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Biomarker responses to estrogen and androgen exposure in the brook stickleback (*Culaea inconstans*): A new bioindicator species for endocrine disrupting compounds

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ABSTRACT

Small-bodied freshwater fish are commonly used in regulatory testing for endocrine disrupting compounds (EDCs) but most lack a sensitive and quantifiable androgen-specific biomarker. Brook stickleback (*Culaea inconstans*) are a North American freshwater fish whose males produce an androgen-regulated glycoprotein in the kidney called spiggin. Although spiggin induction in females has been used as an androgen-specific biomarker of exposure in other stickleback species it has not been characterized in brook stickleback. Therefore, our objective was to develop a bioassay using brook stickleback to measure estrogenic and androgenic responses and establish the sensitivity of traditional and novel biomarkers of exposure. We first developed and optimized a qPCR assay to measure spiggin and vitellogenin transcript levels in kidney and liver tissue, respectively. Basal levels were differentially expressed in mature wild-caught male and female brook stickleback. To determine their sensitivity to EDCs, fish were exposed to nominal concentrations of 1, 10 and 100 ng/L of 17α-methyltestosterone (MT) or 17α-ethinylestradiol (EE2) for 21 days (sampled at 7 and 21 days) under semi-static renewal conditions. MT and EE2 exposure induced spiggin and vitellogenin transcripts in female kidneys and male livers, respectively. Exposure to EE2 also increased hepatosomatic index in both sexes and decreased gonadosomatic index in females. Histopathological alterations were observed in the kidney of EE2-exposed fish and an increase in kidney epithelium cell height occurred in MT-exposed females. Given the sensitivity of these endpoints, the brook stickleback is a promising new freshwater fish model for EDC evaluation and a potential bioindicator for EDCs in North American freshwater environments.

1. Introduction

There is ongoing concern surrounding the release of compounds into the aquatic environment due to their potential to disrupt the endocrine system of many aquatic species. Exposure to endocrine disrupting compounds (EDCs) can induce changes which span multiple levels of biological organization from molecular to whole-organism, including (but not limited to) alterations in transcript and protein levels, changes in concentrations of circulating sex steroids, histopathological changes in target tissues, delayed or altered development, reproductive failure and abnormal behavior in fish (reviewed in Arukwe, 2001; Rotchell and Ostrander, 2003; Hutchinson et al., 2006). More recently, exposure to EDCs has been implicated in population-level effects, including the collapse and recovery of fish populations (Kidd et al., 2007; Blanchfield et al., 2015). To determine effect thresholds for EDCs several small fish have been applied as model species in regulatory testing through the assessment of biomarker responses which range from mechanistic endpoints to ecologically relevant effects (Ankley and Johnson, 2004; Scholz and Mayer, 2008).

In North America, the fathead minnow (*Pimephales promelas*) is the most common small model fish species used to assess estrogenic and androgenic activity of chemicals (Ankley and Johnson, 2004). Several endpoints in this species are responsive to estrogens, including increased expression of vitellogenin, altered sex-steroid production, decreased nuptial tubercle counts in males, delayed gonadal maturation and decreased fecundity and fertility (Bringolf et al., 2004; Pawlowski et al., 2004a; Seki et al., 2006). Similarly, assays of several secondary sex characteristics in the fathead minnow have been developed as biomarkers of exposure to androgenic compounds, including nuptial tubule growth, changes in the size of the dorsal nape pad and alterations in shape, colouration and breeding behavior (Ankley et al., 2003; Pawlowski et al., 2004b; Martinović et al., 2008). However, these biomarkers are often not responsive to low concentrations of androgenic compounds (Ankley et al., 2003; Pawlowski et al., 2004b) and are scored

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using a subjective scale; thus, they may be influenced by measurement bias. Secondary sex characteristics can also be affected by the hierarchical status of the fish (Martinović et al., 2008) making it difficult to attribute a change in appearance to exposure to an EDC. Although the fathead minnow possess unique hormone-responsive traits, there are limitations to using this species for screening and testing of androgenic compounds.

The threespine stickleback (*Gasterosteus aculeatus*) is a small-bodied model fish species that has been more recently used in bioassays to assess the endocrine activity of compounds. Male stickleback produce a unique, quantifiable and androgen-responsive glycoprotein in the kidney called spiggin, which is used to construct nests during the breeding season (Jakobsson et al., 1999). Although female sticklebacks do not normally produce spiggin, exposure to exogenous androgens will induce spiggin production in females, which can be measured in the form of transcript or protein expression (Katsiadaki et al., 2002a,b; Hogan et al., 2008). Spiggin production occurs in the proximal tubules of the kidney, so increased kidney weight and kidney epithelium cell height (KEH) have also been used as biomarkers of androgen exposure (Katsiadaki et al., 2002a). Finally, vitellogenin production by the liver in males can also be assessed as an indicator of estrogen exposure, making threespine stickleback one of the few fish species with biochemical and apical endpoints for both (anti-)androgens and (anti-)estrogens. However, the geographical distribution of this species is mostly limited to coastal and estuarine environments in North America and Europe, which restricts their application as a monitoring species in freshwater systems.

