**Exposure to estrogenic chemicals induces ectopic expression of *vtg* in the testis of
Rainbowfish, *Melanotaenia fluviatilis***

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Abstract

Vitellogenin (Vtg) is the major egg-yolk precursor protein in oviparous organisms normally synthesised only in mature females. In males and juveniles, the *vtg* gene, although present, is silent, but its hepatic expression may be activated by xenoestrogens. Surprisingly, its induction and potential consequences in non-hepatic tissues remain unexplored. Here we test the hepatic and testicular response of *vtg* expression in adult male rainbowfish *Melanotaenia fluviatilis* exposed to either 1, 3, 5 µg/L 17β-estradiol (E2) or 100, 500 µg/L 4-*n*-nonylphenol (NP) for 24 to 96 h. Significant increase in the expression level of *vtg* mRNA in the liver and testes of exposed males was observed. The early (24 h), sensitive and reliable detection of the *vtg* induction using qPCR demonstrates the assay's robustness to monitor xenobiotic exposure particularly in smaller fish like rainbowfish, an emerging indicator species. Ectopic induction of *vtg* mRNA in testes suggests a more complex Vtg pathway.

Keywords: Australian native, Xenoestrogen, Hepatic, Model species

Introduction

Globally, pollution in aquatic environments is increasing due to tremendous population growth. Due to a huge demand for food, many pesticides and herbicides are applied to agriculture crops, enabling high food production with intensive farming practices to meet the demand for the rapid expanding populations. It has been demonstrated that some of these chemicals mimic endogenous hormones and interfere with the endocrine system (Arukwe et al. 1997), and in particular, interfere with reproductive physiology in fishes (Cooper and Kavlock 1997). To understand the impact of these chemicals, many biomarkers have been identified for potential use in screening programs. One such biomarker of exposure to estrogenic chemicals is induction of vitellogenin (Vtg), which has been studied in many teleosts using methods including immunodetection, northern blotting, *in-situ* hybridization and more recently real-time PCR.

Vitellogenin is a glycolipophosphoprotein that is produced in all oviparous species, and its production is under the control of an endogenous hormone [17β -estradiol] (Amdam et al. 2003). In teleosts, it is synthesised in the parenchymal cells of the liver following stimulation of the vitellogenin gene (*vtg*) by estradiol, during oogenesis in mature females, when increasing concentrations of estrogens are produced by ovarian granulosa cells (Denslow et al. 1999; Shi et al. 2006) via estrogen receptors. The production of endogenous estrogens that subsequently leads to the production of Vtg is influenced by environmental cues including water temperature and photoperiod (Arukwe et al. 1997). The primary function of Vtg is to provide nutritional support to the developing embryo rather than playing a major role as a functional protein (Denslow et al. 1999). Moreover,

it has been reported that the Vtg sequence is not conserved among fish species (Lee et al. 1992; Denslow et al. 1999) and comprises multiple sub-units with varying molecular weight between fish species (Lee et al. 1992; Leusch et al. 2005). Normally, Vtg is restricted only to mature females; however, the gene for Vtg is also present in males and juveniles, and thus can be activated upon exposure to xenoestrogens. Activation of the nuclear *vtg* gene receptor, which requires estrogen responsive elements (EREs), results in *vtg* mRNA synthesis, followed by Vtg protein translation (Guellec et al. 1988; Ding et al. 1990). Therefore, the presence of *vtg* mRNA and protein in the liver as well as blood plasma of male and juvenile fishes has been widely utilized as a biomarker of exposure to xenoestrogens in many teleosts (Denslow et al. 1999) including the rainbowfish (Woods and Kumar 2011). However, the induction of *vtg* mRNA expression in gonads and its consequences have received little or no attention to date.

The rainbowfish, *Melanotaenia fluviatilis* is endemic to the Murray Darling basin, Australia, and its wild populations are increasingly under climatic and anthropogenic threats (Balcombe et al. 2011). Because of its amenability to husbandry and short reproductive cycle, the species is emerging as a model indicator of aquatic ecology and ecotoxicology (Pollino et al. 2007; Shanthanagouda et al. 2013). We report here the induction of *vtg* mRNA in liver and testes, and the presence of Vtg protein in the plasma of male rainbowfish as potential biomarkers of exposure to two estrogenic chemicals including the exogenous 17 β -oestradiol (E2) and the estrogen mimic nonylphenol (NP). An earlier study (Woods and Kumar 2011) has explored induction of *vtg* mRNA in this species but were limited to hepatic tissue. This is the first study to test the *vtg* mRNA expressions in both liver and gonads in short term exposures to xenoestrogens.

