Ecotoxicology and Environmental Safety 124 (2016) 50–59

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Evidence of estrogenic endocrine disruption in smallmouth and largemouth bass inhabiting Northeast U.S. national wildlife refuge waters: A reconnaissance study

L.R. Iwanowicz [a,*], V.S. Blazer [a], A.E. Pinkney [b], C.P. Guy [b], A.M. Major [c], K. Munney [c], S. Mierzykowski [d], S. Lingenfelser [e], A. Secord [f], K. Patnode [g], T.J. Kubiak [h], C. Stern [h], C.M. Hahn [a], D.D. Iwanowicz [a], H.L. Walsh [a], A. Sperry [a]

[a] U.S. Geological Survey, Leetown Science Center, National Fish Health Research Laboratory, Kearneysville, WV, United States
[b] U.S. Fish and Wildlife Service, Chesapeake Bay Field Office, Annapolis, MD, United States
[c] U.S. Fish and Wildlife Service, New England Field Office, Concord, NH, United States
[d] U.S. Fish and Wildlife Service, Maine Field Office, Orono, ME, United States
[e] U.S. Fish and Wildlife Service, Virginia Field Office, Gloucester, VA, United States
[f] U.S. Fish and Wildlife Service, Pennsylvania Field Office, State College, PA, United States
[g] U.S. Fish and Wildlife Service, New York Field Office, Cortland, NY, United States
[h] U.S. Fish and Wildlife Service, New Jersey Field Office, Pleasantville, NJ, United States

ARTICLE INFO

Article history:
Received 8 December 2014
Received in revised form
5 August 2015
Accepted 22 September 2015
Available online 19 October 2015

Keywords:
Intersex
Smallmouth bass
Estrogen
Endocrine disruption
National wildlife refuge

ABSTRACT

Intersex as the manifestation of testicular oocytes (TO) in male gonochoristic fishes has been used as an indicator of estrogenic exposure. Here we evaluated largemouth bass (*Micropterus salmoides*) or smallmouth bass (*Micropterus dolomieu*) form 19 National Wildlife Refuges (NWRs) in the Northeast U.S. inhabiting waters on or near NWR lands for evidence of estrogenic endocrine disruption. Waterbodies sampled included rivers, lakes, impoundments, ponds, and reservoirs. Here we focus on evidence of endocrine disruption in male bass evidenced by gonad histopathology including intersex or abnormal plasma vitellogenin (Vtg) concentrations. During the fall seasons of 2008–2010, we collected male smallmouth bass ($n=118$) from 12 sites and largemouth bass ($n=173$) from 27 sites. Intersex in male smallmouth bass was observed at all sites and ranged from 60% to 100%; in male largemouth bass the range was 0–100%. Estrogenicity, as measured using a bioluminescent yeast reporter, was detected above the probable no effects concentration (0.73 ng/L) in ambient water samples from 79% of the NWR sites. Additionally, the presence of androgen receptor and glucocorticoid receptor ligands were noted as measured via novel nuclear receptor translocation assays. Mean plasma Vtg was elevated (>0.2 mg/ml) in male smallmouth bass at four sites and in male largemouth bass at one site. This is the first reconnaissance survey of this scope conducted on US National Wildlife Refuges. The baseline data collected here provide a necessary benchmark for future monitoring and justify more comprehensive NWR-specific studies.

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1. Introduction

Since the early 1980s, endocrine disruption in humans, fish, and wildlife has been recognized as a global environmental concern (Bernanke and Kohler, 2009; Hotchkiss et al., 2008; Kortenkamp, 2007; Lathers, 2002; Rhomberg et al., 2012). Endocrine disrupting compounds (EDCs) are collectively grouped into the category of emerging contaminants (ECs) or chemicals of emerging concern (CEC). They run the gamut of natural and synthetic chemicals, but also include biogenic plant and animal hormones. To date, estrogenic endocrine disrupting chemicals (EEDCs) have received considerable attention due to the perceived risk they pose to vertebrate reproduction and better established biomarkers. In aquatic ecosystems, two dominant sources of EEDCs are agricultural production such as animal feeding operations (AFOs) and crop fields applied with manures and herbicides (Battaglin et al., 2009; Blazer et al., 2012; Ciparis et al., 2012; Gall et al., 2011; Orlando et al., 2003) and wastewater treatment plant (WWTP) effluents (Kusk et al., 2011; Sarmah et al., 2006; Vajda et al., 2008). As a result, aquatic vertebrates including fish can potentially be

* Corresponding author.
E-mail address: liwanowicz@usgs.gov (L.R. Iwanowicz).

http://dx.doi.org/10.1016/j.ecoenv.2015.09.035
0147-6513/Published by Elsevier Inc.

affected by these chemicals. Fish are commonly used as indicators of aquatic ecosystem health and in recent decades have been exploited as resident sentinels in locations where estrogenic endocrine disruption is present (Simmons et al., 2014).

