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Effects of exposure to oestrogenic compounds on aromatase gene expression are gender dependent in the rainbowfish, *Melanotaenia fluviatilis*

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ABSTRACT

This study investigated the influence of two endocrine disrupting chemicals (EDCs)—an exogenous oestrogen 17β-estradiol (E2) and the oestrogen mimic 4-*n*-nonylphenol (NP) on the expression of aromatase transcripts in both sexes of adult Murray river rainbowfish. Reproductively active mature fish were exposed to 1, 3, and 5 μg/L E2 or 100 and 500 μg/L NP for 24, 48, 72 and 96 h. The results show a significant reduction in the expression of *cyp19a1a* isoform in ovarian tissues with complete inhibition at the higher concentrations (3 and 5 μg/L E2; 500 μg/L NP between 24 and 72 h) and at all concentrations after 96 h. There was no expression of the *cyp19a1a* isoform in female brain, male brain or testes in any treatment. E2 significantly increased expression of *cyp19a1b* in female brain except at 5 μg/L after 24 h exposure. In male brain tissue E2 exposure decreased *cyp19a1b* expression except at 1 and 5 μg/L at 24 h. NP significantly upregulated *cyp19a1b* in the female brain (except with 500 μg/L at 72 h) and in testes tissues. NP downregulated expression of *cyp19a1b* in the male brain tissue. Collectively, these observations support the hypothesis that the expression of *cyp19a1b* is regulated via both positive and negative feedback mechanisms, with differential modulation based on the type and concentration of the exposed oestrogens, duration of exposure, fish tissue and gender of the fish. The results also imply that exogenous oestrogens can have a disruptive effect on the steroidogenic pathway and may lead to effects on sex differentiation, sexual behaviour and reproductive cycles in this fish.

1. Introduction

Aquatic as well as terrestrial organisms are exposed to various chemicals, which disrupt their endocrine system and physiology. Presently, over 200,000 varieties of chemicals enter into aquatic systems (Tyler et al., 2008) and some of these mimic endogenous steroid hormones of animals (Colborn et al., 1993). They interfere with the endocrine system of the non-target organisms, mainly fish often leading to trans-generational effects (Kavlock et al., 1996; Vos et al., 2000). These EDCs induce physiological changes by binding to the ERs (Marino et al., 2006; Gao and Wright, 2011). Within the organism, these chemicals act similarly or antagonistically to that of endogenous hormones (Kuhl et al., 2005) resulting in alterations ranging from skewed sex ratios in alligators (Lance and Bogart, 1992), birds (Abinawanto et al., 1996) and reptiles (Wibbels and Crews, 1994) to inter-sex in common carp and roach (Gimeno et al., 1996; Folmar et al., 2001; Jobling et al., 2002) and feminized testes in medaka (Gronen et al., 1999).

It is well recognised that oestrogens play a vital role in neural development, metabolism, growth, sex differentiation, sexual behaviour, and reproduction and also regulate other physiological functions (Gen et al., 2001; Diotel et al., 2010). Oestrogens play a role in the control of cell cycles and proliferation including uterine and neuronal growth and differentiation (Cheshenko et al., 2008). Mechanistically, oestrogens bind to oestrogen receptors (ERs) on the cell membrane as well as in the cytoplasm (Marino et al., 2006; Gao and Wright, 2011) facilitating their activation, dimerization and binding to specific DNA sequences named oestrogen responsive elements (EREs) leading to regulation of target gene transcription (Cheshenko et al., 2007). This mechanism is often disturbed by natural and synthetic Endocrine Disrupting Chemicals (EDCs) (Colborn et al., 1993).

