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Characterization of *vtg-1* mRNA expression during ontogeny in *Oreochromis mossambicus* (PETERS)

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ABSTRACT

The yolk-precursor lipoprotein, vitellogenin (VTG) has been widely recognized as a biomarker for the detection of estrogenic activity in water-borne chemical pollutants. We characterized the expression status of this important constituent of reproduction in the Mozambique tilapia (*Oreochromis mossambicus*), a tilapiine freshwater fish species indigenous to Southern Africa, and investigated its utility in detection of exposure to estrogen using a quantitative real-time polymerase chain reaction (QPCR) assay. We initially isolated a 3 kb upstream promoter region of the *vtg* gene and identified putative binding sites for several regulatory factors including estrogen receptor (ESR). Evidence for the expression of several splice-site *vtg* mRNA variants was found in a number of tissue types. A quantitative real-time polymerase chain reaction (QPCR) assay was subsequently developed based upon a specific primer pair (OMV6/9) that selectively amplified the liver-enriched transcript. The level of this transcript in liver tissue was high in females and lower, but detectable, in males and was significantly increased in male fish following laboratory exposure to 17β-estradiol (E_2). This study further established that juvenile whole body homogenates (WBHs) contained extremely low levels of liver-specific *vtg* mRNA between 5 and 110 days post-fertilization (dpf) compared to adult male liver. Subsequent exposure of 20 dpf juveniles to E_2 showed a substantial increase in this transcript within hours, and when compared to classic male model under same conditions, the juveniles were remarkably more sensitive. We therefore conclude that the quantification, using QPCR methodology, of *vtg* mRNA expression in 20 dpf *O. mossambicus* juveniles has promise for assessing estrogenic EDC activity in aquatic sources.

1. Introduction

Evidence presented over the last century has led to the formulation of the endocrine disruption hypothesis, stating that anthropogenic and natural chemical substances in the aquatic environment have the potential to disrupt the normal functions of the endocrine system and its hormones in wildlife and humans [1,2]. These endocrine disrupting chemicals (EDCs) have been shown to mimic, antagonize or modulate the normal actions of hormones or affect hormonal synthesis and degradation pathways [2–8]. However, because environmental EDCs are mostly found as part of complex mixtures, understanding the context in which EDCs operate remains problematic, and sensitive biological screening platforms are required that are amenable to high throughput methodologies. Tier I screening applications favor aquatic systems, due to the water-borne nature of many chemical pollutants and their accumulation in sediment and groundwater [9]. Various aquatic species, including several small fish species, have been studied and currently serve as sentinels for low concentration chemical exposure to endocrine-modulating chemicals [3,6,10–22].

Initially, most research on endocrine disruption in fish focused on the actions of environmental estrogens: EDCs that elicit an estrogenic response similar to the actions of the natural female hormone 17β-estradiol (E_2) [23]. Assay development included measurement of changes in expression of estrogen-regulated gene products. Synthesis of one such product, vitellogenin (the egg yolk-precursor phospholipoglycoprotein; VTG), is generally associated with oviparous reproduction and forms the basis of many estrogenic screening programs in aquatic systems [24–26]. Egg-producing females produce substantially higher levels of VTG relative to males [24,25]. The vitellogenin gene (*vtg*) is described to be primarily expressed in the liver where its encoded protein is extensively modified post-translationally, secreted into the bloodstream, and sequestered by the oocytes *via* specific vitellogenin receptors in coated pits (endocytosis) [27]. VTG has subsequently been used widely in screening programs involving several small fish

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species as a biomarker for assessing estrogenic activity of EDCs in wastewater effluents and natural water resources [28–30]. Kim et al. [31] illustrated the enhanced and induced effect of E_2 on hepatocyte cultures in male and female tilapia, *Oreochromis mossambicus*, respectively, and thereby confirm the strong VTG-inducing effect by this hormone in this species *ex vivo* [31] and *in vivo* [32]. However, *vtg* expression in males is inducible following exposure to estrogenic substances and represents a useful biological screening platform for estrogen-related EDCs [33,34]. Although immunological methods to quantify circulating VTG protein levels in either juvenile homogenates or adult plasma samples are currently used [32,35–37], the measurement of *vtg* mRNA transcripts using a quantitative real-time RT-PCR (QPCR) assay is not yet widely used. This approach has been shown to provide more sensitive detection than the enzyme linked immunosorbent assay in assessing estrogenic activity [38]. A QPCR-based assessment would provide additional insight related to temporal variation in expression of *vtg* genes during reproductive development which is critical for yolk production and subsequent larval survival. Information obtained on the temporal and tissue-specific expression patterns of *vtg* transcripts during development provides additional highly sensitive tool to assess temporal variation in expression of *vtg* genes as well as show early signs of estrogenic EDC activity.