Evidence of estrogenic endocrine disruption has been observed in resident fish species across the globe for almost two decades, and intersex has been reported in approximately 37 species of fish (Bahamonde et al., 2013). Intersex manifested as the presence of oocytes in the testes (testicular oocytes; TO) in male gonochoristic fish has been used as an indicator of estrogenic exposure. Testicular oocytes have been reported in largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*) inhabiting drainages across the United States (Anderson et al., 2003; Blazer et al., 2014, 2007; Hinck et al., 2009; Ingram et al., 2011; Kellock et al., 2014; Yonkos et al., 2014). Population effects associated with environmentally relevant concentrations of estrogens have been demonstrated in certain short-lived fish species, thus emphasizing the risk of EEDCs (Kidd et al., 2007; Thorpe et al., 2009; Vos et al., 2000). Moreover, skewed sex ratios and reproductive failure have been reported (Routledge and Sumpter, 1996; Walker et al., 1999). Intersex has also been associated with impaired sperm quality and reduced sperm density (Blazer et al., 2012; Harris et al., 2011; Tetreault et al., 2011). Perhaps the most comprehensive research regarding possible causes of intersex in smallmouth bass has included field observations in the headwaters of the Chesapeake Bay drainage. Testicular oocytes are commonly observed in smallmouth bass and there are significant positive correlations between the incidence and severity of this condition with land-use metrics (Blazer et al., 2012, 2007). Wastewater treatment plants seem to be a minor contributor to TOs in locations investigated within the Chesapeake Bay drainage (Blazer et al., 2011, 2007; Iwanowicz et al., 2009) as the density of AFOs and other agricultural practices better correlate with the severity of and estrogenic potential of chemical mixtures in the water (Ciparis et al., 2012). Research 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).

The U.S. Fish and Wildlife Service (USFWS) Refuge system includes 560 National Wildlife Refuges (NWRs) and 38 wetland management districts across the nation (USFWS, 2013a). These 60 million hectares of land and waters are maintained for the conservation, management, and where appropriate, restoration of fish, wildlife, and plant resources and their habitats. Refuges provide habitat for more than 700 species of birds, 220 species of mammals, 250 reptile and amphibian species, more than 1000 species of fish, and protect more than 380 threatened or endangered plants and animals across the U.S. Some of these NWRs are proximate to Canada and Mexico and either share international borders, or aquatic ecosystems. In the USFWS Northeast Region (Fig. 1) there are 71 NWRs comprising 200,000 ha of habitat, with many NWRs located near major urban areas (USFWS, 2013b). Challenges such as climate change, increasing demands for energy development and extraction, habitat fragmentation, urban encroachment, and degradation of water quality may affect the proper refuge function (USFWS, 2013a). Threats to water quality include the issue of environmental EEDCs. Effective management strategies related to water quality threats require an understanding of the current conditions and the factors that may lead to deleterious impacts. Baseline data regarding the status of fish and wildlife health are a necessary metric for land managers to monitor change over time.

The goal of this study was to provide a reconnaissance level survey of estrogenic endocrine disruption in smallmouth and largemouth bass in waters on or adjacent to National Wildlife

