene duplication resulting in multiple types of *Vtg* paralogous genes (Finn and Kristofferson, 2007).

Induction of transcription and/or translation of the *Vtg* gene is widely used as a strong biomarker for endocrine disrupting chemicals (EDCs) (Sumpter and Jobling, 1995; Marin and Matozzo, 2004). Of the diverse xenoestrogens, nonylphenol (NP), octylphenol (OP), and bisphenol A (BPA) are characterized as strong estrogen agonists with estrogenic mimicking actions in binding to the estrogen receptors (ERs), even though these compounds differ slightly structurally from endogenous estrogens (Soto et al., 1991; Krishnan et al., 1993; Arnold et al., 1996; Laws et al., 2000). As Vtg synthesis is under E2 control, efforts to determine the potential effect of EDCs have been expanded to monitor for modulating vitellogenesis.

The self-fertilizing mangrove killifish *Kryptolebias marmoratus* (formerly, known as *Rivulus marmoratus*) is a useful oviparous teleost for diverse experimental studies, as it is relatively small in size (3–5 cm in adult), has a short generation time (3–4 months), and is easily maintained under laboratory conditions (Lee et al., 2008). Their unique reproductive system offers fish biologists the opportunity to study sex determination and differentiation (Soto et al., 1992; Cole and Noakes, 1997). As an androdioecious species, pure females do not exist in this species and greater than 60% of the hermaphrodites transform into secondary males by ovarian atresia in 3 to 4 years after hatching (Harrington, 1967). The hermaphrodites have a marbled brownish color pattern with a caudal peduncle ocellus, while males produce only sperm and have an orange-pink body color with humeral mottling splotch with a caudal peduncle ocellus that is variably faint or absent (Lee et al., 2008). Environmental factors such as low temperature (18–20°C) and daily light control the hermaphrodite to secondary male sex ratio (Harrington, 1967). Primary male can be induced by temperature alteration at the final stages of embryonic development in a laboratory, but artificially induced primary males showed high mortality and abnormality (Harrington, 1967). The gonad (ovotestis) of *K. marmoratus* is composed of ovary-like oogenic and spermatogenic tissues that are not distinctly separated by membrane (Sakakura et al., 2006). Although diverse approaches have been conducted to understand the underlying endocrine regulatory metabolism of *K. marmoratus* (Kanamori et al., 2006), mechanism of sex determination and differentiation mostly remains unknown.

In this study, three *Km-Vtg* genes were identified and characterized with cloning, sequencing, and phylogenetic analysis. Even though one *Vtg* gene (analyzed as *Vtg-Aa* in this study) was cloned in our previous study (Kim et al., 2004), to date the characterization and gene expression profiles of *K. marmoratus Vtgs* (*Km-Vtgs*) have not been studied in response to EDCs. To evaluate their transcriptional sensitivities in response to EDC exposure, transcriptional expression levels were analyzed in different tissues in EDC-exposed hermaphrodite and male *K. marmoratus*. Our results facilitate a better understanding of the molecular mode of action and the effects of estrogenic chemicals on vitellogenesis of *K. marmoratus*.

1. Material and methods

Detailed description on the fish culture condition, *in silico* analysis, and methods for molecular and biochemical techniques were incorporated in the supplemental file as described in our previous studies (Rhee et al., 2011).

1.1. Cloning of three forms of vitellogenin cDNAs

For cloning of partial *vitellogenin (Vtg)* cDNA fragments, we performed ClustalW analysis with multiple alignments including full-length *Vtg* cDNAs across other fish species and designed degenerate oligonucleotides from highly conserved regions. To clone partial cDNA sequence for each *Vtg* gene, hermaphrodite was sacrificed in ice and its liver tissue was used to synthesize cDNA in accordance with the guidelines of the Animal Welfare Ethical Committee of Sungkyunkwan University. Due to their long nucleotide lengths, multiple sets of degenerate primer were used for cloning each *Vtg* cDNA (Suppl. Fig. S1A). These primers were used to amplify the corresponding cDNAs from hermaphrodite liver cDNA. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 2 μM of each primer (Table S1) under the following conditions: 95°C/4 min; 40 cycles of 98°C/25 sec, 55°C/40 sec, 72°C/60 sec; and 72°C/10 min. Same annealing temperature was used for all primer pairs. The amplified PCR products were isolated from 1% agarose gels, cloned into pCR2.1 TA vectors (Invitrogen, Carlsbad, CA, USA), and sequenced using an ABI 3700 DNA analyzer (Bionics Co., Seoul, South Korea).

