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Temporal evaluation of estrogenic endocrine disruption markers in smallmouth bass (*Micropterus dolomieu*) reveals seasonal variability in intersex

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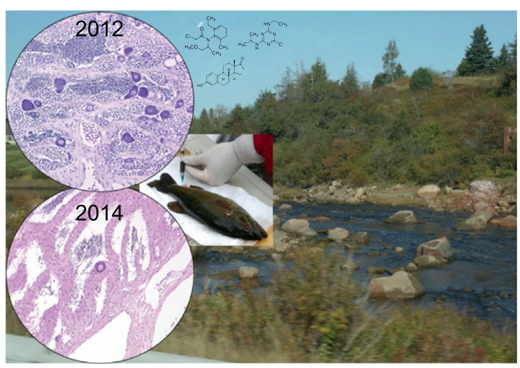
HIGHLIGHTS

- Intersex in smallmouth bass decreased from 92.8% to 28.1% between September 2012 and June 2014.
- Intersex prevalence and severity was not associated with chemical contaminants, fish age or estimated year of recruitment.
- Single, snap-shot sampling for intersex research may not yield representative site-specific data sets

GRAPHICAL ABSTRACT



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ABSTRACT

A reconnaissance project completed in 2009 identified intersex and elevated plasma vitellogenin in male smallmouth bass inhabiting the Missisquoi River, VT. In an attempt to identify the presence and seasonality of putative endocrine disrupting chemicals or other factors associated with these observations, a comprehensive re-evaluation was conducted between September 2012 and June 2014. Here, we collected smallmouth bass from three physically partitioned reaches along the river to measure biomarkers of estrogenic endocrine disruption in smallmouth bass. In addition, polar organic chemical integrative samples (POCIS) were deployed to identify specific chemicals associated with biological observations. We did not observe biological differences across reaches indicating the absence of clear point source contributions to the observation of intersex. Interestingly, intersex prevalence and severity decreased in a stepwise manner over the timespan of the project. Intersex decreased from 92.8% to 28.1%. The only significant predictor of intersex prevalence was year of capture, based on logistic regression analysis. The mixed model of fish length and year-of-capture best predicted intersex severity. Intersex severity was also significantly different across late summer and early spring collections indicating seasonal changes in this metric. Plasma vitellogenin and liver vitellogenin Aa transcript abundance in males did not indicate exposure to estrogenic endocrine disrupting chemicals at any of the four sample collections. Analysis of chemicals captured by the POCIS as well as results of screening discrete water samples or POCIS extracts did not indicate the contribution of appreciable estrogenic chemicals. It is possible that unreported changes

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in land-use activity have ameliorated the problem, and our observations indicate recovery. Regardless, this work clearly emphasizes that single, snap shot sampling for intersex may not yield representative data given that the manifestation of this condition within a population can change dramatically over time.

1. Introduction

Endocrine disruption is a specific form of toxicity in which natural and/or anthropogenic chemicals, defined as endocrine disruptors (EDs), lead to adverse health effects by disrupting the endogenous hormone system (Solecki et al., 2017). In natural environments EDs have been shown to interfere with the endocrine function of fish and wildlife (Matthiessen et al., 2018; Tyler et al., 1998). The consequences of exposure may manifest as the derailment of normal development, metabolism, reproduction, immunocompetence, and behavior (Abdel-Moneim et al., 2015; Blazer et al., 2007; Fuzzen et al., 2015; Harris et al., 2011; Hayes et al., 2011; Iwanowicz et al., 2014; Kidd et al., 2007; Lavelle and Sorensen, 2011; Papoulias et al., 2014; Robertson et al., 2009; Soffker and Tyler, 2012). Sources of endocrine disrupting compounds (EDCs) are widespread, but waste water treatment plants (WWTPs), pulp mills, and agricultural practices (e.g. crop and livestock production) are important, well studied sources of EDCs to the environment (Frye et al., 2012; Hicks et al., 2017; Pollock et al., 2010). Although EDCs have been documented extensively in natural systems, it is often unclear if their presence has demonstrable adverse effects to fish and wildlife (Mills and Chichester, 2005).