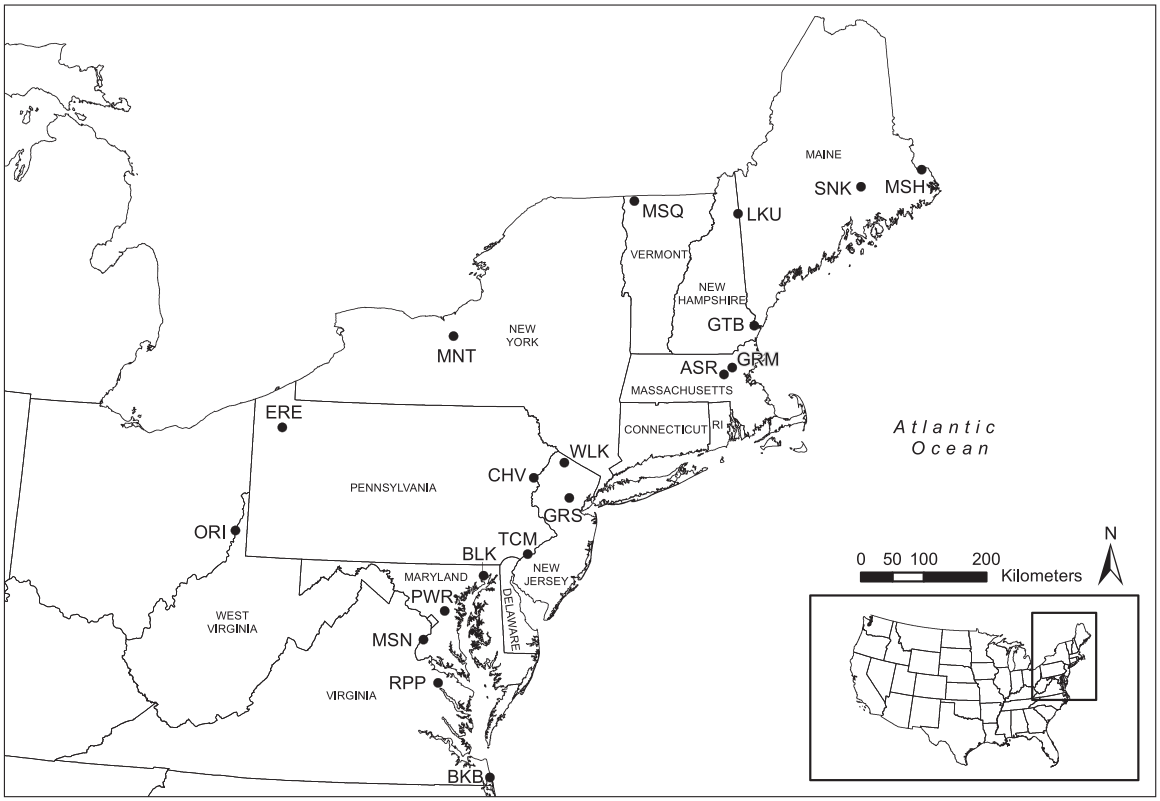

Fig. 1. Northeast Region National Wildlife Refuges sampled during this projectas follows: Assabet River (ASR), Back Bay (BKB), Blackwater(BLK), Cherry Valley (CHV), Erie (ERE), Great Bay (BTB), Great Meadows (GRM), Great Swamp (GRS), John Heinzat Tinicum (TCM), Mason Neck (MSN), Missisquoi (MSQ), Montezuma (MNT), Moosehorn (MSH), Ohio River Islands (ORI), Patuxent (PWR), Rappahannock (RPP), Sunkhaze (SNK), Umbagog (LKU) and Wallkill (WLK). Specific sampling locations are identified in Table 1 and described in greater detail in Supplemental material.

Table 1
Fish sampling locations. SMB: smallmouth bass; LMB: largemouth bass. More detailed site information including maps are provided as Supplemental materials.