1.2. Tissue-preferential mRNA (messenger ribonucleic acid) expression

The expression pattern of *Km-Vtg* mRNAs was studied in seven different tissues (brain/pituitary, eye, gonad, intestine, liver, muscle, and skin) from hermaphrodites and secondary males using real-time RT-PCR with primers (Table S1). Thirty hermaphrodites or secondary males were separated into three groups. Each group was comprised of 10 fish, and three technical replicates of real-time RT-PCR experiments were performed for each pooled tissue.

1.3. Endocrine disrupting chemical (EDC) exposure

To study the effect of EDC exposure on *Km-Vtg* mRNA expressions, adult hermaphrodites and secondary males were exposed to EDCs. Both hermaphrodites and secondary males (length ≈ 3.5 cm) were exposed in separate tanks in an aqueous static renewal culture system. The dimethyl sulfoxide (DMSO) concentration in control and treated groups was maintained at a concentration of less than 0.01%. Thirty hermaphrodites or secondary males were separated into three groups as biological triplicate. Ten fish in each

treatment group were exposed to 17β-estradiol (E_2, 100 ng/L for 96 hr), a known natural estrogen, and tamoxifen (10 μg/L), a nonsteroidal estrogen antagonist, for 96 hr. All tissues used in this study were pooled from 10 fish for analyzing transcript profile. E_2 and tamoxifen concentrations were chosen from previous studies in *K. marmoratus* and other fish (Lerner et al., 2007; Rhee et al., 2009, 2011; van der Ven et al., 2007). The water of treated and control groups was replaced with freshly prepared water every 24 hr. After exposure for 96 hr, fish were dissected, and the different organs were pooled and used for total RNA preparation.

In a time-series experiment, the transcriptional expression of three *Km-Vtg* genes was measured in the liver of hermaphroditic fish. As we described the biomarker potential of *Vtg* genes *via* their rapid induction in a short time period, we decided to analyze the mRNA expressions of *Km-Vtg* gene for 24 hr. Test of *Vtg* inducibility in the secondary males was excluded as the number of secondary male was not enough to validate statistics. In general, ovary atresia would take a long time, and to confirm a pink transitioning secondary male, the fish would have to be sacrificed for gonadal histology. Thirty hermaphroditic fish were separated into three groups as biological triplicate. Each group was comprised of 10 fish. They were placed in a glass tank and exposed for 24 hr to each EDC: linear 4-n-nonylphenol (NP, 300 μg/L), 4-*tert*-octylphenol (OP, 300 μg/L), and bisphenol A (BPA, 600 μg/L). The concentrations of EDCs used for exposure are based on our previous gene expression studies in *K. marmoratus* (Rhee et al., 2009, 2011) and that of Tanaka and Grizzle (2002). After exposure for 24 hr, fish were dissected, and the liver tissues were pooled and used for total RNA preparation.

1.4. Statistical analyses

The SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) software package was used for statistical analyses. Data are expressed as means ± S.D. (standard deviation). Significant differences between control and exposed groups were analyzed using one-way or multiple-comparison analyses of variance (ANOVAs) followed by Tukey's tests. Differences with $P < 0.05$ were considered significant.

2. Results

2.1. Km-Vtg genes

Km-Vtg cDNA information was submitted to GenBank with the accession nos. AAQ16635 (*Km-Vtg-Aa*), KJ917549 (*Km-Vtg-Ab*), and AGA82747 (*Km-Vtg-C*), respectively. In our previous research, we reported solely on one vitellogenin (*Km-Vit*) (AY279214) gene (Kim et al., 2004), but in this study, we confirmed the placement of this *Km-Vit* gene with two *Km-Vtg* genes by phylogenetic analysis. The *Km-Vit* sequence was annotated as *Km-Vtg-Aa*. *Km-Vtg* genomic clones are located in a single scaffold (#Km-0026) (Fig. 1a).