The tilapiine species, *O. mossambicus* (Mozambique Tilapia), is native to Southern Africa and extensively used in aquaculture [39]. Many aspects of the life history and ecology of this mouthbrooding species have been described [40,41], but what remains unclear are many aspects related to the development and reproductive biology of this species; particularly with respect to molecular mechanisms [42–49]. Because of the potential for a sexually undifferentiated phase and the contribution of environmental sex determination of *O. mossambicus*, juvenile fish may serve as a powerful alternative to adult males in the context of an EDC assay. In addition, the assay is simplified through reduced duration, increased ease of husbandry and the lack of a need to separate test populations by sex. Thus quantification of differential expression of *vtg-1* in early ontogeny may hold several advantages for Tier I screening such as sensitivity to changes during early development that may suggest extensive organizational consequences and limited influence of endogenous hormones, short exposure periods, large sample numbers, and small sample volumes.

In order for juvenile tilapia to serve as a good animal model in an EDC assay, it is important to establish the expression pattern of *vtg* mRNA during normal development and determine early developmental stages at which *vtg* expression is suitably low. It is only against this baseline knowledge that an investigation of E_2-responsive nature of *vtg* can be performed. The aim of this study was to develop a quantitative PCR (QPCR) protocol that effectively measures the expression levels of *vtg* transcript (a) in different adult tissue types, (b) during early development and (c) following estrogen exposure in both adult males and juveniles of *O. mossambicus*.

The *vtg* promoter region (approximately 3 kb) and partial cDNA of *O. mossambicus* was isolated and the proximal region (designated as ~1.5 kb) analyzed using computational (*in silico*) tools to facilitate the interpretation of *vtg* gene regulation, with special emphasis on vitellogenin gene regulation and expression in response to estrogen exposure. Experimental results from this study confirmed tissue-restricted expression of the normally spliced *vtg* transcript in the liver of both males and females; whereas putative alternatively spliced transcripts were observed in all other tissue types examined. Adult female liver expressed significantly more *vtg* transcript than males. Induction experiments from the present study confirmed that *vtg* mRNA abundance increased in *O. mossambicus* adult male hepatic tissue following exposure to E_2. Juvenile whole body homogenates (WBHs) displayed much lower levels of liver-specific *vtg* transcript which increased significantly upon E_2 exposure confirming the great potential of juvenile tilapia as a bio-indicator of exposure to estrogenic compounds in aquatic environments in Southern Africa.

2. Materials and methods

2.1. Experimental animals

Adult breeding stock of *O. mossambicus* were obtained from Aquastel (Stellenbosch, South Africa) and maintained in aquaria with tap water under constant aeration that was filtered through activated charcoal. Water temperature was maintained at 27 °C (±1 °C). Fish were fed once daily with Tilapia pellets (AquaNutro, South Africa). The light regime followed a 14:10 light:dark cycle. Offspring production was monitored daily in this mouthbrooding species. Females carrying eggs in their mouths were removed from the breeding aquaria to culturing tanks. Each brooding female was kept alone until the offspring reached the swim-up fry stage, at which time the adult females were removed and re-introduced into the breeding tank. Each batch of offspring was reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage (determined by age in 5 day intervals after fertilization) were collected, euthanized using 0.01% Benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA*later* (Ambion Inc., USA) at 4 °C. At least three different breeding pairs were used to generate offspring that were sampled at each developmental stage. Individual samples of all specimens were analyzed for *vtg* expression individually—no pooling of nucleic acid material between individuals occurred. For the developmental samples, between 5 and 10 specimens were collected per batch ($n \geq 5$ per batch, and $n \geq 15$ per time point). Due to data loss associated with this type of analysis, values of sample sizes used in data analyses changed and are indicated in Section 3. To establish the differential *vtg* mRNA expression pattern amongst different tissues in adults, we dissected at least five males and five females and prepared total RNA as outlined below.

2.2. Genomic DNA isolation

Tilapia finclips of about 25 mm^2 were homogenized in 700 μl extraction buffer (50 mM Tris, pH 8; 0.7 M NaCl; 10 mM EDTA; 1% CTAB; 0.1% β-mercaptoethanol) and genomic DNA was isolated using a CTAB-method according to D'Amato et al. [50].

2.3. Chemical exposure

Animal husbandry, treatment and handling were done according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X). Adult males ($n = 8$) were exposed to 60 μg/l 17β-estradiol (E_2, Sigma, USA) or an equal volume of vehicle (ethanol) alone ($n = 10$) for 12 h. Vehicle was diluted by a factor of 6×10^6. Chemical exposure experiments were all conducted in static water conditions with aeration as described under Section 2.1. Livers were dissected and weighed to calculate the hepatosomatic index (HSI: liver mass/total body mass) in order to compare data from adults to that of juveniles. Juvenile fish which were used in induction experiments were selected at 20 dpf where sex is not morphologically distinguishable but is believed to be already determined [51]. These juveniles were exposed to 0.5, 1.0, and 60 μg/l E_2 ($n = 10$ for each condition) in a temporal experiment with 0, 12 and 24 h time points. Fish were euthanized and total RNA was prepared from adult livers or juvenile whole body homogenates (WBHs) as described below.