Refuge, State	Description/ID	Type	Lat/Long	Date	Comments
Ohio River Islands, OH/WV	Near Wheeling Island (refuge) (ORI)	River	40.079, −80.73761	9/2/2008	Adjacent refuge; 2 minor WWTPs (2–3 km) and 1 major WWTP (7 km)
Ohio River Islands, OH, WV	Ohio River- Pike Island (ORI2a) & Belleville locks (ORI2b)	River	40.13278, −80.70459 & 39.11637, −81.7383	9/3/2008	Sampled 2 reaches believed to be less impacted; ORI2a is below a lock with a minor WWTP located 6 km upstream; no facilities close to ORI2b
Patuxent, MD	Western Branch, PWR2	River	38.79620, −76.72960	9/10/2008	25 km downstream of refuge boundary, adjacent to major WWTP
Montezuma, NY	Seneca River, MNT1	River	42.95747, −76.73786	9/15/2008	Adjacent refuge, agricultural influences include CAFOs, also WWTPs in area
Montezuma, NY	Seneca-Cayuga Canal, MNT2	River	42.89883 −76.88172	9/16/2008	Considered lesser-impacted site; ~10 km upstream of MNT1, 4 locks between the 2 sites
Moosehorn, ME	St. Croix River Upriver, MSH1	River	45.20012, −67.43213	9/22/2008	Reach is above Woodland Dam with no nearby WWTP or pulp mill
Moosehorn, ME	St. Croix River Downriver, MSH2	River	45.13832, −67.36486	9/23/2008	Lower boundary of reach is km upstream of refuge; reach is below Woodland Dam and 0.1 km downstream of pulp mill and major WWTP
Great Meadows, MA	Sudbury River, GRM1	River	42.37498, −71.38211	10/2008	Adjacent refuge; few local sources in or near sampling reach
Great Meadows, MA	Concord River, GRM2	River	42.47412 −71.34384	10/3/2008	Adjacent refuge; sampling reach includes a major WWTP
Wallkill, NJ	Wallkill River, WLK1	River	41.194, −74.5751	10/6/2008	Adjacent refuge, 5 km downstream from major WWTP
Rappahannock River Valley, VA	Rappahannock River at Hicks Landing, RPP1	River	38.18622, −77.23972	10/15/2008	Adjacent refuge;~4 km downstream from a major WWTP
Rappahannock River Valley, VA	Rappahannock R. at Little Falls, RPP2	River	38.25611, −77.41472	10/15/2008	Within 0.1 km of 2 major WWTPs, about 5 km upstream of refuge
John Heinz, PA	Darby Creek, TCM1	River	39.88019, −75.27197	10/27/2008	On-refuge, within 0.1 km of major WWTP
Missisquoi, VT	Missisquoi R., Upstream (5 km reach between Highgate and Swanton Dams), MSQ1	River	44.91142, −73.119569	9/1/2009	5 km reach is upstream of refuge boundary and separated by Swanton Dam; paper mill is 12.6 km upstream
Missisquoi, VT	Missisquoi R.; Downstream (6 km reach between Swanton Dam and refuge), MSQ2	River	44.93716, −73.113478	9/1/2009	Reach extends from 0.1 km downstream of Swanton Dam and WWTP to refuge at Mac's Bend launch; 3 dairy cattle CAFOs
Sunkhaze Meadows, ME	Penobscot R.; Lincoln, SNK1	River	45.35401, −68.55929	9/14/2009	40 km above refuge; within 0.5 km of a pulp mill and a major WWTP
Sunkhaze Meadows, ME	Penobscot R.; Costigan (refuge), SNK2	River	45.01539, −68.64338	9/15/2009	Within 1 km of refuge boundary, no nearby WWTP or paper mills
Mason Neck, VA	Potomac R., Pohick Bay, MSN1	River	38.68394, −77.17577	9/28/2009	~4 km downstream of major WWTP, about 8 km from refuge
Cherry Valley, PA/NJ	Delaware R., Water Gap area, CHV1	River	40.97879, −75.13371	10/5/2009	~0.5 km from refuge boundary; minor WWTP within ~1 km
Cherry Valley, PA/NJ	Delaware R., near Easton, PA, CHV2	River	40.67546, −75.1773	10/6/2009	48 km downriver of refuge boundary, 0.2 km from major WWTP, 1.3 km from a second major WWTP
Blackwater MD	Susquehanna R., near Garrett Island (refuge), BLK1	River	39.56689, −76.07916	10/13/2009	Adjacent to Refuge (Garrett Island), 14 km below Conowingo Dam, 4 km below minor WWTP
Blackwater, MD	Susquehanna R., Conowingo Creek, BLK2	River	39.68312, −76.19688	10/14/2009	Tributary flows into Conowingo Pond above Conowingo Dam; minor WWTP within 3 km
Assabet River, MA	Assabet R.: Reach bordering refuge above the Ben Smith Dam, ASR1	River	42.423623 −71.475032	8/31/2010	Adjacent refuge, above Ben Smith Dam with three WWTPs between A1 reservoir and dam
Patuxent, MD	Triadelphia Reservoir, PWR1	Reserv.	39.19897, −77.01303	9/9/2008	Drinking water reservoir, no WWTPs nearby
Rappahannock River Valley, VA	Motts Reservoir, RPP3	Reserv.	38.31528, −77.55667	10/16/2008	Water supply reservoir, about 20 km upstream of refuge
Mason Neck, VA	Burke Lake, MSN2	Reserv.	38.76417, −77.29809	9/29/2008	Reservoir, fertilized yearly to stimulate the aquatic food chain; dam prevents upstream fish passage from Potomac River
Assabet River, MA	A1 Reservoir (uppermost reservoir, source of river), ASR2	Reserv.	42.26348, −71.63806	9/1/2010	Uppermost reservoir, spillway starts Assabet R.; no local WWTPs
Missisquoi, VT	Gander Bay & Goose Bay portions of Lake Champlain, MSQ3	Lake	44.97911, −73.13574	9/14/2010	2 locations on Lake Champlain close to refuge, nutrients are major concern
Umbagog, NH/ME	Umbagog Lake, LKU1	Lake	44.70228, −71.05508	9/16/2010	On refuge, no nearby WWTPs
Patuxent, MD	Cash Lake, PWR4	Lake	39.03188, −76.78765	10/13/2010	On refuge, near state highway
Great Meadows, MA	Heard Pond, GRM3	Pond	42.35301, −71.38245	8/30/2010	34-ha pond, nearly all shoreline is refuge land
Great Bay, NH	Upper Peverly Pond, GTB1	Pond	43.08720, −70.84049	9/2/2010	On refuge, down gradient from former landfill
Erie, PA	Pool H, ERE1	Pond	41.59512, −79.97153	9/20/2010	On refuge; no known sources
Great Swamp, NJ	Hidden Valley Nursery pond, GRS1	Pond	40.6863, −74.4862	9/28/2010	On refuge, historical evidence of pesticide storage and use
Back Bay, VA	Pond D, BKB1	Pond	36.67296, −75.91916	10/4/2010	On refuge, no known sources
Rappahannock River Valley,	Wilna Pond, RPP4	Pond	38.01660, −76.89208	10/5/2010	On refuge, no known sources

VA						
Rappahannock River Valley, VA	Chandler's Mill Pond, RPP5	Pond	38.09989, −76.84541	10/6/2010	On refuge, no known sources	
Patuxent, MD	Snowden Pond, PWR3	Pond	39.05344, −76.81422	10/13/2010	On refuge, receives nutrient inputs from an off-refuge stormwater pond that is not fully functioning	

Refuges in the USFWS Northeast Region as evidenced by gonad histopathology including intersex or abnormal plasma vitellogenin (Vtg) concentrations. This study was limited to biomarker data (chemical analyses were not conducted), and therefore this report focuses on endpoints in male fishes as their normal physiological background lacks a strong estrogenic regulatory signature. This work was intended to identify NWRs that warrant more intensive study including both biological and chemical sampling.

2. Methods

2.1. Site selection and fish collection

We identified Northeast Region NWRs with populations of largemouth and smallmouth bass on or near NWR lands by consulting NWR managers, local researchers, and State fish and wildlife staff. We also identified possible sources of contaminant loadings into these water bodies by searching State and Federal databases to locate AFOs, WWTPs and industrial facilities such as pulp and paper mills. Because the dominant hydrological features differed across NWR units, we divided the NWR list into non-randomized sampling designs for rivers or pond/impoundment. For the river sampling, we spaced several sampling sites (usually two, sometimes three) such that at least one was on or adjacent the NWR and the other(s) was remote. In some cases, we identified point sources near one or more of the sampling sites. To the extent possible, we attempted to locate sampling sites where barriers such as dams would restrict fish movement between sites. In other cases, we selected sites at least 10 km apart to lessen the probability of bass movement between sites (see Section 4). We did not pair the ponds and impoundments on NWR lands as part of the study design.

