## Division of Science and Research

# Research Project Summary

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# *Reconnaissance of Surface Water Estrogenicity and the Prevalence of Intersex in Smallmouth Bass (Micropterus Dolomieu) Inhabiting New Jersey*


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***Abstract***

The Division of Science and Research (DSR), in cooperation with NJDEP Bureau of Freshwater and Biological Monitoring (BFBM) and the USGS Water Science Center, performed a reconnaissance study of estrogenicity in surface water and the prevalence of intersex in smallmouth bass across the state. This project was initiated after a 2008-2010 study of 19 sites in the Northeastern United States found a high prevalence of intersex smallmouth bass, with one site, the Wallkill River in Sussex County, New Jersey, having a 100% prevalence of intersex smallmouth bass. This project implemented a two-step process to first evaluate the level of estrogenicity in the surface water and then to evaluate the prevalence and the level of intersex in the smallmouth bass population. Surface water samples were collected at 101 (85 river, 16 lake/reservoir) sites across the state during baseflow conditions to be screened for estrogenicity using a bioassay. Levels of estrogenic activity were detectable at 90% of the sites, with 64% of the sites having levels of estrogenicity above the Environmental Protection Agency's 1 ng/L E2Eqblyes trigger value. Median surface water estrogenicity across all sites was 1.8 ng/L and a maximum of 6.9 ng/L $E2Eq_{(BLYES)}$ was observed. Approximately nine male smallmouth bass were collected, pre-spawn, from each of nine of the 101 sites and were evaluated for the presence and number of oocytes detected in the testicular tissue. Intersex was identified in fish at all sites, with the composite intersex prevalence being 93.8%. Prevalence across the sites ranged from 70.6% at Boonton Reservoir to 100% at Splitrock, Canistear, and Yards Creek Reservoirs. This project established a baseline prevalence of intersex in male smallmouth bass in the state of New Jersey at a limited number of select locations, in addition to identifying several waterbodies that showed estrogenic activity above an effects-based threshold.


## Introduction

Endocrine disrupting compounds (EDCs) are a group of natural and anthropogenic compounds that have the ability to disrupt normal organismal hormone signaling networks (Solecki, 2016). EDCs represent a broad class of compounds that include organochlorinated pesticides, plasticizers, fuels, and many other chemicals that are present in the environment or are used widely (Diamanti-Kandarakis, 2009). Endocrine disruptors and estrogenic activity in surface waters is widespread and has been detected even in remote locations (Conley et al., 2017; Stavreva et al., 2012; and Bradley et al., 2017). However, although EDCs have been documented in many natural systems, establishing cause and effect relationships can be difficult.

Fish are routinely used as sentinels of endocrine disruption in the aquatic environment. Disruption of endocrine activity has been reported in fishes for several decades (Jobling et al, 1998; Abdel-Moneim et al, 2015). The manifestation of exposure to estrogenic endocrine-disrupting chemicals (EEDCs) in gonochoristic male fishes generally include the morphological presentation of testicular oocytes (immature egg cells; TOs), which is known as "intersex". Numerous surveys have been conducted that provide the prevalence of TOs in smallmouth bass (*Micropterus dolomius*) and largemouth bass (*Micropterus salmoides*) (Blazer et al., 2007; Hinck et al, 2009; and others*). Several of these studies have shown significant associations between the incidence and severity of TOs (Blazer et al., 2007; Lee et al, 2016; and Blazer et al, 2012), although other studies have not been as clear (Kadlec et al., 2017; Iwanowicz et al., 2019).

During a 2008-2010 reconnaissance of the US Fish & Wildlife Service Northeast Region National Wildlife Refuges (NWR), the prevalence of TOs were ascertained for 118 smallmouth bass collected from 12 NWR waterbodies, including rivers, lakes, impoundments, ponds, and reservoirs from Maine to Virginia. Intersex in male smallmouth bass was observed at all sites and ranged from 60%-100%. Estrogenicity, quantified through the bioluminescent yeast assay, was detected above the probably no effects concentration of 0.73 ng/L in 79% of the samples. One site where smallmouth bass were collected was the Wallkill River located in Sussex County, New Jersey. The conditions of intersex were identified in 100% of the smallmouth bass at this site. The sample size of that survey was small (n=5), so a more comprehensive strategic sampling approach was created to evaluate additional waterbodies around New Jersey.