2.4. Total RNA isolation and cDNA preparation

Anaesthetized fish were decapitated, dissected and appropriate tissues/whole bodies were homogenized in TRIzol reagent (Invitrogen, USA) for 5 s, working on ice. Total RNA was prepared from specific adult tissues or from WBHs of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water (50 μl), samples were treated with one unit of DNase I (Promega, USA) for 30 min at 37 °C and precipitated at −20 °C following the addition of 0.1 volumes of 3 M sodium acetate pH 5.6 and 2.5 volumes of 95% ethanol. The RNA pellets were washed with 70% ethanol and redissolved in 30 to 60 μl of DEPC-treated water. RNA yields were quantified spectrophotometrically at $Absorbance_{260\,nm}$ and stored at −70 °C. First strand cDNA was prepared from 2 μg of total RNA using oligo $d(T)_{15}$ primers and SuperScript III RNase H^- MMLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Each cDNA sample was diluted 40-fold and stored at −20 °C prior to gene expression analysis or used as template for *vtg* cDNA isolation as described below.

2.5. Isolation of O. mossambicus vitellogenin cDNA and partial gene sequences

To obtain partial *O. mossambicus* vitellogenin gene sequence, PCR was performed using template cDNA derived from *O. mossambicus* liver tissue dissected from three adult females and primers designed against the related *O. aureus* vitellogenin sequence (GenBank accession no. AF072686). Each 25 μl reaction included 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 μM of each primer (VTGforw/rev or OMV6/9, Table 1) and 2.5 units of in-house Taq polymerase. To each reaction, we added 50 ng nucleic acid. The PCR thermocycle included an initial denaturation for 2 min at 94 °C, after which followed 30 cycles of 1 min at 94 °C, 30 s at 65 or 56 °C (respectively for the primer pairs) and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Amplified DNA fragments of interest were cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α. Plasmid DNA was isolated from positive clones detected by colony PCR [52] and insert DNA was sequenced using SP6 and T7 primers. The resulting *O. mossambicus* vitellogenin sequence information was deposited in GenBank (accession no. AJ889835 or FJ756399 for the respective primer pairs, Table 1). We performed sequence analysis of the DNA and derived amino acid sequences using ClustalW software according to Chenna et al. [53]. Graphic illustrations were prepared with software available in Bioedit Sequence Alignment Editor v7 [54].

The resulting *O. mossambicus* vitellogenin cDNA sequence information from AJ889835 was used to design a new species-specific primer pair (iVTG1a and iVTG2a, Table 1) for use in inverse PCR (iPCR) to isolate an upstream *vtg* promoter region from genomic DNA [55]. Three micrograms of genomic DNA were digested in individual reactions with EcoRI, EcoRV, BamHI, XbaI restriction enzymes (Promega Corporation, Madison, USA) overnight. Digested DNA (1 μg) was resuspended in a ligation mix containing 9U T4 DNA ligase, 40 μl ligase buffer (Promega Corporation, Madison, USA) to a final volume of 400 μl at 4 °C for 12 h. Circularised DNA was phenol/chloroform (1:1) extracted, precipitated and resuspended in 40 μl distilled water. Digestion and re-ligation of genomic DNA were visualized in ethidium bromide-stained 0.8% (w/v) agarose gels. iPCR reactions were performed with 150 ng re-ligated genomic DNA in a final volume of 50 μl containing 1 μl Elongase enzyme mix (Invitrogen, USA), 10 μl total volume of Buffer A and B, 0.2 mM of each dNTP and 0.2 mM of each primer. PCR samples were denatured at 94 °C for 30 s followed by 35 cycles of amplification (94 °C denaturation, 1 min; 50 °C annealing, 1 min; 68 °C polymerization, 5 min). All iPCR reactions were carried out using a PerkinElmer GeneAmp Thermocycler 9700. The 5′-flanking region of the VTG gene was cloned into pGEM-T Easy vector (Promega Corporation, Madison, USA) and sequenced in both directions (ABI PRISM 3100 genetic analyser) using the ready reaction kit with AmpliTaq DNA polymerase (The PerkinElmer Corporation, Norwalk, USA). Two clones were isolated which displayed 100% overlap within the 5′ flanking area of the *O. mossambicus vtg* gene. These initial sequence results provided information for the design of additional primers, iVTG1d/2d (Table 1) at the 5′ and 3′ ends of the putative promoter region which were used in the amplification and generation of a third *vtg* promoter-associated clone.

2.6. Computational analysis of the vtg promoter region

Comparative genomic analysis was conducted using the rVISTA computational platform (http://genome.lbl.gov/vista/index.shtml). The vitellogenin proximal promoter sequence (~1500 bp including ATG start codon) of *O. mossambicus*, designated as OmVtg (GenBank accession no. AJ889574) was used as reference sequence and compared to the *vtg* proximal (ranging from 1 kb to 1.5 kb upstream of the ATG start codon) promoter regions of chicken (*Gallus gallus*: GgVtg2 X04593; GgVtg3 M28125), African clawed frog (*Xenopus laevis*: XlVtg Y00354), blue tilapia (*Oreochromis aureus*: OaVtg AF072686) and to other fish species; zebrafish (*Danio rerio*: DrVtg1 BC094995; DrVtg2 BC154732; DrVtg5 BC097081; DrVtg6 BC153381), medaka (*Oryzias latipus*: OrVtg1 AB064320; OrVtg2 AB074891), fugu (*Takifugu rubripes*: TrVtg ensemble gene ID ENSTRUG00000017475), stickleback (*Gasterosteus aculeatus*: GaVtg ENSGACG00000009769) and tetraodon (*Tetraodon*

Table 1
Primers used in the isolation and QPCR of *O. mossambicus* vitellogenin cDNA.