The 19 selected NWRs (Fig. 1) included the most comprehensive geographical coverage possible within the region. The proposed sites were refined by ground truthing, often attempting limited sampling to verify that sufficient bass were present, before committing to the site. Table 1 lists the NWR names and abbreviations, sample sites, dates and a brief description of site location in reference to NWR boundaries, and point sources near the sites. More detailed site descriptions and maps are in the Supplemental material.

Electroshocking was the preferred method of fish capture; however, at some locations hydrological or geomorphological conditions precluded this approach and fish were captured by angling. When possible both bass species were collected from the same site for an interspecies comparison; however, typically only one species was observed at each sampling location. At several NWRs, largemouth bass were present in impoundments while smallmouth bass were present at the river collection sites. These fish spawn in the spring and sexual recrudescence initiates during the late summer or early fall depending on the geographic location. Fish collections occurred between late August and early November during 2008, 2009 and 2010 in an attempt to evaluate individuals during this window (Table 1). Water quality parameters—temperature, conductivity, pH, and dissolved oxygen—were measured at all sites using a handheld YSI multimeter (YSI Inc. Yellow Springs, OH) (Supplemental Table 1).

2.2. Sample processing and analytical methods

2.2.1. Fish processing

All fish capture, handling and euthanasia protocols were approved by the USGS, Leetown Science Center, Institutional Animal Care and Use Committee. Fish were held in aerated coolers containing water from the collection site until processed (usually less

than 1 h). Fish ≥ 250 mm were euthanized with tricaine methanesulfonate (Finquel, Argent Laboratories, Redmond, WA) and bled from the caudal vasculature using heparinized 3cc syringes fitted with 23 gauge needles. This size selection assured that we sampled sexually mature fishes. Blood was expressed into heparinized vacutainer tubes containing 62 units sodium heparin (Fisher Scientific, Pittsburgh, PA) and held on wet ice. Blood was centrifuged within four hours of collection for 10 min at 1000*g* for plasma separation. Plasma was removed, aliquoted into cryovials and stored at −80 °C. Each fish was weighed (to the nearest g), measured (to the nearest mm), observed for gross lesions and abnormalities, and liver and gonad removed and weighed to the nearest 0.01 g. Otoliths were removed and used for aging the fish. Condition factor (Ktl) was calculated by the formula: ((body weight – gonad weight in g)/length^3 in mm) × 10^5. The gonadosomatic index was calculated by the formula: (gonad weight in g/body weight in g) × 100. Pieces of gonad were excised and fixed in Z-Fix™ (Anatech Ltd., Battle Creek, MI). Gonadal tissues were processed for routine histopathological evaluation, embedded into paraffin, sectioned at 6 µm and stained with hematoxylin and eosin (Luna, 1992).

2.2.2. Reproductive endpoints

Intersex was defined as the presence of immature oocytes within the testes. At least five cross-sections along the length of the testes were evaluated for testicular oocyte prevalence and severity. The intersex severity score assignments used criteria previously defined for centrarchids (Blazer et al., 2007). In short, 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 (Suppl. Fig. 1).

Plasma Vtg concentrations were measured using a direct enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody 3G2 (Caymen Chemical, Ann Arbor, MI) as previously described (Blazer et al., 2012; Denslow et al., 1999). In brief, plasma samples were diluted as necessary in PBSZ-AP (10 mM phosphate, 150 mM NaCL, 0.02% azide, 10 KUI/ml aprotinin, pH 7.6). Smallmouth bass Vtg was used as a standard for all plasma analyzed (including that from largemouth bass). The vitellogenin standards were prepared at the University of Florida, Department of Physiological Sciences from plasma of 17β-estradiol exposed male fishes. All analyses were performed in 96-well plates. Samples and standards adsorbed to plates overnight at 4 °C in a humidified chamber. Detection reagents included monoclonal antibody 3G2, goat anti-mouse IgG-biotin (115-065-003; Jackson ImmunoResearch Laboratories, West Grove, PA), strepavidin-alkaline phosphatase, and p-nitro-phenyl phosphate. Optical density was measured on a multiwell plate reader (Vmax, Molecular Devices Inc., Sunnyvale, CA) at 405 nm. Concentrations of the unknowns were determined from the standard curves and using the Softmax Pro TM Program (Molecular Devices). The limit of detection was 0.001 mg/ml. Inter and intra-assay variability were < 10%.