Materials and Methods

Adult rainbowfish were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Melbourne, Australia) and reared at $24\pm 1^{\circ}\text{C}$ in 16 h light/8 h dark regime in flow-through aquaria with carbon filtered aerated water. Throughout the maintenance, water quality parameters including temperature, dissolved oxygen, pH and electric conductivity (Shanthanagouda et al. 2013) were regularly monitored (TPS Ionode, Victoria, Australia). Fish were maintained in holding tanks for ~2 weeks before they were transferred to experimental tanks.

Adult male rainbowfish were exposed to either 1, 3, 5 $\mu\text{g/L}$ E2 [$\geq 98\%$ purity] (CAS 50-28-2, Sigma Aldrich) or 100, 500 $\mu\text{g/L}$ estrogenic mimic 4-*n*-nonylphenol [NP, $\geq 98\%$ purity] (CAS 104-40-5, Sigma Aldrich) for 24, 48, 72 and 96 h and unexposed females were used as a positive control. Both control and solvent controls were also run in parallel. All chemicals used in this study were purchased from Sigma-Aldrich Pty. Ltd. Ethanol was used to dissolve and dilute both chemicals to obtain appropriate stock solutions. Respective stock solution was then dispensed at the rate of 10 $\mu\text{L/L}$ of water to obtain required exposure concentrations. During the experiment, exposure water was renewed daily until termination of the experiment. From each treatment, three different individuals were sampled (one from each replicate) after 24, 48, 72 and 96 h and euthanased in 80 mg/L AQUIS [Iso-eugenol] (Lower Hutt, New Zealand). The length (cm) and weight (g) of individual fish were measured and the condition factor was calculated using the formula $K = \text{body weight}/(\text{total length})^3 \times 100$ (Shanthanagouda et al. 2013). Blood samples were collected using 0.3 mL heparinised syringes with 30 gauge needles (BD Microlane™, Ireland) by either caudal puncture or by severing of the dorsal artery. Blood samples were spun at 5,000 g for 15 min at 4°C to separate plasma from red blood cells. All samples were stored at -80°C for further assays.

Gene-specific oligonucleotides were designed on primer express software (Applied Biosystems Pty. Ltd.) and synthesized by Sigma-Proligo (Sigma–Aldrich Pty. Ltd.). Primers were based on a published cDNA sequence of *M. fluviatilis* vitellogenin (Woods 2007). GAPDH was used as an internal control to normalize *vtg* cDNA abundance, and *gapdh* primers were based on *M. fluviatilis* sequence previously described by Ponza (2006). Primer sequences are presented in Table 1. The expression of *gapdh* transcripts did not show significant differences (in controls and treated fish) as stated by Shanthanagouda et al. (2013), thus demonstrating its suitability as a reliable reference gene. Primers were designed to amplify 126, and 100 bp for *vtg* and *gapdh* respectively. Then, total RNAs were extracted from each tissue using RNeasy lipid tissue mini kits including a DNase step to eliminate eventual genomic DNA contamination (Qiagen Pty. Ltd.) and further expression analyses were conducted as described by Patil and Gunasekera (2008) and Shanthanagouda et al. (2012).

The size of Vtg proteins varies among fishes, so 8% acrylamide gels were used for electrophoresis. Immunodetection was conducted using a commercial antibody raised against Vtg of Japanese medaka (CK-4B3, Biosense Laboratory) as described by Ponza (2006).

Real-time data was collected, compiled and the cycle threshold was calculated automatically using a CFX software package (Bio Rad Pty. Ltd.). Data for *vtg* expression was analysed using the publicly available Q-gene Excel script package (Muller et al. 2002). Data for *vtg* expression from each sample was averaged and shown as Mean Normalized Expression ($MNE \pm SE$) and analysed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons between the treatments and time of exposure, and the interaction between chemical dose and different

time points using SPSS17.0 (SPSS Inc. 2008). Data means were considered significantly different from each other at $P < 0.05$.