Application	Gene	Primer name	Primer sequence
Cloning	Vitellogenin	VTG fwd	TCGAGCTGGGGTTAAAATC
Cloning		VTG rev	TGGCAGTGGTTCAGGTC
Cloning		iVTG1a	CATGGAAGGCACTGCCAAGC
Cloning		iVTG2a	GACCTGAACCACTGCCA
Cloning		iVTG1d	AAAAGTCAATAAGCCAACAC
Cloning		iVTG2d	AGGTGCTCTTGGTCATGG
Cloning/QPCR		OMV4	TAACTACATCATGAAGCCAGCACCC
Cloning/QPCR		OMV6	GTTGGAGTGAGGACTGAGGG
Cloning/QPCR		OMV9	GGTCCACTGGCAAACTGGATAAGC
Cloning/QPCR		OMV10	AGTGCTGACAATCTGAGCCTCGGC
QPCR	β-Actin	OMBA1	TGTGATGGTGGGTATGGG
QPCR		OMBA2	CTGTGGTGGTGAAGGAGTAG
QPCR	Ribosomal protein L8	PAC L8 up	AGAGCCCATGTAAAGCAC
QPCR		PAC L8 dn	CCTGTAAGGGTCACGGAA

nigroviridis: TnVtg ENSTNIG00000000613). Promoter sequences were retrieved from the Ensembl Genome Browser (archive version at http://jul2008.archive.ensembl.org/index.html).

Putative transcription factor (TF)-binding sites (residing in highly conserved promoter regions) were predicted using the TRANSFAC database at stringency settings: 0.75 matrix similarity and 0.95 core similarity cut-off via rVISTA and the MATCH™-platform (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi). All sequence similarity search and alignment analyses were conducted using BLAST (http://blast.ncbi.nlm.nih.gov).

2.7. Quantitative gene expression analysis

Primers for analysis of gene expression by quantitative real-time PCR (QPCR) were designed using Primer Premier Version 5.00 software (Premier Biosoft International, USA). These included vitellogenin based upon *O. mossambicus* and *O. aureus* sequences (GenBank accession nos. AJ889835, AF072686, respectively, Table 1) and the potential normalizer control gene targets β-actin (*O. mossambicus* BA, GenBank accession no. AB037865) and ribosomal protein L8 (rpl8) for which cross-species primers were found to provide specific amplification. Gene expression was quantified using either Stratagene MX3000P or MX4000 real-time quantitative PCR systems (Stratagene, USA) or an Applied Biosystems 7500 (Applied Biosystems, CA, USA). Each 15 μl QPCR reaction contained 2 μl of diluted first strand cDNA, 7.5 μl 2× SYBRgreen mix (Sigma, Germany), 0.08 μl ROX reference dye (Sigma, Germany) and 0.33 μM of each primer. The thermocycle program included 95 °C (9 min), followed by 40 cycles of 95 °C (15 s), 56 °C for (30 s) and 72 °C (45 s). Each sample was evaluated in at least triplicate amplification reactions and each QPCR run included control reactions containing no cDNA template and a standard concentration of each target DNA amplicon (4×10^4 copies/reaction). Amplification DNA products were quality checked using melting curve analysis. C_t values obtained across independent amplification runs for a given gene target were used to determine relative mRNA abundance by the $\Delta\Delta C_t$ method [56–59]. Two gene transcripts, β-actin and rpl8, were used in this study, and normalization using either gene transcript yielded highly similar results. We therefore present only the data normalized with rpl8. Expression data generated from juvenile fish samples indicating poor RNA quality or suboptimal amplification were not included in further analyses.

2.8. Statistical analysis

Statistical analyses were performed using the STATISTICA software package v8 (StatSoft Inc., USA). Data were tested for normality and equal variance among groups. For parametric data-sets, we used analysis of variation (ANOVA) to test for significant variation ($p < 0.05$) together with Holm-Sidak's Multiple Comparison test to determine significance among groups. In cases of non-parametric data-sets, we used Kruskal–Wallis analysis of variance (ANOVA) followed by a Mann–Whitney *U* test to establish significance between treatment groups.