Brook stickleback (*Culaea inconstans*) could potentially be used as an additional small fish model for regulatory testing and ecological monitoring of EDCs. This species has similar reproductive behaviors and physiology as the threespine (Stewart et al., 2007) and is therefore hypothesized to have similar measurable responses to exogenous hormone exposure. Brook stickleback are widely distributed in freshwater systems in North America (Wootton, 1984) and have been previously studied for reproductive behavior (Reisman and Cade, 1967; McKenzie, 1969; McLennan, 1993). Recently, brook stickleback were used as a sentinel species to evaluate the effects of municipal wastewater effluents in an effluent-dominated stream in Saskatchewan, Canada (Tetreault et al., 2012). Therefore, the objective of this study was to develop a bioassay using brook stickleback to measure estrogenic and androgenic responses and determine the sensitivity of various traditional and novel biomarkers of exposure. To achieve this, we first developed and validated a real-time qPCR method to assess the expression of spiggin in the kidney and vitellogenin in the liver of brook stickleback. Sex differences in basal transcript abundance were assessed using reproductively mature males and females. Short-term exposures to 17α-methyltestosterone or 17α-ethinylestradiol (ng/L range) were employed to determine the hormonal responsiveness of these transcripts in addition to apical endpoints such as organosomatic indices, condition factor and histopathological changes in kidney tissues. Sampling was conducted after 7 and 21 days of exposure to determine the response of these biomarkers over time.

2. Material and methods

2.1. Animals

Brook stickleback used in this study were collected from the Cranberry Flats Conservation Area near Saskatoon, Saskatchewan, Canada. Cranberry Flats is a marshy wetland that connects to the South Saskatchewan River during the spring snowmelt runoff season. All fish were collected with dip nets. Fish used to assess basal transcript levels of spiggin and vitellogenin in males versus females were collected in mid-breeding season in July 2014 while stickleback used for the steroid hormone exposures were collected during September 2013. Fish were transported to the Aquatic Toxicology Research Facility (Toxicology Centre, University of Saskatchewan, Canada) and placed in an 84″ × 24″ × 14.5″ Min-O-Cool supplied with a constant flow of filtered facility water. Animals were maintained at a temperature of 16 ± 1 °C with a photoperiod of 12:12 (light:dark). Fish were fed twice daily with frozen blood worms (Sally's Bloodworms, San Francisco Bay Brand, CA, United States). All methods used in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board (AUP #: 20130105) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2. Laboratory exposures and sampling

The synthetic steroids, 17α-methyltestosterone (MT; ≥98% purity) and 17α-ethinylestradiol (EE2; ≥98% purity), used for fish exposures were purchased from Sigma-Aldrich (Oakville, ON, Canada). Male and female brook stickleback were transferred from a holding tank to 10-gallon glass tanks one week prior to exposure with a fish loading rate in compliance with OECD recommendations for the 21 day fish assay at 0.5 g fish/L water (OECD, 2009; MT: 20 fish/tank; EE2: 18 fish/tank). Fish were exposed to three nominal concentrations (1, 10 and 100 ng/L) of either MT or EE2, or to an acetone-carrier control (0.002%; henceforth referred to as control). Exposures were conducted in a semi-static renewal system with two-third water renewal and re-dose of chemical every 48 h. Previous exposures to MT using a similar semi-static exposure regime have elicited androgenic responses in the threespine stickleback and measurement of MT in water revealed actual concentrations that were approximately 80% of nominal (Katsiadaki et al., 2002a, 2006). All treatments were run in triplicate. Water temperature was maintained at 16 ± 2 °C, photoperiod at 16:8 (light:dark) and water quality measurements were measured each week using API Aquarium test strips (pH: 7.5–8.4; ammonia: 0.25–0.5 mg/L).

Sampling was conducted on days 7 and 21 with half of the fish randomly sampled each day (MT: 30 fish/treatment/day; EE2: 27 fish/treatment/day). Fish were stunned with a blow to the head, weighed and total fork length measured (to the nearest 0.1 mm) prior to being killed by spinal severance. The liver, kidney and gonad were excised, weighed (to the nearest 0.01 g), immediately flash frozen on dry ice and stored at −80 °C until RNA extraction. A subset of samples (4–9 fish/treatment/day) were collected from one tank in each exposure group for kidney histology on days 7 and 21 (MT exposure) and day 21 (EE2 exposure). Fish selected for kidney histology were placed whole (incision made in the abdomen) into 10% neutral-buffered formalin and later transferred to 70% ethanol for long-term storage prior to processing.

2.3. RNA extraction and reverse transcription

Total RNA was obtained from kidney and liver using E.Z.N.A. MicroElute Total RNA Kit following manufacturer protocols (Omega BioTek, Norcross, GA, United States). RNA was treated with DNase to eliminate gDNA contamination using Turbo DNA-free kit (Ambion, Burlington, ON, Canada). RNA concentration was quantified on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States) and quality was verified by gel electrophoresis on a 1% tris-borate EDTA agarose gel. Reverse transcription was performed with an RNA input of 1 μg for liver and 0.25 μg for kidney using iScript cDNA synthesis kit according to the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, United States). cDNA was diluted at 10- and 100-fold for liver and 25-fold for kidney for subsequent gene expression analysis.

2.4. Primer design, optimization and real-time PCR analysis of spiggin and vitellogenin

Spiggin and vitellogenin primers for qPCR were designed from partial sequences amplified using degenerate primers. Degenerate primers were designed against previously identified sequences from other fish