A number of genes and gene pathways are affected by EDCs, among which aromatase (*cyp19a*) is a key target for either direct or indirect EDC action, due to its central role in sex steroid biosynthesis (Marino et al., 2006; Gao and Wright, 2011). The Cyp19a aromatase is a key steroidogenic enzyme, catalysing the final and critical step in oestrogen biosynthesis, thus controlling many physiological processes (Simpson et al., 2002). Unlike higher vertebrates (with the exception of porcines), most teleosts have two different *cyp19a* genes—one predominantly expressed in the ovary (*cyp19a1a*) and the other

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preferentially in the brain (*cyp19a1b*) (Tchoudakova and Callard, 1998; Blázquez and Piferrer, 2004; Guigen et al., 2010). *Cyp19a1a* is believed to play a role in sex differentiation and gonad development and *cyp19a1b* is presumed to play a role in neuroprotection, neurogenesis, shaping and mating behaviour in fish (Tchoudakova and Callard, 1998; Barney et al., 2008; Patil and Gunasekera, 2008; Guigen et al., 2010).

Fish are considered sentinel for evaluating effects of aquatic pollution as their endocrine system is well understood and they are often exposed to various sources of EDCs in the aquatic environment (Matthiessen, 2003). Aromatase expression can be an indicator of the effects of EDCs on the endocrine system of fishes as demonstrated in many teleosts (Hahlbeck et al., 2004; Kazeto et al., 2004; Kallivretaki et al., 2006; Sun et al., 2007; Villeneuve et al., 2009; Diotel et al., 2010). However, majority of the earlier toxicological studies focus on the response of *cyp19a1a* and not *cyp19a1b*, despite the observations that it is the latter that responds more readily to exogenous oestrogens (Diotel et al., 2010). Ready response of *cyp19a1b* compared to *cyp19a1a* is also supported by the presence of full ERE elements in the promoter region of the former and not the latter in many teleosts (Guigen et al., 2010). We therefore hypothesised and tested that *cyp19a1b* is a better reporter of oestrogenic EDC exposure in teleosts, noting that *cyp19a1a* is not expressed in the testes of this species (Shanthanagouda et al., 2012). In the current study we investigate the ability of the exogenous oestrogens (E2 and NP) to ectopically induce *cyp19a1a* expression in testes and its suitability as a reporter of xenoestrogenic toxicity using this model species. The sensitivity of the rainbowfish, *Melanotaenia fluviatilis* to toxicants is well established (Pollino et al., 2007), including molecular responses (Woods et al., 2009; Woods and Kumar, 2011). The cDNA sequences for *cyp19a* as well as their basal expression in the species were recently established (Shanthanagouda et al., 2012).

The specific objective of this study was to investigate the effect of an exogenous E2 and the oestrogen mimic NP on the expression of aromatase mRNA in brain and gonads in both sexes of *M. fluviatilis*. The experiments were designed to test if the chemicals would elicit effects consistent with their presumptive mode of action and if so, which of the isoforms responds readily to the EDCs and whether these responses would be time, concentration and gender dependent in a sexually dimorphic fish.

2. Material and methods

2.1. Experimental fish

Reproductively active mature adult Murray river rainbowfish (*M. fluviatilis*, Atheriniformes: Melanotaeniidae) were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Melbourne, Australia) and held at 24 ± 1 °C in 16:8 h light: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 were monitored (TPS Ionode, Victoria, Australia). Fish were maintained in holding tanks for ~2 weeks before they were transferred to experimental tanks. During holding they were fed commercial fish pellets (Tetra colour™, Blacksburg, VA, USA) twice daily.