The complete cDNA sequence of *Km-Vtg-Aa* is 5229 bp in length, including a 59 bp 5′-untranslated region (UTR), a 5133 bp open reading frame (ORF), and an 88 bp 3′-UTR with a poly (A) tail (Fig. S2A). The promoter region contains one putative estrogen responsive element (ERE) and four putative ERE-half sites (Fig. 1b). The ORF encodes a polypeptide of 1711 amino acids. The predicted molecular weight and theoretical

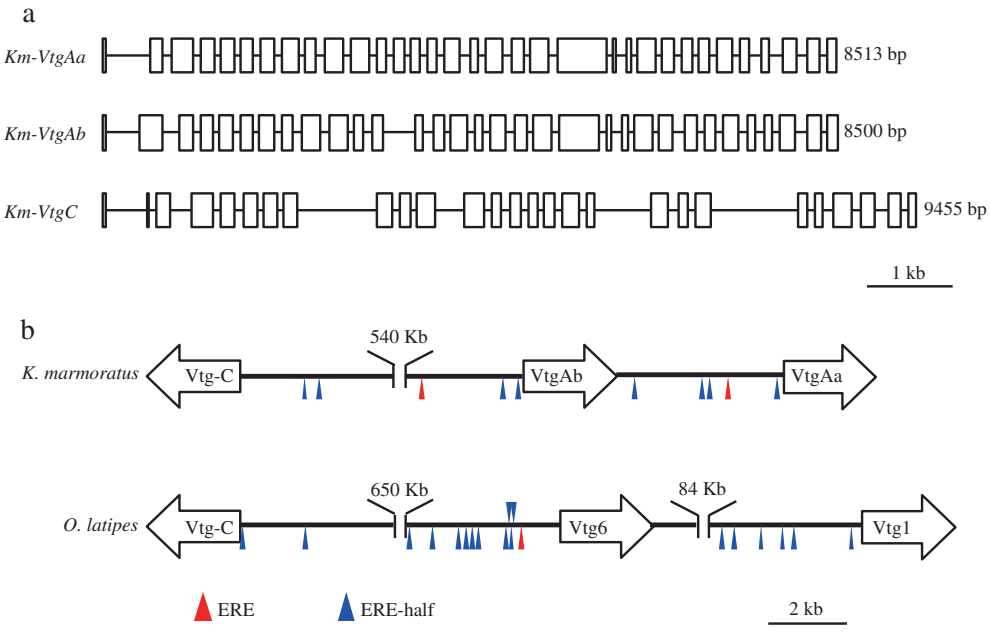

Fig. 1 – (a) Schematic diagram of the genomic structure of *K. marmoratus* vitellogenin genes. Box represents exon and is drawn to scale. Black line represents introns and is shown at scale. Each length is indicated in the number of nucleotides, (b) genomics organization and putative estrogen responsive elements (EREs) analyzed in each vitellogenin gene of *K. marmoratus* and Japanese medaka. Putative ERE and ERE-half sites are marked with red and blue inverted triangles, respectively.

pI of Km-Vtg-Aa protein were calculated to be 189 kDa and 9.24, respectively.

The *Km-Vtg-Ab* gene is 5201 bp in length including a 5100 bp ORF (Fig. S2B). One putative ERE and two putative ERE-half sites were observed in the promoter region of the *Km-Vtg-Ab* gene (Fig. 1b). The ORF encodes a polypeptide of 1700 amino acids with a pI value of 8.9 and a molecular weight of 188 kDa. The full-length *Km-Vtg-C* contained a 3771 bp coding region for 1257 amino acid residues with a molecular weight of 142 kDa and pI of 5.72 (Fig. S2C). Three putative ERE-half sites were identified in the promoter region of the *Km-Vtg-C* gene (Fig. 1b). The similarity of the Km-Vtg-C protein to both Km-Vtg-Aa and Km-Vtg-Ab was high in the N-termini but low at the C-termini, as the Km-Vtg-C protein lacks the polyserine phosvitin region, even though it shared the highly conserved lipovitellin I and II regions (Fig. S3; lipovitellin domains marked with blue vertical bars) with the two *Vtg* sequences (Km-Vtg-Aa and Km-Vtg-Ab) (Fig. S3; phosvitin region marked with a red bar). In addition, phylogenetic analysis showed that Km-Vtg proteins were separated into three groups, Vitellogenin Aa, Ab, and C, which form distinctive clades in fishes (Fig. S4).