3. Results

3.1. Isolation and in silico analysis of the O. mossambicus vtg promoter

We isolated a 3061 bp *vtg* genomic fragment from *O. mossambicus* (*Omvtg*; GenBank accession no. AJ889574). Vitellogenin comparative promoter analysis revealed the *O. mossambicus* (OmVtg) reference sequence to be 96% identical to a 1335 bp 5′-flanking sequence (including ATG) of *O. aureus* (OaVtg) (Fig. 1), but showed no conservation (using rVISTA at 70% default level) to other species. Sequence analysis between OmVtg and OaVtg

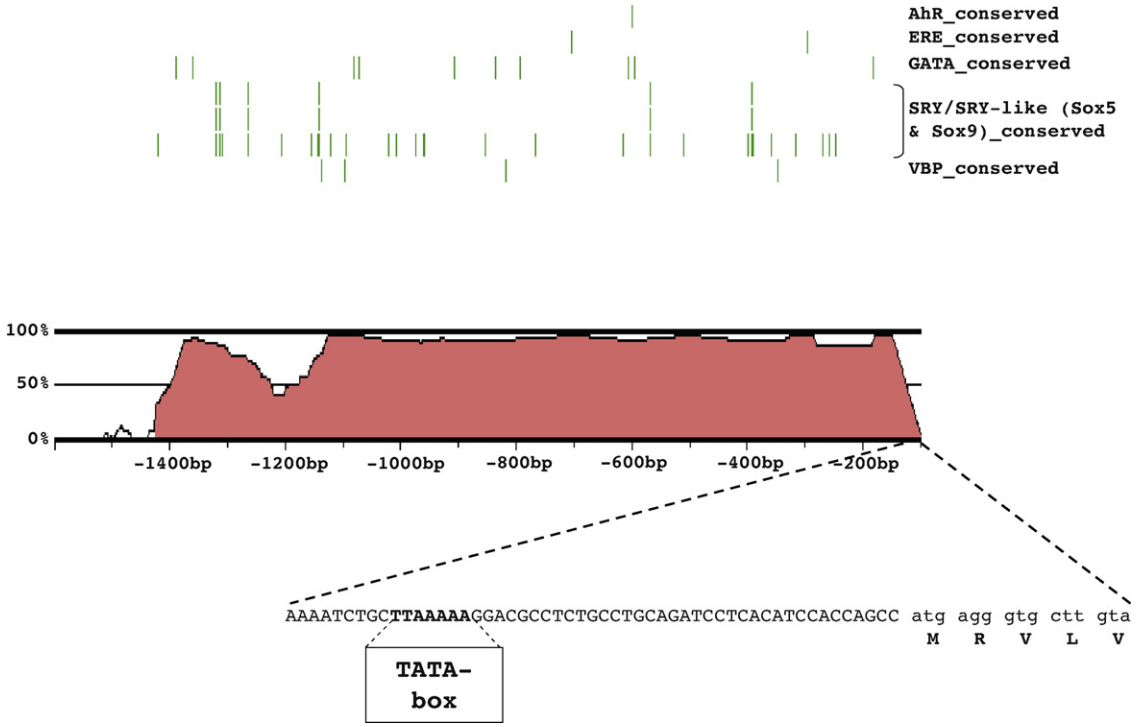

Fig. 1. Representation of highly conserved proximal *vtg* promoter regions between *O. mossambicus* and *O. aureus*. The nucleotide sequence is indicated at the conserved TATA-box and core promoter area including part of the first exon. Putative TF binding sites identified by TRANSFAC are indicated as vertical lines above the conserved regions at the indicated position within the promoter region. The *x*-axis indicates the base sequence (1500 bp) and nucleotide position upstream of the ATG start codon. The *y*-axis represents percent identity of the conserved area.

showed identical positions of the TATA-box with the core sequence of TTAAAAA (Fig. 1) as previously identified in OaVtg [60]. The OmVtg fragment included 45 bp of exon 1 which was 93% identical to the *O. aureus* cDNA sequence. Initial analyses, using the TRANSFAC database (at default parameters), revealed the presence of several different putative motifs within the *vtg* promoter sequence of *O. mossambicus*. However, for this study, we focused specifically on estrogen responsive elements (EREs) and the synergistic interplay of other *cis*-acting elements involved in estrogen-dependent transcriptional regulation of *vtg*. From the *in silico* analysis (at stringent 0.95 core similarity TRANSFAC parameters) of the 1500 bp (including start codon) proximal promoter region we could identify putative *cis*-motifs for: ERE half site with the consensus sequence GGTCA (TRANSFAC accession no. T00258), aryl hydrocarbon receptor (AhR; T00018), sex determining protein (SRY; T00996) and SRY-related (Sox-5; T01429 and Sox-9; T01853), GATA-box (T00267, T00305, T00307, T00308, T00314) and vitellogenin binding protein (VBP; T00881) (Fig. 1).

3.2. Identification of putative differentially spliced vtg mRNA and QPCR assay development

Using sequence information garnered from the isolated *O. mossambicus vtg* partial cDNA and the known *O. aureus vtg* gene organization, we generated several DNA primer sets that could amplify different regions of *O. mossambicus vtg* mRNA. Collectively, these regions represented approximately 17% of the *vtg* gene (Fig. 2A). Primer positions spanned putative intronic regions, based upon the *O. aureus vtg* gene structure, and could be used to assess the presence of genomic DNA contamination during RT-QPCR analysis. Indeed, the lack of amplification of an intron-containing fragment using OMV6/9 primers confirmed the absence of genomic DNA template in the assessed samples (Fig. 2A and B).

