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Assessment of the sensitivity of three North American fish species to disruptors of steroidogenesis using *in vitro* tissue explants

Shawn C. Beitel[a,b,*], Jon A. Doering[a,b], Sarah E. Patterson[a,b], Markus Hecker[b,c,*]

[a] Toxicology Graduate Program, University of Saskatchewan, Saskatoon, SK, Canada
[b] Toxicology Centre, University of Saskatchewan, Saskatoon, SK, Canada
[c] School of the Environment and Sustainability, University of Saskatchewan, Saskatoon, SK, Canada

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ABSTRACT

There is concern regarding exposure of aquatic organisms to chemicals that interfere with the endocrine system. One critical mechanism of endocrine disruption is impairment of steroidogenesis that can lead to altered hormone levels, altered or delayed sexual development, and ultimately reproductive failure. With the current large gap in knowledge and a high degree of uncertainty regarding the sensitivity of fishes native to northern ecosystems to endocrine disrupting chemicals (EDCs), the aim of this study was to develop an *in vitro* gonadal explant assay enabling the assessment of EDCs on sex-steroid production in wild fish species native to North America. Northern pike (*Esox lucius*), walleye (*Sander vitreus*), and white sucker (*Catostomus commeroni*) were sampled from a reference location in Lake Diefenbaker, Saskatchewan, Canada, at spawn and multiple post-spawn time points. Gonads were excised and immediately exposed for 24 h to a model inducer (forskolin) or inhibitor (prochloraz) of steroidogenesis in L-15 supplemented media. Furthermore, seasonal profiles of plasma 11-ketotestosterone (11-KT) and 17-β estradiol (E2) concentrations were characterized. Enzyme-linked immunosorbent assays were used to quantify hormone concentrations in plasma and media. The seasonal profile of plasma hormones was significantly correlated with basal *in vitro* hormone production. Gonad tissue exposed to forskolin showed a concentration-dependent increase in E2 and a general increase in 11-KT. Gonad tissue exposed to prochloraz resulted in a decrease of concentrations of 11-KT and E2. These results illustrated that gonadal tissue is undergoing steroidogenesis in an *in vitro* setting that is comparable to *in vivo* hormone profiles, and which is responsive to chemical exposure in a concentration-dependent manner. The seasonal time point during which gonad explants were excised and exposed had an impact on the potency and magnitude of responses, resulting in a seasonal effect on sensitivity. Male and female white sucker showed greatest sensitivity to forskolin, while male and female walleye showed greatest sensitivity to prochloraz. Also, gonad explants from these species were found to have greater sensitivity than responses previously reported for *in vitro* explants of other fish species such as the fathead minnow (*Pimephales promelas*), and stable cell lines currently used as screening applications to detect chemicals that might disrupt the endocrine system. Therefore, current approaches that use stable cell lines or tissue explants from standardized small bodied laboratory species might not be protective of some wild fish species. Future research is required that investigates whether this *in vitro* gonadal explant assay is predictive of *in vivo* effects in wild species of fishes.

1. Introduction

Numerous chemicals in the aquatic environment are known to interact with the endocrine system of aquatic vertebrates (Kavlock et al., 1996; Jobling et al., 2006; Tyler et al., 2005; Gerbron et al., 2010; McMaster et al., 2005). Exposure to these endocrine disrupting chemicals (EDCs) has been linked to a wide variety of developmental and reproductive effects in multiple fish species throughout the world (Scholz et al., 2013; Vos et al., 2000; Palace et al., 2009; Jobling and Tyler, 2003). Numerous regulatory bodies including the United States Environmental Protection Agency (US-EPA) have recognized the importance of identifying chemicals that can affect the endocrine systems of fish and other wildlife. To address these needs, screening programs such as the US-EPA

* Corresponding authors at: University of Saskatchewan, Toxicology Centre, 44 Campus Drive, Saskatoon, SK, Canada S7N 5B3. Tel.: +1 306 966 8837; fax: +1 306 966 4796.
E-mail addresses: scb839@mail.usask.ca (S.C. Beitel), markus.hecker@usask.ca (M. Hecker).

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Endocrine Disruptor Screening Program (EDSP) were developed and implemented (Fenner-Crisp et al., 2000). These programs aim to identify chemicals with specific endocrine disrupting properties such as interaction with the estrogen receptor (ER), androgen receptor (AR), and steroidogenic pathways, which might ultimately cause an adverse effect on reproduction.

To enable more objective risk assessments of EDCs, it is critical to identify the sensitivity of species native to the environments of concern. However, the majority of data used to date in support of environmental risk assessments of EDCs in aquatic systems relies on standard, small bodied, laboratory species such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) that often are not representative of the environments of concern. The sensitivity to the exposure with EDCs has only been studied in a small proportion of wild freshwater fish species, with the majority of the data having been derived from few selected cyprinids and salmonids (Jobling and Tyler, 2003). This leaves a large gap in knowledge and a high degree of uncertainty regarding the sensitivity of fishes native to northern ecosystems such as pike (Esoxidae), perch (Percidae), suckers (Catostomidae), and others to EDCs.

To date, most focus in context with EDCs has been on exogenous chemicals causing endocrine disrupting effects by agonistically or antagonistically binding to sex steroid receptors, mainly the estrogen and androgen receptor (Goksoyr, 2006; Hecker and Giesy, 2008; Snyder et al., 2001). However, there are a number of other equally relevant non-receptor mediated processes that are known to significantly disrupt endocrine functions. These can include disruption of enzymes involved in synthesis, as well as transformation, transportation, and elimination of steroid hormones (Hecker et al., 2002; Hecker and Giesy, 2008; Villeneuve et al., 2007, 2009; Yeung et al., 2011).