2.2.3. Water sampling and estrogenicity bioassay

Single, discrete grab water samples were collected at all of the fish sampling locations. In addition, they were collected from WWTP effluent discharge points on the days of fish collection at sampling sites proximate to such discharges at or near Moosehorn (MSH), Patuxent (PWR), Rappahannock River Valley (RPP), and John Heinz at Tinicum (TCM) NWR locations. Grab water samples were collected in 500 ml pre-cleaned amber glass bottles (I-Chem, Rockwood, TN). Water was acidified to pH 3 within 4 h of collection, held on ice, and stored at 4 °C. Within one week of collection, 400 ml of the preserved water samples was filtered through a GF/F filter (0.7 µm) using a solvent-washed all-glass apparatus. Filters were rinsed with 1 ml of methanol to liberate compounds from the retained suspended solids. Filtered samples and blanks were subjected to solid phase extraction (SPE) using OASIS® HLB (200 mg) glass cartridges (Waters Corporation, Milford, MA), following established methods (Ciparis et al., 2012). In short, cartridges were sequentially pre-conditioned and 400 ml of filtered samples were loaded onto the cartridge at a flow rate of 5–6 ml/min (continuous vacuum). Analytes were eluted from the cartridge with 100% methanol. Half of the sample was blown to dryness and shipped to collaborators at the National Cancer Institute, Laboratory of Receptor Biology and Gene Expression for development of a novel *in vitro* screening platform (Stavreva et al., 2012).

The OASIS-HLB extracts were analyzed for total estrogenicity using the bioluminescent yeast estrogen screen (BLYES) (Sanseverino et al., 2005) as described previously (Ciparis et al., 2012). Strain BLYES was maintained in modified Yeast Minimal Media (YMM leu-, ura-) (Routledge and Sumpter, 1996). Preparation of the screening assay involved the expansion of the strain BYLES to early stationary phase in YMM leu-, ura- at 30 °C on a rotary shaker to an appropriate OD_{600} of 0.750. The assay was performed in sterile, clear-bottom, black polystyrene 96-well assay plates (Costar, Corning Inc., Corning, NY). Sample extracts previously solubilized in methanol were diluted to 10% in YMM and 100 µl of the diluted sample was added to triplicate wells. An equal volume of yeast in YMM was added to each well, resulting in a final sample dilution of 5%. All assay plates included a 12-point standard curve consisting of 17β-estradiol and blanks. Blanks and standards all contained 5% methanol to account for known effects of this solvent. Plates were incubated in the dark at 30 °C for 6 h on an orbital shaker. Luminescence was quantified using a SpectraFluor Plus plate reader (Tecan Group Ltd., Durham, NC) in luminescence mode (1 s integration time/well, gain 180). Quantitation limit was 0.31 ng/L 17β-estradiol equivalents.

2.2.4. Translocation bioassays for androgen and glucocorticoid activity

Estrogens are the most commonly measured nuclear receptor (NR) ligands in environmental water samples via *in vitro* bioassays. Ligands that interact with other NRs are present in environmental samples, but less commonly screened. Androgens are reported to be the most common sex steroid(s) in the environment and ligands that interact with the glucocorticoid receptor have also been reported. The latter are most commonly associated with wastewater effluent. Translocation assays to screen for the presence of human androgen receptor (hAR) and human glucocorticoid receptor (hGR) agonists were performed at the National Cancer Institute, Bethesda, MD using OASIS-HLB extracts solubilized in dimethyl sulfoxide. These methods and data are published elsewhere (Stavreva et al., 2012). Briefly, the 3617 and 3108 cell lines that express green fluorescent protein- tagged hGR and hAR respectively under the control of a tetracycline-repressible promotor were grown overnight in Dulbecco's minimal essential medium containing 10% charcoal stripped serum without tetracycline. Cells were seeded in 96 or 386 well plates and treated with a vehicle control (DMSO), steroid hormone (100 nM) or DMSO solubilized water sample and incubated for 30 min at 37 °C. Cells were then fixed with 4% paraformaldehyde and stained with DRAQ5 and imaged on a Perkin Elmer Opera Image Screening system. Segmentation analysis was performed and values were normalized to the control (DMSO) sample.

2.2.5. Statistical analyses

Student *t*-tests or one-way analysis of variance (ANOVA) were used to compare endpoints between sites for each NWR sampling pair or trio. For these pairs or trios, Fisher's Exact Tests were used

to compare ordinal data sets (ie. measurements reported as a percent). Data collected from NWRs with only one river site (or from impoundments) were only included in correlation analyses. Relationships between metrics were not assumed to be linear and were analyzed using Spearman rank order correlations. All statistics are reported at $\alpha=0.05$ and were performed using SigmaPlot 11 (SPSS Inc., Chicago, IL). Correlation analyses were conducted using pooled data for each species, pooled data by species where evidence of EEDCS was observed or a site-by-site basis. Comparisons of relationships between intersex severity, GSI, Vtg, age and length were evaluated.

3. Results

3.1. Collections

A total of 118 male smallmouth bass from 12 sampling locations and 173 male largemouth bass from 27 sample sites were collected from the 19 NWRs. Morphometric data for each collection are summarized in Supplemental Table 2. Comparison of largemouth and smallmouth bass was only done at the Ohio River Islands NWR (ORI2), the only site where adequate samples sizes of both species were attained.