2.2. Tissue-preferential mRNA expression

All three *Km-Vtg* transcripts were abundantly expressed in the liver tissues of both hermaphrodite and secondary males, as well as in the gonads and intestines (Fig. 2), but the expression patterns were different for each gene. Generally, the hermaphroditic adult fish expressed all *Vtg* genes more than the secondary males (ovary atresia stage). The expression patterns of *Km-Vtg-Aa* and *Km-Vtg-Ab* were similar, and their patterns were distinctive compared to those of *Km-Vtg-C* gene. In the case of *Km-Vtg-Aa* and *Km-Vtg-Ab*, extrahepatic mRNA expressions were observed in the brain/pituitary, gonad, and intestine, while the *Km-Vtg-C* gene was expressed dominantly in liver tissues of both sexes, but low expression levels also detected in the gonad and intestine (Fig. 2).

2.3. Induced transcriptional modulation of Km-Vtg genes in different tissues

E2-exposed fish showed significant upregulation of all *Km-Vtg* transcripts in the liver tissues of both hermaphrodites and secondary males (Fig. 3). The *Km-Vtg-Ab* transcript was slightly increased in the gonad tissue from hermaphrodites (Fig. 3) compared with secondary males. In E2-exposed hermaphrodites and secondary males, *Km-Vtg-C* gene expression in gonad ($P < 0.05$) and intestine ($P < 0.05$) tissues was significantly elevated compared to the control group (Fig. 3). Tamoxifen (10 μg/L), an E2 antagonist, also upregulated transcriptional levels of three *Vtgs* genes in liver tissues of both *K. marmoratus* genders ($P < 0.05$).

2.4. EDC-induced transcriptional modulation of Km-Vtg genes in the liver tissues of hermaphrodites

All tested EDCs caused upregulation of the three *Km-Vtg* transcripts in liver tissues from hermaphrodites after exposure for 24 hr (Fig. 4). Interestingly, the expression patterns of

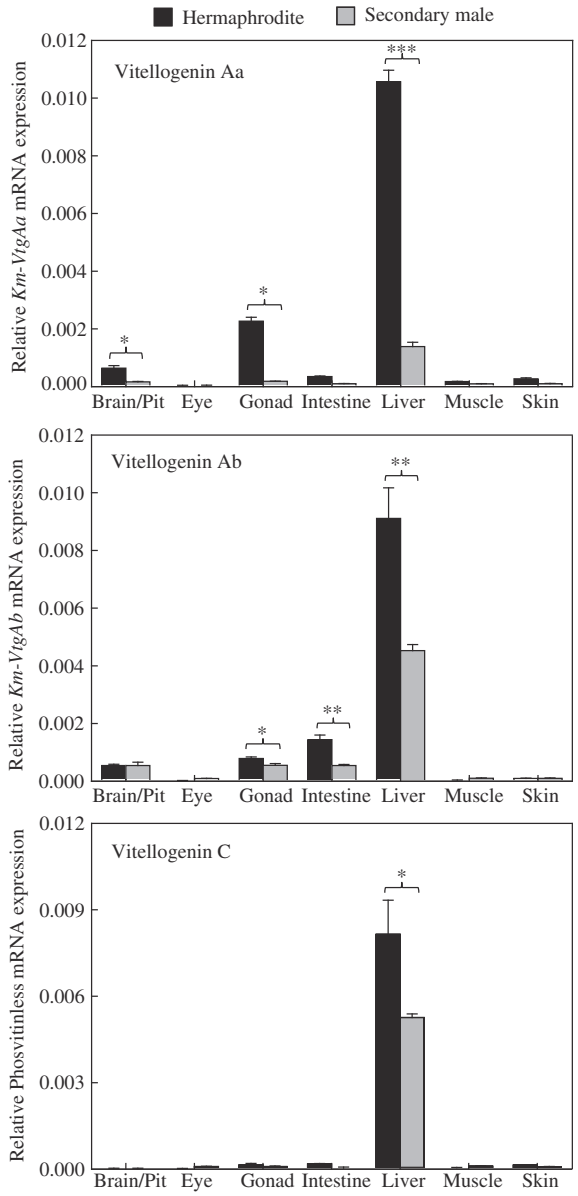

Fig. 2 – Tissue-preferential mRNA expression of *K. marmoratus Vtg* genes in the hermaphroditic and secondary male fish. The *K. marmoratus 18S rRNA* gene was used as a reference gene to normalize the expression. Each value is the average of three technical replicate samples, and data are shown as means ± S.D. Significant differences of the means of mRNA expression between hermaphrodites and secondary males were analyzed using the paired Student's *t*-test. Asterisks (* and *) indicate significant change ($P < 0.05$ and $P < 0.001$, respectively). mRNA: messenger ribonucleic acid; rRNA: ribosomal ribonucleic acid; S.D.: standard deviation.**