Evidence of estrogenic endocrine disruption has been reported in resident fish species since the 1990's (Abdel-Moneim et al., 2015; Jobling et al., 1998). It is well accepted that estrogenic endocrine disrupting chemicals (EEDCs) affect the endocrine system of individuals in a manner that may alter sexual development and fertility (Bernanke and Kohler, 2009). Biological evidence of EEDC exposure in resident species of fishes typically includes the histological presentation of intersex manifested as testicular oocytes (TO) in gonochoristic, male fishes. Extensive research has been conducted specific to the prevalence of TO in largemouth bass (*Micropterus salmoides*) and smallmouth bass (*M. dolomieu*) inhabiting waters within the United States (Abdel-moneim et al., 2017; Anderson et al., 2003; Blazer et al., 2007; Hinck et al., 2009; Ingram et al., 2011; Iwanowicz et al., 2016; Kadlec et al., 2017; Kellock et al., 2014; Lee Pow et al., 2017; Yonkos et al., 2014). Testicular oocytes are frequently observed in smallmouth bass, and significant positive correlations between the incidence and severity of this condition with agricultural land-use metrics have been documented (Blazer et al., 2012; Blazer et al., 2007). In the upper-Chesapeake Bay watershed, this strong association with land-use provides the greatest evidence at present that this physiological phenomenon is the result of drivers originating from land-use activities. Notably, in locations investigated within the Chesapeake Bay drainage, WWTPs seem to be a minor contributor to TOs (Iwanowicz et al., 2009), whereas the density of animal feeding operations and other agricultural practices better correlate with this observation and estrogenic potential of the water (Ciparis et al., 2012). Investigations in other geographic areas with other species have noted associations between the occurrence and degree of intersex and the extent of urban land-use (Tanna et al., 2013). Evidence of an association between watershed development an intersex in smallmouth bass inhabiting northeastern waters of Minnesota is ambiguous, thus suggesting that complex interactions in that geographic area may explain this observation (Kadlec et al., 2017).

Previously we reported the results from a reconnaissance study that focused on biological indicators of estrogenic endocrine disruption in smallmouth and largemouth bass in waters on or adjacent to Northeast Region National Wildlife Refuges (NWRs) (Iwanowicz et al., 2016). The prevalence of TO in males ranged from 60% to 100% in smallmouth bass and from 0% to 100% in largemouth bass. In addition, the egg yolk precursor protein, vitellogenin (Vtg), normally absent or below assay detection levels in plasma of male bass, was detected in bass from several refuges. During 2009, plasma vitellogenin concentrations exceeded 1 mg/mL in some males inhabiting the Missisquoi River, Vermont (an order of magnitude higher than concentrations measured in males from most other rivers investigated). The purpose of that reconnaissance study was to prioritize refuges for more intensive follow-up research if necessary.

Based on results from that reconnaissance study, the Missisquoi River which runs through the Missisquoi National Wildlife Refuge was selected for a comprehensive reevaluation. The Missisquoi River originates near Lowell, VT, flows northward into Canada and then returns to the United States at East Richford, VT and flows west before draining into Lake Champlain. This river is approximately 130 km long and fed by a 2214 km^2 watershed of which ~186 km^2 is in Vermont. Primary land-use along the river is rural agriculture, and this region has been an area of intensive environmental monitoring primarily due to the observed eutrophication of Lake Champlain (Asim et al., 2016). Total nitrogen (TN) input into the river has been the primary contributor or eutrophication, and while decreases in TN have been recently observed they are not the result of decreased fertilizer use (Smeltzer et al., 2012). The health of the Missisquoi has been evaluated using macroinvertebrate fingerprint metrics, and in general river health has been in decline since 1999 based on biological community assessments (Missisquoi 2015 Assessment Report).

Here we report multi-season, multi-year data that include biological and chemical analysis of water samples for chemicals of emerging concern including EEDCs to more comprehensively investigate other aspects of river and fish health. Our objectives were to reevaluate the prevalence and severity of TO and elevated Vtg across multiple sampling years and seasons, and identify the presence of known EEDCs and possible sources. We then sought to specifically test the hypothesis that these measured biotic and abiotic variables were predictive of biological indicators of endocrine disruption.

2. Methods

2.1. Site selection and fish collection

2.1.1. Sampling locations

Three sampling reaches were selected along the Missisquoi River to capture a gradient of inputs from the landscape. The reaches were separated by dams that precluded upstream transit of smallmouth bass. The uppermost reach (Reach 1) extended from the Canadian border downstream to Richford, VT and was a 9.1 km section of river that has the most forested habitat of the three reaches. It was above all known WWTPs with the exception of a plant in Troy, VT. The middle reach (Reach 2) included 12.8 km of river between Sheldon Springs Dam to Highgate Falls Dam (Fig. 1). This section of the river was downstream from two WWTP with significant agricultural areas both within and upstream of the reach. Point sources included the Rock-Tenn Paper Mill (that utilizes recycled paperboard as source material), the Richford WWTP (48 km upstream) and Enosburg Falls WWTP (16 km upstream). Both were small facilities with an average discharge of ~0.95 million liters per day; MLD. The percent contribution of these facilities to stream flow is unknown given that absence of stream gauges in this location. The downstream reach (Reach 3) extended from the Swanton Dam to the Missisquoi River Delta. This was an 11.9 km section of river that includes the Swanton WWTP (1.2 to 1.8 MLD) during 2012–2014, located just