## Methods

### Site Selection- Water Sampling Locations

Discrete grab samples of surface water were collected under baseflow conditions at 101 sites during the fall of 2016. Sites included both rivers and lake/reservoirs. These waterbodies were selected based on previous chemical and/or biological monitoring studies, current sampling networks, or other areas of concern. Surface water sampling sites can be seen in Figure 1.

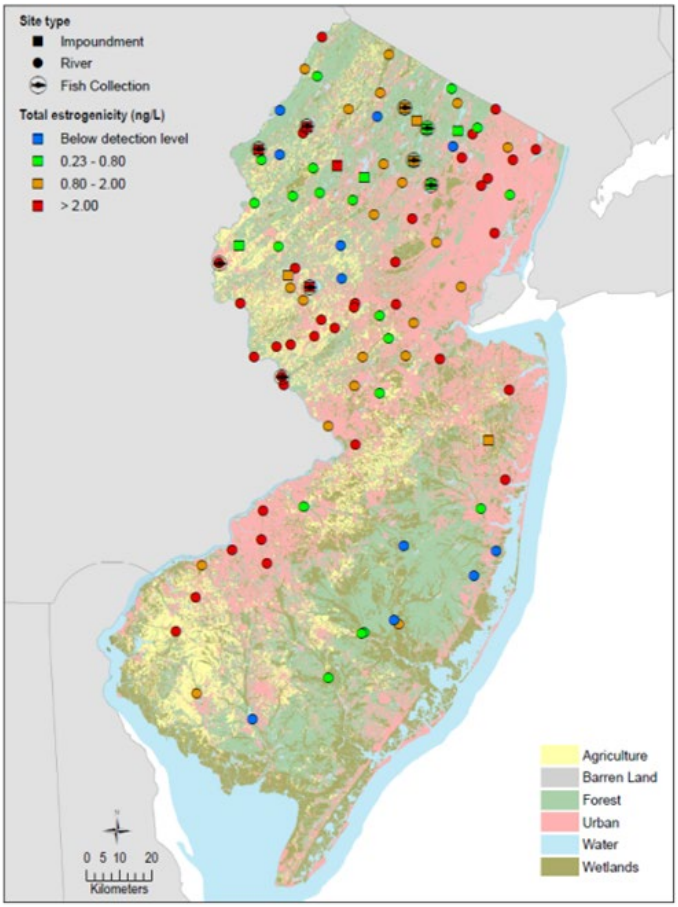


*Figure 1: Surface water and fish sampling locations throughout NJ, with binned ranges of total estrogenicity and land use*

### Water Sample Collection and Estrogenicity Bioassay

The 101 surface water samples were analyzed for total estrogenicity using a bioluminescent yeast screening assay at the USGS Leestown laboratory in WV. Duplicate samples were collected at 19% of the original sampling sites. In addition, water samples were collected again at the time of fish collection and again later in the fall of 2017 to establish a repeated seasonal measure of estrogenicity. Surface water was collected in a pre-cleaned, 1-L amber glass bottle, acidified to a pH of 3 within 4 hours of collection and stored at 4°C. Within one week of collection, 800 mL of the preserved sample was filtered through a GF/F filter (0.7 µm) using a solvent-washed all-glass apparatus, and then filters were rinsed with 1 ml of methanol. Filtered samples and blanks were subjected to solid phase extraction (SPE) using OASIS®HLB (200 mg) glass cartridges (Waters Corporation, Milford, MA), following published methods (Ciparis et al., 2012). The SPE cartridges were sequentially pre-conditioned, and 800 ml of filtered samples was loaded onto the cartridge at a flow rate of 5-6 ml/minute. Analytes were eluted from the cartridge with 100% methanol.

The OASIS®HLB were analyzed for total estrogenicity using strain BLYES (Sanseverino et al., 2005) as previously described (Ciparis et al., 2012). The assay was performed in sterile, clear-bottom, black polystyrene 96-well assay plates (Costar, Corning Inc., Corning, NY). All assay plates included a 12-point standard curve consisting of 17β-estradiol and blanks. Plates were incubated in the dark at 30° for 6 hours on an orbital shaker. Estrogenicity was reported as 17β-estradiol equivalents specific to the screening assay ($E2Eq_{(BLYES)}$). The level of quantitation for these discrete samples was 0.16 ng/L $E2Eq_{(BLYES)}$.