Tissue-specific QPCR analysis revealed alternatively spliced *vtg* transcripts (Fig. 2A and B). OMV1/2 primer-based amplification from adult female and male resulted in a larger than predicted 397 bp product expressed in all tissues examined (brain, gonad, spleen, gill, heart and muscle) with the exception of the liver, where a 297 bp product of the predicted size was obtained (Fig. 2A and B). DNA sequencing of each amplified cDNA revealed that the larger fragment contains exon IV, exon V and the intervening intronic sequence. This intron is absent in the DNA amplicon obtained from the liver (Fig. 2B). Putative translation of this transcript isoform region results in the presence of stop codons in all 3 frames (Fig. 2A) suggesting that it may not encode a functional VTG protein.

Expression of a second alternatively spliced *vtg* mRNA product was highlighted by the use of the downstream primer pair, OMV4/10, which identified a 365 bp fragment displaying inclusion of the intronic sequence in *vtg* mRNA. This intronic region was absent in *vtg* mRNA expressed within the liver, as represented by a 279 bp amplified DNA product (Fig. 2B). The liver-specific mRNA containing this region of the *vtg* transcript expressed at higher levels in females compared to males.

Using primer pair OMV6/9 cDNA was isolated from female *O. mossambicus* liver (GenBank accession no. FJ756399). NCBI BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated OMV6/9 amplified *Vtg-1* according to Davis et al. [61]. In contrast to the OMV1/2- and OMV4/10-amplified cDNA sequences, primer pair OMV6/9 did not detect multiple *vtg* mRNA transcripts and demonstrates a tissue-restricted pattern of expression in the livers of both male and female *O. mossambicus*.

In the case of all three *vtg*-specific primer sets, the female liver showed higher levels of mRNA expression compared to the male livers. These data suggest that alternate *vtg* transcripts are expressed in tissues other than the liver, while a liver-specific *vtg* transcript is present in *O. mossambicus*. Thus, development of a *vtg*-specific

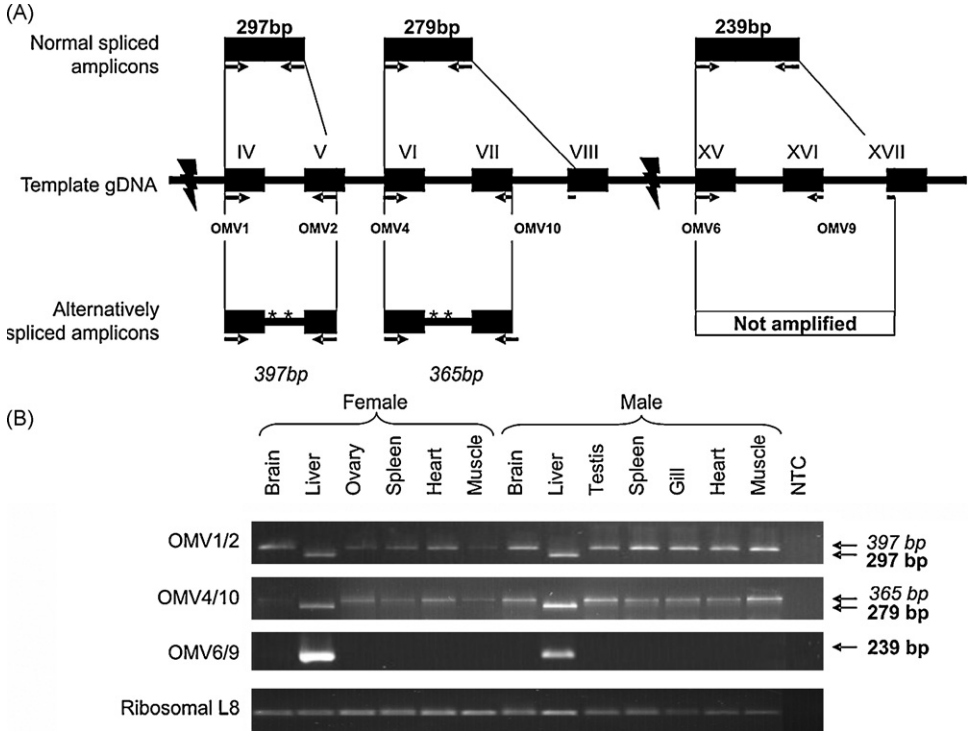

Fig. 2. Identification of *O. mossambicus vtg* mRNA isoforms in various tissues and QPCR primer design. (A) Organization of the related *Oreochromis aureus vtg* gene (gDNA) displaying the relative locations of exons and intronic sequences. The QPCR amplicons generated from *O. mossambicus* are shown with products from normally spliced mRNA indicated above while those generated from alternate transcripts are indicated below. Asterisks indicate the presence of putative in-frame stop codons. (B) Agarose gel analysis showing the various DNA amplicons generated with RNA isolated from the indicated adult male and female tissues is shown. Amplification of rpl8 was included to assess sample integrity and as a loading control. The relative band sizes are indicated in base pairs (bp). NTC, no template control.