the *Km-Vtg* genes differed in response to the mode of action of the exposed chemicals. NP and OP showed a greater inducing effect on *Km-Vtg* transcripts than did BPA. Additionally, each *Vtg* gene showed a different pattern of expression over time. For example, the liver tissue induction of *Km-Vtg-Aa* 12 hr after exposure in response to NP was similarly significant to that observed after a 24 hr exposure to OP and BPA (Fig. 4). The

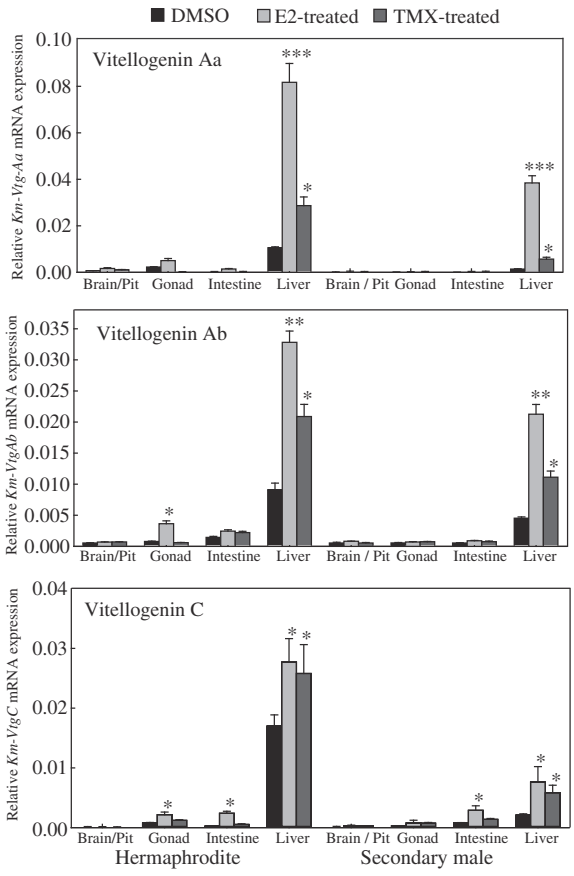

Fig. 3 – Effect of 17β-estradiol (E2, 100 ng/L for 96 hr) and tamoxifen (TMX, 10 μg/L) on *K. marmoratus Vtg* mRNA expressions in hermaphrodites and secondary males. Control fish were treated with solvent DMSO. The expression of each *Km-Vtg* mRNA was analyzed using real-time RT-PCR. The *K. marmoratus 18S rRNA* gene was used as a reference gene to normalize expression. Each value is an average of three technical replicate samples, and data are shown as means ± S.D. The symbols (*, **, and *) indicate significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) compared with control values. mRNA: messenger ribonucleic acid; DMSO: dimethyl sulfoxide; *Km-Vtg*: *Kryptolebias marmoratus Vtgs*; RT-PCR: reverse transcription-polymerase chain reaction; rRNA: ribosomal ribonucleic acid; S.D.: standard deviation.**