### Site Selection- Smallmouth bass

Based on the results of the estrogenicity bioassay in addition to evaluation of the land use within the watershed contributing to the waterbody and the availability of viable smallmouth bass populations, nine sites were identified for the collection of smallmouth bass. These locations included reservoirs, lakes and sites along the Delaware River. The locations of fish collection sites are shown in Figure 1.

### Fish Collection

Smallmouth bass were collected pre-spawn from the nine sites between April and May of 2017. An attempt was made to collect 10 male and 10 female smallmouth bass larger than 250 mm through electroshocking at each sample location. Fish were held in aerated coolers containing water from the collection site until processing. Fish processing is fully explained in Iwanowicz, 2020, and included the euthanasia of the fish with tricaine methanesulfonate (Finquel Argent Laboratories, Redmond, WA). They were then bled from the caudal vasculature and the blood was expressed in microcentrifuge tubes pre-loaded with 1000 units of sodium heparin, and placed on wet ice, then centrifuged within 4 hours of collection for plasma separation. Each fish was weighed, measured, observed for gross lesions or malformations, and the livers and gonads were removed and weighed to the nearest 0.01 g. Otoliths were removed and used for aging the fish.

Gonadal tissues were processed for routine histopathological evaluation, embedded into paraffin, sectioned at 6 µm and stained with hematoxylin and eosin (Luna 1992).

**Reproductive Endpoints**
Intersex was defined as the presence of immature oocytes within the testes. Five cross-sections along the length of the testes were evaluated for testicular oocyte prevalence and severity. The assignment of an intersex severity score was based on criteria previously defined for centrarchids (Blazer et al., 2007). Briefly, the testicular oocyte severity was ranked as follows: (1) single oocyte per field of view, (2) multifocal, more than one oocyte per field of view, but oocytes not closely associated, (3) cluster, groups (2-5) of oocytes closely associated with each other and (4) zonal, multiple clusters or more than five closely associated oocytes.

Plasma vitellogenin (Vtg) concentrations were measured using a direct enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody 3G2 (Caymen Chemical, Ann Arbor, MI) as previously described (Iwanowicz et al, 2016; Blazer et al, 2012; 29, 36). Reverse transcription quantitative PCR (RT-qPCR) for vitellogenin Aa (*vtgAa*), was conducted on a Vii7 (Applied Biosystems) as previously described (Hahn et al., 2016)

**Land Use Summary**
A drainage basin was delineated for each sampling site using ArcGIS. The allocation of land use types in each delineated area was based on the 2011 National Land Cover Database (NLCD, 2011). All known discharges to surface water and groundwater were also compiled through the New Jersey Department of Environmental Protection's downloadable shapefiles of NJPDES permits. The number of contaminated sites, as well as the number of landfills were summarized for each basin using similar GIS data available from the NJDEP Bureau of GIS.

**Statistical Analysis**
Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Differences in estrogenic activity between water collected from rivers versus impoundments were evaluated with an unpaired t-test and Welch's correction. Differences in detection frequency between these water sources was compared via Fisher's Exact Test. Differences in composite estrogenicity across years for the subset of nine fish sampling sites was conducted using the Friedman test and Dunn's multiple comparison.

Differences in intersex prevalence across sites was evaluated using Fisher's Exact Test. Intersex severity was compared across sites using one-way ANOVA followed by Tukey's *post-hoc*. Plasma Vtg and hepatic Vtg transcript abundance were compared across sites separately for males and females using the Kuskal-Wallis test followed by Dunn's multiple comparison *post-hoc* analysis. Plasma Vtg was compared between males and females at each site using the Mann-Whitney U-test. Associations between age, intersex severity, plasma Vtg and *vtgAa* were analyzed using Spearman-rank correlation analysis.