Results

Induction of *vtg* mRNA in the liver of male fish exposed to E2 was observed throughout the experiment, and the expression remained significantly above the controls (Fig. 1A). Exposure of fish to 1 $\mu\text{g/L}$ E2 resulted in *vtg* induction that was significantly higher than controls. Fish exposed to 3 $\mu\text{g/L}$ E2 had *vtg* mRNA levels which fluctuated—initially increasing at 24 and 48 h, decreasing at 72 h and again increasing at 96 h. Fish exposed to 5 $\mu\text{g/L}$ E2 showed elevated *vtg* level at 24 h and reached a peak at 48 and 72 h, then declined at 96 h. Despite these fluctuations, *vtg* in male fish treated with all three concentrations (1, 3 and 5 $\mu\text{g/L}$ E2) consistently remained significantly higher than controls. In unexposed female livers (positive control) the level of *vtg* was significantly higher than the livers of exposed and control males (Fig. 1A). These results implicate the significant effect of treatment and exposure on *vtg* expression in liver tissues in response to E2. In fish exposed to 100 $\mu\text{g/L}$ NP, *vtg* mRNA showed somewhat cyclic expression at the four sampling time points (Fig. 1B), increasing at 24 and 72 h, and decreasing at 48 and 96 h respectively. Nonetheless, the induction was significantly greater than controls throughout the experimental period. In slight contrast, with exposure to 500 $\mu\text{g/L}$ NP, the expression level of *vtg* mRNA remained elevated but comparable at both 24 and 48 h exposure. Significant reduction was observed at 72 h, followed by a significant increase at 96 h. Similar to E2 exposure, the expression level remained significantly higher in NP exposed fish compared to that of controls (Fig. 1B). Results showed a significant effect of treatment and exposure time on *vtg* level in liver tissues in response to NP.

Table 1. List of primers used in real-time PCR for *vtg* expression

Primer Name	Sequence (5' - 3')	Amplicon
<i>VtgF</i>	GAAAGCGGTTGTGCTTGCCCTGACTCTG	<i>vtg</i>
<i>VtgR</i>	GGCATTCCTCCTGGGAGGTCTGCC	<i>vtg</i>
<i>GAPDHf</i>	ACACCCACTCCTCCATCTTT	<i>gapdh</i>
<i>GAPDHR</i>	GTTGCTGTAGCCGAACATCAT	<i>gapdh</i>

Fig. 1. Expression of *vtg* mRNA in rainbowfish exposed to E2 and NP. A) Vitellogenin mRNA in livers of male fish exposed to E2 ($F_{0.05, df=19, 40}=6.45$), B) Expression of *vtg* mRNA in livers of male fish exposed to NP ($F_{0.05, df=15, 32}=10.658$), C) Expression of *vtg* in testes of male fish exposed to E2 ($F_{0.05, df=19, 40}=4.057$), D) Expression of *vtg* in testes of male fish exposed to NP ($F_{0.05, df=15, 32}=3.191$). X-axis labels: F.Liver-Female Liver, M.C.Liver-Male Control Liver, M. SC. Liver-Male Solvent Control Liver. C. Testes—Control Testes, SC testes-Solvent Control Testes. Values described by the same letter are not significantly different from each other ($n=3$, $P<0.05$).

Following exposure to E2, the *vtg* expression in the testes of all exposed fish (all concentrations) was significantly higher compared to control testes and ovary of females, generally exhibiting an initial increase in expression, followed by a decrease in

expression by 96 h (Fig. 1C). ANOVA results showed a significant effect of treatment and exposure on *vtg* expression in testes tissues with exposure to E2. Similarly, exposure to both concentrations of NP also resulted in a significant increase in *vtg* expression throughout the exposure, exhibiting initial increase in expression, followed by a decrease in expression by 96 h (Fig. 1D). Results demonstrated a significant effect of treatment and exposure on *vtg* expression in testes tissues in response to NP.

Vitellogenin protein expression was observed only at 3 and 5 µg/L E2 at 48 h (Fig. 2) whilst no protein expression was observed at 72 and 96 h (data not shown). The size of the band was ~75 kDa, and corresponded to the size of one of the bands observed in plasma from female rainbowfish (positive control) (Fig. 2). While for NP (100 and 500 µg/L) exposed fish, no Vtg protein expression was detected at all throughout the experiment (data not shown).

Discussion

The results of this study illustrated that the induction of *vtg* gene expression occurs not only in liver but also in testes of male rainbowfish following exposures to E2 and NP. The elevated expression of *vtg* transcripts in the liver and testes of exposed males was strongly evident by qPCR analysis, while the detection of Vtg protein in plasma was not consistent.

An increase in *vtg* mRNA expression was observed in male livers exposed to either E2 or NP at all concentrations tested. Similar results were also observed in male sheephead minnows exposed to both E2 (0.089, 0.71 µg/L) and *p*-NP (5.6, 59.6 µg/L) in individual exposures (Hemmer et al. 2002), medaka exposed to either E2 (0.1–10µg/L), EE2 (25–100 ng/L) or nonylphenol (20–50 µg/L) (Islinger et al. 2002; Yamaguchi et al. 2005),

mosquitofish exposed to 250 ng/L E2 (Leusch et al. 2005) and Japanese whiting exposed to 50 ng/L E2 and 40 µg/L 4-*tert*-octylophenol (Yoon et al. 2008). In a recent study on *vtg* in adult male rainbowfish exposed to either E2 or EE2 (1 to 100 ng/L), a dose dependent increase in hepatic mRNA induction was observed for both chemicals (Woods and Kumar 2011). As these examples indicate, expression of *vtg* is consistently induced in the livers of male teleosts in response to E2 or NP, and hence, *vtg* serves as a suitable biomarker of exposure to (xeno) estrogenic chemicals, as shown in the present study.