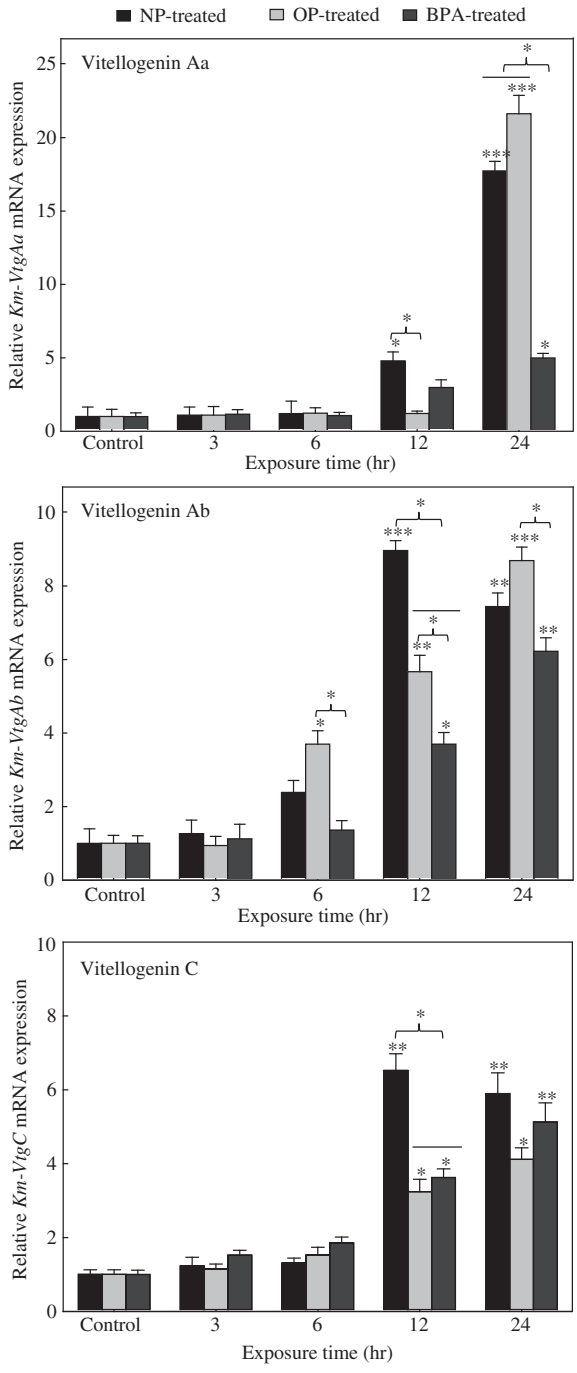

Fig. 4 – Transcriptional expression of *K. marmoratus Vtg* genes in the livers of hermaphroditic fish after 96 hr exposure to 4-n-nonylphenol (300 μg/L), bisphenol A (600 μg/L), and 4-*tert*-octylphenol (300 μg/L). The expression of each *Km-Vtg* mRNA was analyzed using real-time RT-PCR. The *K. marmoratus 18S rRNA* gene was used as a reference gene to normalize expression. Each value is an average of three technical replicate samples, and data are shown as means ± S.D. The symbols (*, **, and *) indicate significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) compared with control values. RT-PCR: reverse transcription-polymerase chain reaction; rRNA: ribosomal ribonucleic acid; S.D.: standard deviation.**

expression of *Km-Vtg* transcripts was not significantly affected in response to EDCs up to 3 hr.

3. Discussion

Acanthomorpha, or ray-finned fish (*i.e.*, medaka, stickleback, *Takifugu*, tetraodon), have more than three *Vtgs* genes, and all of the *Vtgs* genes are located in a single chromosome in each species (Finn and Kristofferson, 2007; Babin, 2008). *VtgAa* is located next to *VtgAb* and is transcribed in the same direction, while *VtgC* lacks the phosvitin domain and is found apart from both *VtgAa* and *VtgAb*; it is also transcribed in the

opposite direction (Babin, 2008). Although the main function of phosvitin domain is still unclear, previous studies suggested its potential receptor binding role during receptor-mediated endocytosis in vertebrates (Miller et al., 1982; Woods and Roth, 1984; Wang et al., 2000). As shown in other oviparous vertebrates, a well-conserved gene orientation of the *Vtg* cluster was identified in *K. marmoratus* as the three *Vtgs* genes spanned 550 kb in a single scaffold with the conserved genomic orientation compared to other fishes in the same class, Acanthomorpha. Phylogenetic analysis supported the similar phylogenetic distance identified in Acanthomorpha in addition to the gene order. During vertebrate whole genome duplication (WGD) with lineage-specific gene duplication of teleosts, teleostean Vtgs were divided into three groups: VtgAa, VtgAb, and VtgC. In the phylogenetic tree, Km-Vtg proteins were distinctly clustered into Vtg-Aa, Vtg-Ab, and Vtg-C, as has been reported in other teleosts (Matsubara et al., 2003; Finn and Kristoffersen, 2007; Reading et al., 2009). Of these, the VtgA group of the spiny ray-finned teleosts including *K. marmoratus* was further separated into VtgAa and VtgAb except for salmonoid (Protacanthopterygii) Vtgs, while the duplicated VtgB and VtgD were lost during WGD (Finn and Kristoffersen, 2007). Thus, VtgAsa and VtgAsb observed in salmonoid were not identified in the *K. marmoratus* genome. These results are consistent with teleost Vtg genealogy.