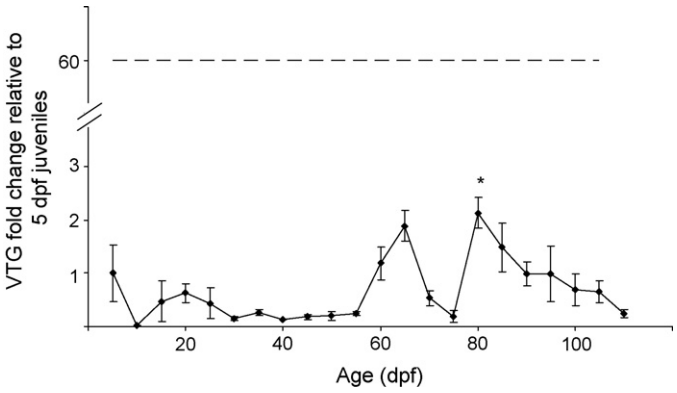

Fig. 3. Expression of liver-specific *vtg* mRNA during early development of *O. mossambicus*. Liver-specific *vtg* transcript levels were investigated in WBHs of juveniles at the indicated days post-fertilization (dpf). All data were normalized to the invariant endogenous rpl8 transcript (n = 3–15 per age group).

QPCR-based biomarker for *O. mossambicus* must incorporate this novel information in the design of appropriately positioned gene-specific primers and OMV6/9 was selected in subsequent QPCR assessment of *vtg* expression patterns. The standard curve for quantification using OMV6/9 was linear over 3 orders of magnitude with linear correlation (R^2) between C_t and the number of target copies $\geq$ 0.98 in each case with reactions done in quadruplicate (data not shown).

3.3. Normal and induced expression of vtg mRNA in juvenile O. mossambicus

Expression of the liver-specific form of *vtg* mRNA was assessed using the OMV6/9 primer set. Overall, the steady-state levels of *vtg* transcript in juvenile liver were found to be extremely low over an early developmental period (5–110 dpf) compared with an approximately 60-fold higher abundance measured in adult male liver after correction for WBH (Fig. 3). The level of *vtg* mRNA remained relatively steady until 65 dpf where a 2-fold increase compared to 5 dpf was observed. However, a significant increase in *vtg* transcript levels was only attained at 80 dpf ($p < 0.05$, Mann–Whitney *U* test), while abundance levels remained non-significant at successive time points (Fig. 3).

Juveniles at 20 dpf developmental stage were chosen for E_2 induction analysis of *vtg* mRNA expression. The exposure concentrations of E_2 employed were at levels similar to or lower than those used with adult males in which we found ~4-fold induction (Fig. 4). Following 12 h of exposure to 1.0 and 60 μg/l E_2, juveniles displayed a significant increase in liver-specific *vtg* transcript abundance relative to the time-matched controls (3- and 13-fold, respectively; Fig. 5).

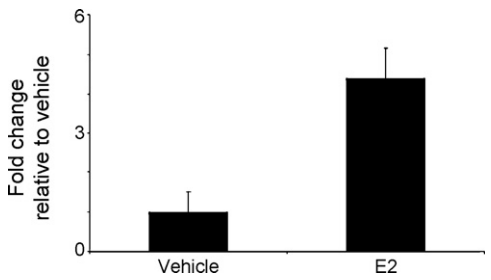

Fig. 4. Induction of *vtg* mRNA expression in *O. mossambicus* adult male liver. Relative *vtg* transcript levels were examined in livers from adult males exposed to ethanol vehicle or 60 μg/l E_2 for 12 h (n = 10 and 8, respectively) using the OMV6/9 primer set. Expression data were normalized to the invariant endogenous rpl8 transcript. The observed increase in liver-specific *vtg* mRNA abundance was significant at $p < 0.05$, Mann–Whitney *U* test. The error bars represent standard error of the mean.

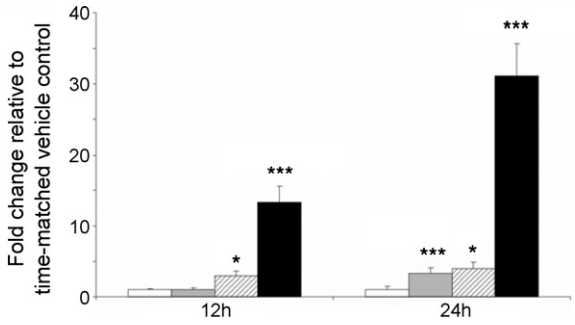

Fig. 5. Estrogen-dependent induction of liver-specific *vtg* mRNA in juvenile *O. mossambicus*. Relative levels of *vtg* transcripts were determined from WBH of 20 dpf animals exposed to ethanol vehicle (white bars), 0.5 (grey bars), 1.0 (hatched bars) or 60 μg/l E_2 (black bars) for the indicated times. Error bars represent standard error of the mean. Significance is indicated relative to the time-matched control by a single ($p < 0.05$; Mann–Whitney *U*) or triple asterisk ($p < 0.001$; Mann–Whitney *U*). All data were normalized to the invariant endogenous rpl8 transcript (n = 10 for each bar).