Throughout this study, *vtg* expression in treated male gonads (testes) was higher than both control testes as well as ovarian tissues. The expression of *vtg* mRNA in the testes indirectly indicates the presence of estrogen receptors as was observed in spotted ray, *Torpedo marmorata* testes (Giudice et al. 2011). Such extra-hepatic expression of the *vtg* gene in testes following exposure to estrogenic chemicals indicates that the liver is not the only site for Vtg induction. Conceivably, the ectopic expression of *vtg* in the testes of exposed *M. fluviatilis* may lead to subsequent production of Vtg protein. This could potentially impair gamete development or damage the testicular architecture, via accumulation of Vtg in blood vessels and surrounding tissues. Interestingly, we also observed basal low level expression of *vtg* in unexposed gonads (both testes and ovaries). In case of females, ectopic detection of basal *vtg* in the ovaries could be due to the transportation of mRNA from the liver to the ovaries along with Vtg protein. It is unknown as to what role such basal expression play in the gonads, but imply a much more complex regulatory and synthetic *vtg* pathway than previously known.

In this study, the minimum concentration of E2 that led to induction of Vtg protein in male rainbowfish was 3 µg/L. In contrast, 100 or 500 µg/L NP was not sufficient to induce Vtg protein synthesis in male rainbowfish and hence, it is concluded that the 17β-

estradiol is a more potent estrogenic chemical compared to the nonylphenol. As expected, Vtg protein was detected in female plasma, but the response was not consistent. This may reflect individual differences or inconsistency in reproducibility of the technique adopted. Given that the medaka Vtg protein (positive control) generated consistent banding patterns in Western blot analysis, we believe the inconsistent response is not due to error in the protocol used, but rather that the antibody used did not have a high affinity for rainbowfish antigens. Such issues have been commonly reported where heterologous testing systems are employed (Arukwe and Goksøyr 2003) and highlight the need of species-specific antibody generation for sensitive and quantitative measurement of Vtg protein induction.

Fig. 2. Immunodetection of Vtg protein in plasma of adult Murray River rainbowfish with exposures to xenoestrogens. Immunodetection was carried out using Japanese Medaka anti-Vtg monoclonal Antibody (CK-4B3). The values at the left indicate protein marker and the lane numbers indicate 1: Protein marker, 2: Female plasma (unexposed), 3: Male plasma (fish exposed to 3 µg/L E2 after 48 h), 4: Male plasma (fish exposed to 5 µg/L E2 after 48 h) 5: Male plasma (solvent control), 6: Medaka purified Vtg protein. Arrow marks on the figure indicate vitellogenin bands.

Although the medaka Vtg antibody appears to cross-react with *M. fluviatilis* plasma Vtg, the banding pattern observed between different individuals was inconsistent.

Unfortunately, the low volume of plasma obtained from each fish limited the amount of analysis that could be done. In a similar study using Murray rainbowfish, Woods and Kumar (2011) used a different Vtg antibody (Anti-Seabream), and observed Vtg banding patterns at a molecular weight of 250 kDa. This supports the notion that in the present study, the smaller fragments observed at ~75 kDa may represent partial degradation products as opposed to intact Vtg sub units.

In contrast to the observations in this study, the ability of nonylphenol (NP) to induce Vtg induction in immature fish and in males has been reported in a number of teleosts including male zebrafish exposed to 100 and 500 µg/L NP (Yang et al. 2006), immature rainbow trout exposed to 20.3 µg/L NP (Jobling et al. 1996), and male fathead minnows exposed to 100 µg/L NP (Harries et al. 2000) for 3 weeks. Perhaps the shorter exposure duration in this study (96 h) was insufficient to induce Vtg protein expression in livers. Therefore, studies incorporating longer exposure periods may be required to detect any Vtg responses following exposure.

Conclusions

Detection of *vtg* mRNA in liver and testes using qPCR is suitable, sensitive, quick and reliable. With further development to improve the consistency in response over different concentrations and different time periods, it could potentially be utilized as a biomarker to detect exposure to xenoestrogens such as E2 and NP, particularly in both liver and testes of male *M. fluviatilis*. This unscheduled *vtg* expression detected at mRNA level could lead to reproductive dysfunction in the physiology of male *M. fluviatilis*. Collectively, we conclude that measurements of Vtg expression at transcript level could be used as a robust indicator of exposure to estrogenic contaminants using qPCR.

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