In each *Km-Vtgs* genes, different numbers of putative ERE and/or ERE-half sites were observed. *Vtg* is a well-known estrogen-inducible gene that is regulated by ERE in the promoter region (Gruber et al., 2004). Traditionally, Vtg is regarded to be synthesized in the female liver tissue under the control of estrogen (or E2) *via* estrogen responsive elements (EREs) that bind to estrogen receptor (ER) and E2-mediated genes (Klein-Hitpaß et al., 1986; Burch et al., 1988). To date, ERE-mediated transcriptional regulation of the *Vtg* genes in vertebrates has been reported in several teleosts including *Oreochromis aureus* (Teo et al., 1998), *Cyprinidon variegatus* (Denslow et al., 2001), *Danio rerio* (Wang et al., 2005), and others. ERE-half sites also could be regulators for the function of estrogen inducible genes (Tora et al., 1988; Kato et al., 1992). Thus, a different composition of ERE and ERE-half sites in the promoter regions of three *Km-Vtgs* can influence the role of each *Km-Vtg* gene in oocyte maturation in response to estrogenic compounds.

In the livers of hermaphroditic *K. marmoratus*, transcriptional levels of all *Vtg* genes were highly expressed, showing that the liver is the major organ for Vtg production. In the case of *Km-VtgAa* and *-VtgAb* mRNAs, both genes were significantly expressed in extrahepatic organs including the brain, gonad, and intestine, while *Km-VtgC* gene expression was not detected. In general, vtg is expressed in the liver; however, their transcripts can be expressed in extrahepatic tissues without any estrogenic compound treatment as shown in several teleosts (Islinger et al., 2003; Wang et al., 2005, 2010; Ma et al., 2009; Zhong et al., 2014). Interestingly, three *Km-Vtg* transcripts were quite highly expressed in secondary males' livers compared to the other tissues from hermaphroditic fish under ordinary conditions. In *K. marmoratus*, the phenotypic characteristics of secondary males develop due to atresia of the ovary from hermaphrodites, while the liver tissue remains unchanged phenotypically during hormonal changes, suggesting that transcriptional levels of *Vtg* genes in *K. marmoratus* are maintained at a relatively high level in the secondary male liver. In fact, both hermaphrodites and primary males secreted E2 and 11-ketotestosterone (11-KT), and mean plasma E2 level of hermaphrodite was significantly lower than that of primary male (Minamimoto et al., 2006). Thus, transcriptional regulation of three *vtg* genes would be highly maintained in the secondary male, as they also secrete estrogen, androgen, and progestin synchronously as shown in hermaphrodites. Investigations of unique tissue-specific expression of *Vtg* genes with a transcriptional regulation study would be interesting to better understand the species-specific mode of action of *Vtg* genes.

In *K. marmoratus*, significant *Vtg* mRNA induction in response to E2 treatment was observed predominantly in the liver in parallel with strong elevation of choriogenins (Chgs) (Rhee et al., 2009), indicating that the liver plays a major role in hormone-mediated yolk accumulation in the growing ovary; Chgs are precursors of the inner layer of the egg envelope that are synthesized in hepatocytes under estrogen control. Changes in endogenous hormone levels and the transcriptional modulations of endocrine regulatory genes in response to estrogenic compounds were continuously reported in *K. marmoratus* (Kanamori et al., 2006; Minamimoto et al., 2006; Rhee et al., 2011). Therefore, *K. marmoratus* is very susceptible for exogenous estrogenic compounds and would have highly sensitive endocrine regulatory system including vitellogenesis. In teleosts, a similar inducibility in hepatic *Vtg* mRNA with extrahepatic expression and plasma Vtg synthesis was extensively shown in response to E2 or 17α-ethinylestradiol (EE2) (Bowman et al., 2000; Wang et al., 2000; Takemura and Kim, 2001; Woods and Kumar, 2011; Söffker and Stevens, 2012; Humble et al., 2013, 2014; Zhong et al., 2014). Thus, *Vtg* gene expression has an interspecific difference that presumably affects the role of organs and is involved in vitellogenesis in response to estrogenic compounds.