2.2. Chemicals and experimental design

All chemicals (molecular biology grade) used in this study were purchased from Sigma-Aldrich Pty. Ltd. Reproductively active, ~18 months old male (5.49 ± 0.25 cm) and female (5.32 ± 0.32 cm) fish were used for exposure studies in 10 L water (in 12 L glass tanks) and to each of the three replicates 6 individuals of single sex fish were transferred. Stock solutions and dilutions were prepared in absolute analytical grade ethanol for both chemicals and stored in the dark and 10 μL (0.0001%) of the stock was added to each replicate to obtain 1, 3, and 5 μg/L E2 and 100 and 500 μg/L oestrogen mimic 4-*n*-nonylphenol (NP). Similar concentrations of these chemicals have been shown to elicit vitellogenin mRNA expression in key experimental fish models including medaka (Islinger et al., 2002) and zebrafish (Hinfray et al., 2006; Yang et al., 2006). Analytical grade ethanol was used as a solvent to dissolve both chemicals at a concentration of 0.0001%. Exposure of fish to both chemicals (E2 and NP) was conducted in parallel; therefore the controls were common for both chemical treatments. However, males and females were exposed separately. In this study, only the nominal concentrations were used and throughout the exposure experiment, with 25% renewal daily until the termination of the experiment after 96 h of exposure. These chemicals are known to be relatively stable in water over a period of 8–14 days (Rose et al., 2002; Pickford et al., 2003) and hence would be stable over the exposure period of 96 h used in the current study in which nominal concentrations are reported. During the experiments, fish were fed frozen brine shrimp and rotifers (Kyorin Co. Ltd.) once daily at 4% (wet weight) of body weight. A total of three individuals were sampled (n = 3) from each treatment (1 from each replicate) after 24, 48, 72 and 96 h and anaesthetised at 80 ppm of AQUI-S (Iso-eugenol) (Lower Hutt, New Zealand) and decapitated. The length (cm) and mass (g) of individual fish were measured and the condition factor was calculated using the formula $K = \text{body weight}/(\text{total length})^3 \times 100$. Samples of brain and gonads were collected and were stored in RNA*later* (Sigma-Aldrich Pty. Ltd.) according to the manufacturer's instructions for total RNA extraction and real-time analysis. All experimental procedures were conducted under the Royal Melbourne Institute of Technology; Animal Ethic Committee (RMIT AEC) approved project number 0732.

2.3. Water quality parameters

General water quality parameters were monitored daily until the termination of the experiment. Throughout the exposure period, the water quality parameters including pH, DO_2, temperature and conductivity were consistent between treatments and the results are presented in Table 2.

2.4. cDNA synthesis, oligonucleotides and qPCR

Total RNAs were extracted from each tissue using RNeasy lipid tissue mini kits including DNase step to eliminate eventual genomic DNA contamination (Qiagen Pty. Ltd. Australia). The tissues were homogenized in 1 mL QIAzol lysis buffer using a sterile disposable syringe head. The RNA integrity was checked by electrophoresis on a 1.0% agarose gel in TAE (40 mM Tris, 40 mM EDTA, 1 mM glacial acetic acid) buffer and visualised under UV light. Absorbance ratio at 260/280 nm was also quantified using a spectrophotometer. Subsequently, cDNA synthesis was carried out for quantitative Real-Time PCR (qPCR) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Pty. Ltd. Australia). The qPCR was carried out on a MJ MiniOpticon system version 3.1 (Bio-Rad Pty. Ltd.) with SYBR green fluorescent label. The cycling parameters for the qPCR were as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 62 °C for 1 min. A melting curve analysis was performed at the end of the amplification phase with a minimum of 30 °C to a maximum of 95 °C, with 0.2 °C increases for every 0.02 s to test the specificity and identity of the qPCR products. Primers for qPCR for *cyp19a1a* (Genbank Accession no. GU723457), *cyp19a1b* (GU723458) and *gapdh* (Ponza, 2006) were designed (Sigma-Aldrich Pty. Ltd. NSW or Gene-Works Pty. Ltd. SA, Australia) using primer express (ABI) software based on the cDNA sequences obtained. The primers (Table 1) were designed at the 3′ end of the cDNA sequences and amplified 229, 161 and 100 bp for *cyp19a1a*, *cyp19a1b* and *gapdh* respectively.

Expression of *cyp19a1a* and *cyp19a1b* isoforms was measured in the gonad and brain tissues of both sexes in triplicates for each individual