At the 24 h time point, all E_2 concentrations examined evoked a significant increase in *vtg* transcript (3-, 4- and 31-fold for 0.5, 1.0, and 60 μg/l E_2, respectively; Fig. 5).

4. Discussion

Comparative multi-species analysis of promoter regions is a powerful strategy to identify evolutionary conserved *cis*-motifs that are highly likely to be functional [62,63]. Promoter analysis using OmVtg as reference sequence showed a high degree of conservation with the *vtg* proximal promoter of related *O. aureus*, however, we could not extrapolate additional information on *cis*-motifs that may have reserved functionality in conserved sequences between other species. In this study we detected putative *cis*-motifs that have previously been shown to act synergistically with ERE and/or estrogen response [64]. In addition, we identified a putative aryl hydrocarbon receptor (AhR) element. This may have important implications towards analysis in response to EDCs as it has been shown that there is regulatory cross-talk between AhR and ER in response to chemical induction and this led to the identification of genome-wide zebrafish biomarkers for AhR and ER agonists in response to chemical toxicity [65].

To have induction of mRNA transcription mediated through the *vtg* responsive elements, a basal level of vitellogenin is required. Since juveniles at 5–20 dpf are still feeding on their yolk sac, detection of low levels of *vtg* mRNA as early as 5 dpf was not unexpected. However, the translation of these transcripts into functional VTG has not been confirmed. There is some evidence that *O. mossambicus* utilizes a heterozygous male sex determination mechanism (XX female/XY male) [66]. The presence of putative ER and SRY-related (Sox-5, Sox-9) binding sites in the *vtg* promoter region indicates that sexually dimorphic modulation of gene expression may also occur involving these transcription factors, although such a regulatory mechanism remains undetermined at present. These data indicate higher expression of *vtg* mRNA in adult females when compared to males, leading us to speculate that this sex-dependent difference may arise from a combination of regulatory mechanisms that include ER and SRY action.

Results on putative differentially spliced *vtg* mature RNA argues strongly for ensuring the use of correct primer sets when using transcripts as indicators of estrogen exposure. A recent publication by Davis et al. [61] describes the cloning of three *vtg* fragments that appear to encode functional VTG protein in *O. mossambicus*. The partial cDNA fragments that were cloned by Davis et al. [61]

do not overlap with the ones identified in the present work and were only presented for adult male livers. The present study looked beyond this tissue type and developmental stage and specifically shows that that various splice alternatives may exist in different tissues. The two partial clones we sequenced which retained the "conventional" introns had stop codons when translated in any frame. This implies that a protein product is not expected from these transcripts and, thus, would not be detected by immunoblotting protein homogenates consistent with the lack of observed alternate proteins in Davis et al. [61].

Two biological platforms were assessed for the detection of estrogen-dependent induction of gene expression. The first instance represented a widely used system in which the low background expression of *vtg* in adult male liver forms the basis of the screening assay. Examples of this can be found for adult tilapia (*O. aureus* and *O. mossambicus*) and other teleost males [27,33,61,67–71]. In accordance with the present work on *O. mossambicus*, these studies demonstrated relatively low levels of *vtg* expressed in the liver prior to estrogen-associated induction. Upon E_2 exposure for 12 h (60 μg/l E_2), the steady-state levels of *O. mossambicus vtg* mRNA show a significant increase (4-fold). Lim et al. [27] reported a similar 20-fold increase in *vtg* mRNA in *O. aureus* at 72 h post-treatment with E_2.

While this adult-based assay clearly demonstrates an estrogen-dependent induction of *vtg* expression, it requires husbandry of large numbers of adult males and the isolation of liver tissue for analysis. The second assay platform investigated involves a simpler method that employs WBH preparation from 20 dpf juvenile *O. mossambicus* exposed to estrogenic substances for different exposure periods. The use of a QPCR primer set that recognizes only the liver-specific *vtg* transcript (Fig. 2) along with the application of juvenile stage animals increases assay sensitivity by allowing for the establishment of a low baseline expression when compared to adult male liver. Notably, fish at this early developmental stage were responsive to E_2 within a relatively short time frame (Fig. 5). Significant induced levels of *vtg* transcript were found following 12 h of exposure to 1.0 μg/l E_2 whereas exposure to lower concentrations of E2 (0.5 μg/l) resulted in increased *vtg* transcript after 24 h. These results confirm that *O. mossambicus* juveniles are responsive to a range of E_2 concentrations and suggest that exposure to lower concentrations indicative of environmental levels should be evaluated at the 24 h or later time points. The fact that no mortality was observed for exposed animals along the E_2 concentration gradient illustrates this species' robustness to assay conditions.

5. Conclusion

The present study identifies assay criteria, describes highly sensitive QPCR tools, and establishes important baseline information required to establish the indigenous *O. mossambicus* as a sentinel in Tier I screening for estrogenic activity in Southern African aquatic systems. The substantially lower pre-induction abundance levels of liver-specific *vtg* found in juveniles compared to adult males and the inducibility of *vtg* in WBH of juveniles suggests that the development of an assay based upon indigenous juvenile *O. mossambicus* has promise for detecting estrogenic EDCs in complex water sources.

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