In *K. marmoratus*, 10 μg/L of TMX (E2 antagonist) caused a slight, but not complete, up-regulation of all three *Km-Vtg* transcripts in the livers of both genders compared with E2-exposed *K. marmoratus*, suggesting that transcriptional susceptibility in response to TMX would be different, depending on the TMX concentration used. TMX acts as a direct anti-estrogen by forming a relatively stable complex with the estrogen receptor (ER), reducing affinity of subsequent estradiol binding to receptors (USEPA, 2002). There is still controversy surrounding the role of TMX in the formation of fish yolks as TMX has mixed estrogenic/antiestrogenic actions (MacGregor and Jordan, 1998; Sun et al., 2011; Leaños-Castañeda and Van der Kraak, 2007; USEPA, 2002). For example, in both male and female zebrafish, *Vtg1* and *Vtg2* mRNAs were induced at low concentrations of TMX (30 μg/L), while their transcription levels were reduced in a dose-dependent manner when the fish were exposed to over 30 μg/L of TMX (Sun et al., 2010). In Japanese medaka (*Oryzias latipes*), *Vtgs* expression showed a similar pattern to zebrafish males, while *Vtg1* and *Vtg2* transcripts were strongly suppressed in females in response to TMX in a dose-dependent manner (Chikae et al., 2004; Sun et al., 2011). Agonistic effect

of TMX would be dependent on cell type, ERE-promoter context, and ER types (Watanabe et al., 1997). Taken together, these results suggest that TMX is differentially effective with different compositions of ER/ERE across species in the regulation of *Vtg* expression.

In *K. marmoratus*, all xenoestrogen compounds induced the transcriptional level of three *Km-Vtg* genes sensitively. Similar exposure conditions of E2 and BPA strongly induced *Vtg* transcription with significant increases in mRNA levels of estrogen receptor (ER) α/β, aromatases, and choriogenins (Rhee et al., 2011). In teleosts, similar elevated patterns were reported, particularly when using male fish to understand effects of EDCs on Vtg production in teleosts with obvious sexual dimorphism (Hemmer et al., 2002; Pait and Nelson, 2003; Miracle et al., 2006; Lerner et al., 2007). OP was a stronger inducer of vitellogenin than either NP or BPA in goldfish, and its *Vtg* inducibility was confirmed in the male fish of freshwater cichlid (Li et al., 2012; Genovese et al., 2014). A bisphenol analogue BPAF exposure caused significant upregulation of the *Vtg* transcription in the liver of male zebrafish (Yang et al., 2015). Thus, induction of *Vtg* gene expression confirms the potential for xenoestrogens compounds to exert endocrine-disrupting effects in *K. marmoratus*. Regarding putative induction pathway with differential sensitivities of three *Km-Vtg* genes, their upregulation would be triggered by direct induction *via* ER activation with ERE binding in response to EDC exposure. *In silico* analysis revealed a putative ERE in the promoter region of both *Km-VtgAa* and *VtgAb*, suggesting that EDCs exert their endocrine-disrupting effects *via* ERE-mediated transcriptional regulation of *Vtg* gene. Even though *Km-VtgC* has no putative ERE site in the promoter region, its mRNA levels also upregulated in response to each of the three EDCs. Thus, these results in *K. marmoratus* would shed light on the regulatory role of ERE and ERE-half sites for the induction of *Vtg* genes as shown in several teleosts (Tora et al., 1988; Kato et al., 1992; Teo et al., 1998; Denslow et al., 2001; Wang et al., 2005). The underlying induction mechanism or subsequent physiological outcomes should be clarified in further study.

In conclusion, *K. marmoratus* is a functional hermaphroditic fish with both ovaries and testes in its body. Under normal conditions, *K. marmoratus* expresses sex specific hormones such as estrogen and testosterone (Minamimoto et al., 2006). Thus, to maintain hermaphroditic function, balanced hormonal levels must be maintained. Although the information on the effect of transcriptional changes in *Km-Vtg* genes in response to EDC exposure is unclear as yet, it is likely that the sensitivity of *Vtg* genes to EDCs may serve as an early detector for EDC contamination in the aquatic environment. In addition, our work provides a better understanding of the mechanistic view of the differentially-modulated transcriptional expression of three *Km-Vtg* genes in response to EDC exposure in *K. marmoratus*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.08.006.

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