3.2. Comparisons of TO, plasma vitellogenin and GSI

Male smallmouth bass were collected at a total of 12 sites from 7 NWRs (Tables S1 and S2). Intersex males were observed at all sites and the prevalence ranged from 60% to 100% (Fig. 2). The lowest observed prevalence of intersex (60% at MSQ2) was considerably higher than the lowest reported prevalence of intersex for this species of 10–14% in the Northeast region with an adequate sample size (Blazer et al., 2014, 2007). There was no relationship between intersex severity and age, length, or GSI when evaluated across all sites as determined by Spearman's rank-order correlation. Similarly this was the case when analyses were performed at the level of site even when clear biomarker responses were identified. There were no differences in intersex prevalence between paired and trio locations based on Fisher's exact test. Vitellogenin was detected in males at 8 of 12 sites (Fig. 3). When comparing between sites within a NWR, statistical differences in plasma Vtg were only observed between ORI1 and ORI 2 ($P<0.001$). Differences were not noted in intersex severity between those sites however. One-way ANOVA analyses identified statistical differences in intersex severity between Moosehorn samples MSH1 and MSH2 ($P<0.001$) and Sunkhaze Meadows samples SNK1 and SNK2 ($P=0.008$). A significant negative correlation was observed between plasma Vtg and intersex severity ($n=118$, rho$=-0.617$, $P=0.03$). Statistically significant differences in the gonadosomatic index were observed between males from Ohio River Islands ORI1 and ORI2 ($P=0.019$), Missisquoi samples MSQ1 and MSQ2 ($P=0.031$) and SNK1 and SNK2 ($P=0.001$). Statistically significant relationships were not observed between the other metrics.

Male largemouth bass were collected at 27 sites from 13 NWRs (Tables S1 and S2). Intersex males were observed at 20 of these sites. The range (including the sites with no intersex males) was 0–100% (Fig. 4). Differences in the prevalence of intersex between paired NWR sites were observed for Great Meadows samples GRM1 and GRM2 ($P=0.030$). Plasma Vtg was only detected in males at 6 of 27 sites and there were no significant differences observed between up and downstream locations (Fig. 5). Significant differences in intersex severity were only observed between GRM1 and GRM2 ($P=0.011$), although differences between

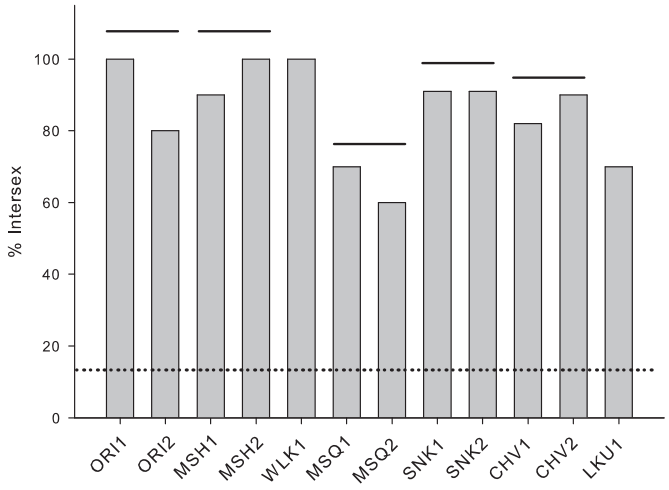

Fig. 2. Prevalence of intersex in male smallmouth bass. No significant differences were noted between sites at or near the same NWR (solid line). Intersex was observed at all sites. Dotted line indicates the lowest published value of intersex in smallmouth bass (Blazer et al., 2007). Sources of fish are denoted graphically: river (white dotted) or lake (gray dotted). Abbreviations are defined in Table 1 and Fig. 1 caption.

