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Simultaneous determination of androgenic and estrogenic endpoints in the threespine stickleback (*Gasterosteus aculeatus*) using quantitative RT-PCR

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ABSTRACT

A method to evaluate the expression of three hormone responsive genes, vitellogenin (estrogens), spiggin (androgens), and an androgen receptor (ARβ) using real-time PCR in threespine stickleback is presented. Primers were designed from previously characterised spiggin and ARβ sequences, while a homology cloning strategy was used to isolate a partial gene sequence for stickleback vitellogenin (Vtg). Spiggin mRNA was significantly higher in kidneys of field-caught males compared to females by greater than five orders of magnitude while ARβ levels were only 1.4-fold higher in males. Female fish had four order of magnitude higher liver Vtg expression than wild-captured males. To determine the sensitivity of these genes to induction by hormones, male and female sticklebacks were exposed to 1, 10 and 100 ng/L of methyltestosterone (MT) or estradiol (E2) in a flow-through exposure system for 7 days. Spiggin induction in females, and Vtg induction in males were both detectable at 10 ng/L of MT and E2, respectively. MT exposure did not induce ARβ expression in the kidneys of female stickleback. In vitro gonadal steroid hormones production was measured in testes and ovaries of exposed stickleback to compare gene expression endpoints to an endpoint of hormonal reproductive alteration. Reduction in testosterone production in ovaries at all three MT exposure concentrations, and ovarian estradiol synthesis at the 100 ng/L exposure were the only effects observed in the in vitro steroidogenesis for either hormone exposure. Application of these methods to assess both androgenic, estrogenic, and anti-steroidogenic properties of environmental contaminants in a single fish species will be a valuable tool for identifying compounds causing reproductive dysfunction in fishes.

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1. Introduction

There is widespread concern that effluents from industrial and municipal sources can affect the development and reproduction of fishes. Exposure to these endocrine disrupting compounds has been linked to altered hormone levels, abnormal gonad morphology, and delayed sexual development (Goksoyr, 2006; Rempel and Schlenk, 2008). Altered circulating hormone levels have been shown to be linked to decreased capacity of the gonad to synthesise sex steroid hormones (Van der Kraak et al., 1992). Underlying the physiological responses are changes in the expression of key genes and proteins responsible for hormone signalling (Arukwe, 2001). Recently, evidence has been presented that links exposure to such compounds to the collapse of fish populations (Kidd et al., 2007).

Although the majority of research has focused on the occurrence of environmental estrogens, it is now apparent that effluents can also possess androgenic and anti-androgenic properties. Masculinization of female mosquitofish by pulp and paper effluent was documented over 25 years ago (Howell et al., 1980) and since then there have been reports of masculinization of fish associated with pulp and paper mill effluent elsewhere (Ellis et al., 2003). Also of potential ecological significance is evidence of male-biased sex ratios in individuals collected from or exposed to effluent-contaminated waters (Larsson and Forlin, 2002; Orn et al., 2006), demonstrating a potential for androgenic impacts at the population level. Another emerging androgen receptor agonist is trenbolone acetate, a synthetic growth promoter in beef cattle and excreted in animal waste (Ankley et al., 2003; Sone et al., 2005).

Given that effluents can contain compounds with both estrogenic and androgenic activity, the ability to simultaneously assess estrogenic and androgenic potential is an important characteristic for a potential monitoring species. The threespine stickleback (*Gasterosteus aculeatus*) is relatively unique in that it possesses a well-characterised androgen responsive gene encoding for the

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spiggin protein. Spiggin is a glycoprotein synthesised in the kidney of male sticklebacks and produced as part of a glue-like secretion, which is used for nest-building during the breeding period (Jones et al., 2001). Hence, the expression of spiggin in kidney from female stickleback is an excellent indicator of exposure to androgenic compounds.

Two enzyme-linked immunosorbent assay (ELISA) methods have been developed for quantifying spiggin protein in stickleback kidneys (Katsiadaki et al., 2002a; Sanchez et al., 2008a). In Katsiadaki et al. (2006) the authors describe the development of a bioassay to assess the anti-androgenicity of model compounds and environmental samples. It is also possible to simultaneously assess both androgenic and estrogenic responses in vivo using juvenile fish (Hahlbeck et al., 2004) and in vitro using a kidney cell culture assay (Bjorkblom et al., 2007; Jolly et al., 2006). Recently, spiggin protein was measured in wild-caught sticklebacks to assess the androgenicity of a French river (Sanchez et al., 2008b). However, techniques for spiggin quantification are not commercially available and there is little information regarding transcript-level responses for these androgenic and estrogenic biomarkers in stickleback. A recent paper by Nagae et al. (2007) appears to demonstrate an induction of spiggin mRNA following exposure to a single high dose of androgen; thus the sensitivity of this technique has not been thoroughly tested. Simultaneous measurement of spiggin and Vtg transcript levels could have significant application in short-term laboratory bioassays and field-based biomonitoring.