The steroidogenic pathway involves the production of a number of different steroid hormones, including sex steroids, glucocorticoids and mineralocorticoids. During production of sex steroid hormones, the precursor compound cholesterol is being converted into active hormones through a series of enzymatic reactions involving multiple cytochrome P450 enzymes and hydroxysteroid dehydrogenases (Parker and Schimmer, 1995; Skolness et al., 2013; Arukwe, 2008; Leusch and MacLatchy, 2003; Hogan et al., 2010). This process is tightly regulated through the hypothalamus-pituitary-gonadal (HPG) axis with positive and negative feedback loops (Ankley et al., 2009; Yeung et al., 2011). The complexity of this pathway encompasses numerous potential targets for disruption. Disruption of certain steroidogenic enzymes can result in alteration of the production of the primary sex steroid hormones 17-β estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT). Proper homeostasis of sex steroid hormones is critical for successful growth, development, and reproduction in fishes (Noris, 1997). Therefore, alterations in hormone levels due to effects on steroidogenesis can lead to disruption of gonadal maturation, abnormal gonad morphology, impaired sexual development, and ultimately reproductive failure (Kime, 1998; Cooper and Kavlock, 2001; Hecker et al., 2002; Nadzialek et al., 2011). There are numerous compounds known to alter steroid hormone synthesis through induction or inhibition of specific or multiple enzymes in the steroidogenic pathway, including fadrozole, ketoconazole, prochloraz, forskolin, and vinclozolin (Powlin et al., 1998; Gray et al., 1997; Villeneuve et al., 2007; Hecker et al., 2006).

Multiple challenges exist in context with assessing the effects of contaminants to native species of interest. These challenges include difficulties in maintaining wild fish species under laboratory conditions, ethical concerns when working with endangered species (which can be of particular interest with regard to their sensitivity to environmental contaminants), and high investments in time, labour and cost involved with *in vivo* assays. Therefore, *in vitro* assays are increasingly used as tools to investigate the toxicity of chemicals because they often have greater sensitivity to low concentrations, specificity of response, high throughput, and have a lesser cost than *in vivo* assays (Gray et al., 1997). Additionally, *in vitro* assays require fewer numbers of animals compared to *in vivo* assays, which is of growing interest in regulatory toxicity testing. One method for *in vitro* testing involves the use of tissue explants. Testes and ovarian tissues maintain some of their natural functions, including steroidogenesis, outside their natural environment (*e.g.* the body of the fish), as all the necessary machinery required for the cell- or tissue-specific function is present (Powlin et al., 1998; Gray et al., 1997). It is hypothesized that species-specific tissue function is preserved within these tissues, and therefore, a test system using gonad explants could be used to identify sensitivity to EDCs which disrupt steroid synthesis. It should be acknowledged, however, that though numerous advantages exist for *in vitro* assays, there are remaining uncertainties regarding their use as surrogates for *in vivo* assays. For example, adsorption, distribution, metabolism and excretion of the test chemical are often not, or only partially, accounted for by *in vitro* assays. This can lead to false positive or false negative results (Gray et al., 1997).

Northern pike (*Esox lucius*), walleye (*Stizostedion viteum*), and white sucker (*Catostomus commeroni*) are ecologically and economically relevant species in northern ecosystems that are at risk of exposure to EDCs. These three species are found throughout North America, are from three different taxonomic orders (Esoxidae, Percidae, Catostomidae), and comprise two trophic levels (predator, bottom feeder). Northern pike and walleye are predatory fish positioned high in the food chain. While they fill an important ecological niche, they can be at a greater risk of accumulation of contaminants. Along with their ecological relevance, pike and walleye are fished for sport, adding economic value to these species. White sucker are bottom feeders, which, through close contact with the sediment, can have an increased risk of exposure to contaminants that accumulate in sediments and/or sediment dwelling organisms. Little is known about the sensitivity of northern pike, walleye or white sucker to exposure to EDCs. Therefore, the aim of this study was to develop an *in vitro* assay to enable the assessment of species-specific sensitivity of these three wild species of fish to disruptors of steroidogenesis. Specifically, gonads were excised and exposed to forskolin or prochloraz, model inducers and inhibitors of steroidogenesis (Hecker et al., 2011), respectively. Sex-steroid production (E2, 11-KT) was used as the endpoint to identify differences in species sensitivity by use of gonad explants. The ultimate goal of this research is to generate information that will allow more objective future risk assessments of EDCs to wild fish species native to northern ecosystems.

2. Materials and methods

2.1. Chemicals

Forskolin from *Coleus forskohlii*, (CAS 66575-29-9; purity: ≥98%), and prochloraz (CAS 67747-09-5; grade: analytical standard), were purchased from Sigma-Aldrich (Oakville, ON, Canada). Serial dilutions of forskolin and prochloraz were prepared in dimethyl sulfoxide (DMSO).

2.2. Field sampling and tissue collection

Sexually mature northern pike (*E. lucius*), walleye (*S. viteum*), and white sucker (*C. commeroni*) ranging from 0.5 to 6.1 kg, 0.6 to 4.7 kg, and 0.6 to 1.6 kg in mass, respectively, were sampled using gill nets from a reference location in Lake Diefenbaker,