Relations among estrogenic activity, intersex severity, plasma Vtg and liver *vtgAa* and a suite of watershed-level land-use predictor variables were evaluated using Spearman-rank order correlations. The watershed level predictor variables included major land-use categories (as a percent), watershed area, the number of groundwater and surface water discharge permits, the number of contaminated sites and the number of landfills (Tables 2 and 3). For all statistical analyses any estrogenicity value reported as below the limit of quantitation was assigned a value of 0.1 ng/L $E2Eq_{(BLYES)}$.

## Results

**Surface Water Estrogenicity**
Estimated estrogenicity levels from the 101 discrete surface water samples ranged from below detection (6% of all samples) to a maximum of 6.9 ng/L of $E2Eq_{(BLYES)}$. The median estrogenicity was 1.8 ng/L and estrogenic activity was at or above the USEPA effects-based trigger (EBT) value of 1 ng/L of $E2Eq_{(BLYES)}$ for most sites. There were no statistical differences between estrogenic activity between river or impoundments for the fall 2016 samples, nor were there differences in detection frequency.

In addition to the fall 2016 sampling, water samples collected from the nine fish sampling locations in the spring of 2017 and the fall of 2017 were found to have some qualitative differences, although statistical differences could not be ascertained due to study design. Composite samples based on season identified seasonal differences in estrogenic activity, with the fall of 2016 being higher than the fall of 2017. Estrogenicity and site metadata are available at https://doi.org/10.5066/P9LZKY6Z.

**Prevalence and severity of testicular oocytes**
A total of 174 (115 males, 59 females) smallmouth bass were evaluated. Some level of Intersex conditions (testicular oocytes) was identified in male smallmouth bass at all sites. On a per site basis, intersex prevalence ranged from 70.6% to 100%. The composite intersex prevalence across sites was 93.8%. The greatest intersex severity (ISS) value was a single fish at the Yards Creek site, with a level of 2.4. Intersex severity was statistically lower in fish collected from Boonton Reservoir compared to Splitrock Reservoir (P=0.002). There were not statistical differences in intersex severity across other sites and no significant differences in the prevalence of intersex across sites. Intersex severity was not correlated with age, length, weight, or condition factor.

**Plasma Vitellogenin and differential expression of hepatic transcripts**
Plasma vitellogenin was detected in all fish, both male and female, at all collection sites and was statistically greater in females than males inhabiting the same site (P=0.005). Differences in plasma Vtg were observed across sites, with females collected from Splitrock Reservoir having lower plasma Vtg than in females collected from Yards Creek Reservoir (P=0.003).

Plasma Vtg was detected in all males and ranged from 119-918 µg/mL across all sites. Median plasma Vtg at each site ranged from 272-703 µg/mL, with the lowest value observed at Boonton and the highest value observed at the Round Valley Reservoir, respectively. Significant positive correlations were determined between plasma Vtg and age (n=111; ρ=0.421;P<0.001) and intersex severity (n=112; ρ=0.26; P=0.006). There was a significant negative correlation between plasma Vtg and condition factor (n=115; ρ= -0.330; P< 0.001).

There were also statistical differences observed in hepatic expression of *vtgAa* in male fish. Unlike the relationship between plasma Vtg and hepatic transcription observed in females, there was no significant relationship between these measures in male fish. Significant positive correlations were identified between hepatic *vtgAa* expression and weight (n = 114; ρ= 0.188; P =0.045), length (n = 114;ρ= 0.204; P = 0.029), gonad weight (n = 114; ρ= 0.243; P = 0.009) and GSI (n = 114; ρ = 0.306; P < 0.001) in male fish.

**Associations with Land Use**

Since there were no statistically significant differences in intersex prevalence across sites, correlation analysis with land use was not applied to that biological endpoint. Associations were identified between land uses and male plasma Vtg or liver transcript expression of *vtgAa*. Percent cultivated crops was the only land use parameter that was not statistically correlated with male plasma vitellogenin, with other land uses all negatively correlated with plasma Vtg. Expression of *vtgAa* was positively correlated with present urban and percent altered land use and negatively correlated with percent forest.

