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

M.M. Esterhuyse[a,*], M. Venter[b], N. Veldhoen[c], C.C. Helbing[c], J.H. van Wyk[a]

[a] *Ecophysiology Laboratory, Department of Botany and Zoology, Private Bag X1, Matieland, University of Stellenbosch, Nature Sciences Building, Stellenbosch 7602, South Africa*
[b] *Institute for Plant Biotechnology, Department of Genetics, Private Bag X1, Matieland Department of Genetics, University of Stellenbosch, Stellenbosch 7602, South Africa*
[c] *Department of Biochemistry and Microbiology, P.O. Box 3055 Stn CSC, University of Victoria, Victoria, BC V8W 3P6, Canada*

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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

* Corresponding author. Tel.: +27 21 808 3222; fax: +27 21 808 2405.
E-mail address: mme2@sun.ac.za (M.M. Esterhuyse).

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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.