The present paper describes the development and validation of a real-time PCR method to assess the expression of spiggin, Vtg and an androgen receptor in the threespine stickleback. Sex differences in transcript abundance were assessed using reproductively mature males and females. A short-term exposure to low concentrations (ng/L range) of the model compounds, methyltestosterone (MT) and estradiol (E2), was then employed to determine the hormonal responsiveness of these transcripts. In addition, we investigated the effects of these hormones on gonadal steroid hormone production using an in vitro steroidogenesis bioassay.

2. Materials and methods

2.1. Animals

Threespine stickleback used to assess sex differences in the expression of spiggin, androgen receptor and Vtg were collected during their early summer breeding period in June, 2007 in the Stanley River Estuary, Prince Edward Island, Canada, using a 1.5 m × 25 m, 3 mm mesh seine net. Adult male and female stickleback for steroid exposure experiments were captured from the Flat River on Prince Edward Island, Canada. The Flat River is a largely forested watershed (~80% of land area) with some mixed row crop and livestock agriculture and no point sources of pollution. Animals for exposure experiments were collected using electric shocking during December 2007. Groups of 50 fish were housed in 20 L glass aquaria initially containing 5 °C brackish water (5 ppt) to reduce stress and prevent disease. Water temperature was increased to 15 °C at approximately 1 °C per day and salt water was gradually replaced with fresh water over the same time period. The initial photoperiod was 10:14 (light:dark) and was increased to gradually to 14:10 by mid January. All stickleback were exposed to the lengthened photoperiod for at least 3 weeks prior to experimentation. The water in the tanks was aerated continuously and 50% of the water was renewed every 3 days. Throughout this acclimation period and during the exposure period, the fish were fed daily with a combination of dried flake food, dried blood worms (Nutrafin, Montreal, QC) and live *Artemia* nauplii (Ward's Scientific, Rochester, NY, USA).

2.2. Laboratory exposures and sampling

The chemicals 17α-methyltestosterone and 17β-estradiol, used for the fish exposures, were purchased from Sigma (Oakville, ON, Canada). Male and female adult sticklebacks were transferred from holding tanks to 80 L gel-coated fibreglass tanks for 1 week prior to experimentation (50 fish/tank). Fish were exposed continuously to chemicals for 7 days in a flow-through system. Water was supplied continuously into the tanks at a rate of 80 mL/min corresponding to a 75% replacement time in 24 h and a fish loading of 0.5 g/L/d. Chemicals were added to the diluent water flow from acetone stock solutions at a rate of 4 μL/min by a peristaltic pump. The resulting nominal flow-through waterborne steroid exposure concentrations were 0 (acetone carrier control), 1, 10 and 100 ng/L of steroid and an acetone solvent concentration of 0.005% in the exposure tanks. A single tank of fish was exposed at each concentration. Flow rates were checked each day and stock solutions were monitored to ensure that a constant volume of solution had been used across treatments.

At the end of the exposure, the weight and length of each fish was recorded. Fish were stunned by a blow to the head and killed by decapitation. The gonads, kidneys and liver were excised and weighed. Kidney and liver were placed in 500 μL RNAlater solution (Sigma) and were stored at 4 °C overnight and then at −20 °C until RNA extraction. Ovarian and testicular tissues were placed in Medium 199 supplemented with 25 mM Hepes, 4.0 mM sodium bicarbonate, 0.01% streptomycin sulphate and 0.1% bovine serum albumin (pH 7.4) in 24-well plates and kept on ice as per in vitro steroid incubations described below.

Kidney and liver tissues were collected in order to validate the real-time PCR protocol for measurement of spiggin, ARβ and Vtg mRNA and to compare their relative expression between sexes. Eight male and eight female individuals were sacrificed and tissues were stored for RNA extraction as described above.

2.3. RNA extraction and reverse transcription

Total RNA was obtained from kidney and liver using the QIAGEN RNeasy Mini Kit (including the RNase-free DNase set) as described by the manufacturer (Qiagen, Mississauga, ON, Canada). The RNA was resuspended in RNase-free water and stored at −80 °C. The RNA concentrations were quantified with the Qubit Fluorometer (Invitrogen, Burlington, ON, Canada). The Quant-iT RNA Assay Kit (Invitrogen) was used, and calibration was performed using a two-point standard curve according to manufacturer's instructions. Total cDNA was prepared from 1 μg total RNA using the iScript cDNA Synthesis Kit as described by the manufacturer (BioRad Laboratories Inc.). The 20 μL reaction volume was diluted 10-fold prior to PCR amplification.

2.4. Cloning of stickleback Vtg

A species-specific cDNA sequence for Vtg was cloned from stickleback liver cDNA. Degenerate primers were designed against conserved sequences from various fish species obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and constructed using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) programs. Primer sequences were: sense primer 5′-TG(ATC)TGAAGCT(CT)(AG)TGGA(ACTG)CCT-3′; antisense primer 5′-AA(TG)GGG (TG)(CT)GCTGAAGAA(TG)(CT)TC-3′. The PCR mixture (25 μL final volume) contained 1.0× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM forward and reverse primers, 0.02 U Taq® DNA Polymerase, and 2 μL of cDNA template. All PCR reagents and primers were from Invitrogen. PCR amplifi-

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