Surface water estrogenicity from the fall of 2016 sampling was statistically correlated with land-use (Table 2). When all water samples were collectively analyzed (rivers and impoundments) percent urban (n = 101; ρ= 0.206; P = 0.039) and percent altered land (n = 101; ρ = 0.306; P = 0.002) were positively correlated with estrogenicity. Notably, when rivers and impoundments were evaluated separately it was clear that the observed associations were driven by the river samples. No statistically significant relationships were observed between land-use and estrogenicity within impoundments. Positive correlations were identified among river sites between estrogenicity and percent urban (n = 85; ρ= 0.277; P = 0.011), percent altered (n = 85; ρ= 0.402; P = 0.002), number of contaminated sites (n = 85; ρ = 0.253; P = 0.020), and number of surface water discharge permits (n = 85; ρ = 0.249; P = 0.023).

## Discussion

This main purpose of this study was to quantify the prevalence and severity of estrogenic endocrine disruption in fish in New Jersey, using the smallmouth bass population as a potential indicator. The estrogenic activity in 101 surface water sites across New Jersey were used as an endpoint and as a potential contributing factor to the prevalence of intersex and to evaluate relationships between the fish, water, and land-use drivers. Fish collection sites were selected based on the gradient of estrogenicity values determined from surface water samples collected in the fall prior to biological sample collection. No specific EEDCs were measured.

Levels of estrogenicity were detected at 90% of the sampled sites, with 64% above the US EPA effects-based trigger values (EBT) of 1 ng/L. Previous studies have identified highly variable estimates of estrogenic activity in stream water, including a report of up to 10 ng/L in the Chesapeake Bay watershed. Other studies have documented intersex smallmouth bass with much lower levels of estrogenicity than was measured in this study (Iwanowicz, 2016 and Blazer, 2012). There is currently no species-specific estrogen equivalent value that is ascribed to the induction of intersex.

This study identified variation in estrogenicity measured in different seasons, for the 9 biological sampling sites. This range of values can be attributed to sources of the estrogenicity, and the mechanisms and dynamics of fate and transport. The variability in estrogenicity measurements impacts the assumption that this value is representative of the overall estrogenicity experienced by the fish. In addition, expression of *vtgAa* can be a measure of exposure to estrogens within hours or days prior to capture, whereas plasma Vtg is a measure of estrogen exposure days or weeks prior to capture. The observation of testicular oocytes captures a larger temporal window. If the high intersex prevalence seen in this study was due to exposure to EEDCs, it is possible that the bioassay data poorly predicted this endpoint due to an artifact of the temporal snapshot. In this study, surface water estrogenicity was not predictive of intersex prevalence or severity. However, since there was essentially no difference in intersex prevalence or severity across the limited number of sites that were sampled, predictive relationships could not be expected.

## Conclusion

The condition of intersex has been assessed in black basses for several years, but the specific drivers that lead to the presence of testicular oocytes has not yet been determined. It is likely that multiple factors that include biotic and abiotic risk factors of natural and anthropogenic origin may contribute to this endpoint. While land use has provided some predictive ability, there are wide variations across geographical river basins. In this study of New Jersey waters, there is clearly a high prevalence of testicular oocytes in smallmouth bass populations. The surface water estrogenicity, while at or above the EBT at a frequency of 90% during base flow conditions, did show a great variation over time. If estrogenicity or other chemical stimuli are associated with intersex, it is possible that the timing of sample collections missed these windows of exposure. Future studies that includes the collection of estrogenicity at more frequent intervals, along with evaluation of the water quality through analytical chemistry, are recommended to examine the potential causes of intersex. In addition, it should be noted that there is evidence that intersex in the environment is not a permanent condition (Hicks et al., 2016) and that it can decrease with the removal of the EDCs (Iwanowicz et al., 2019).

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***For a full list of references, see:** Iwanowicz, Luke R., Kelly L. Smalling, Vicki S. Blazer, Ryan P. Braham, Lakyn R. Sanders, Anna Boetsma, Nicholas A. Procopio, Sandra Goodrow, Gary A. Buchanan, Daniel R. Millemann, Bruce Ruppel, John Vile, Brian Henning and John Abatemarco, Reconnaissance of Surface Water Estrogenicity and the Prevalence of Intersex in Smallmouth Bass (Micropterus Dolomieu) Inhabiting New Jersey, International Journal of Environmental Research and Public Health 2020, 17, 2024.

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## RESEARCH PROJECT SUMMARY

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