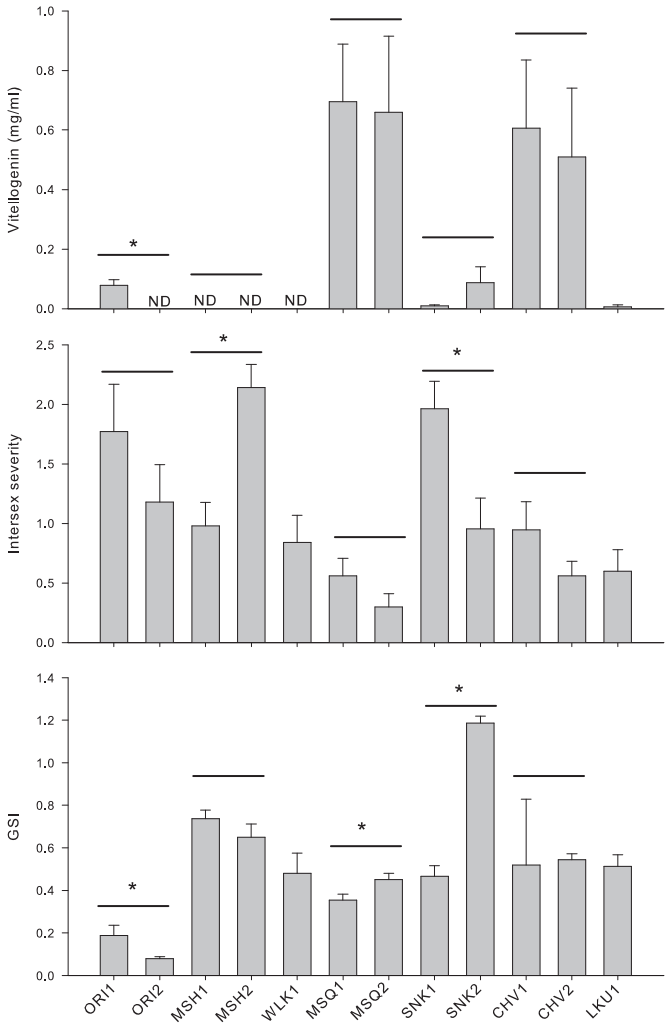

Fig. 3. Reproductive endpoints of male smallmouth bass. Statistical comparisons were between sites at or near the same NWR (solid line). Measures that were statistically different are denoted with an asterisk ($P<0.05$), ND=not detected. Sources of fish are denoted graphically: river (white dotted) or lake (gray dotted). Abbreviations are defined in Table 1 and Fig. 1 caption.

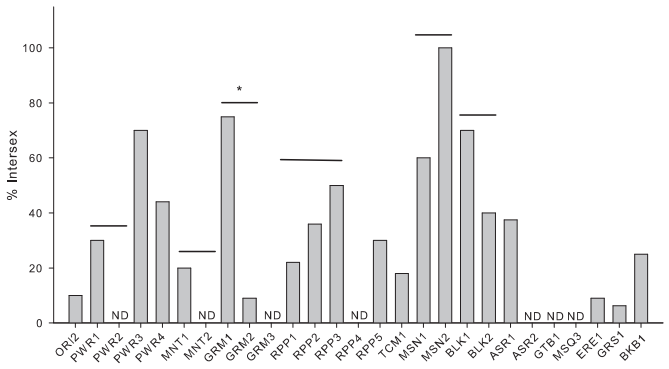

Fig. 4. Prevalence of intersex in largemouth bass males. Statistical comparisons were between sites at or near the same NWR (solid line). Measures that were statistically different are denoted with an asterisk ($P<0.05$), ND=not detected. Sources of fish are denoted graphically: river (white dotted) reservoir (white), lake (gray dotted) or pond (gray). Abbreviations are defined in Table 1 and Fig. 1 caption.

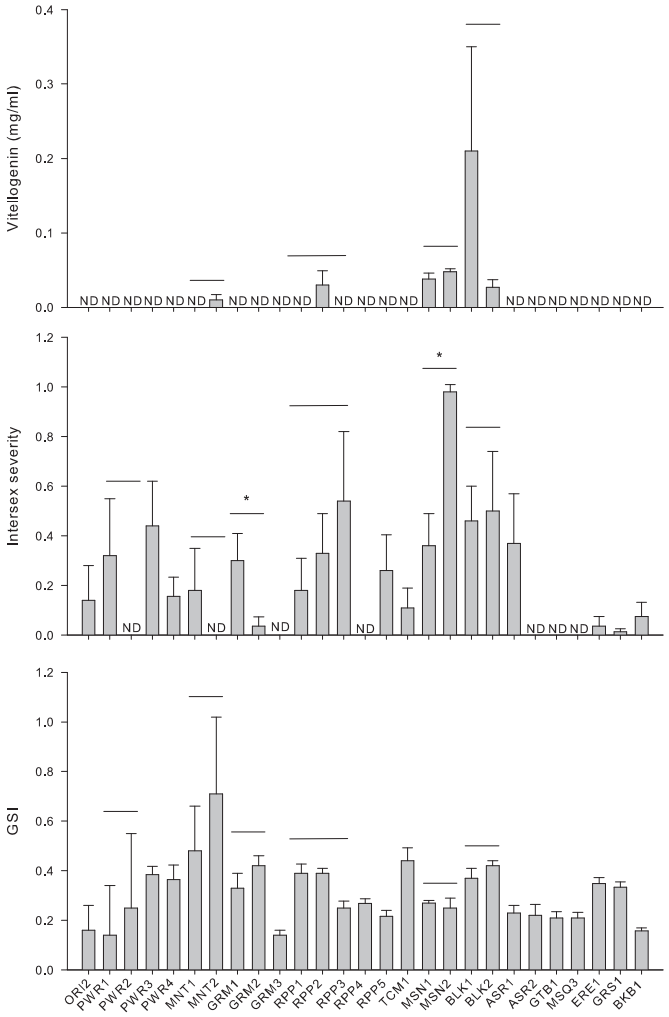

Fig. 5. Reproductive endpoints of male largemouth bass. Statistical comparisons were between sites at or near the same NWR (solid line). Measures that were statistically different are denoted with an asterisk ($P<0.05$), ND=not detected. Sources of fish are denoted graphically: river (white dotted) reservoir (white), lake (gray dotted) or pond (gray). Abbreviations are defined in Table 1 and Fig. 1 caption.

ASR1 and ASR2 approached statistical significance ($P=0.054$). There was no relationship between intersex severity and age or length when evaluated across all sites. A significant positive correlation was observed between plasma Vtg and intersex severity ($n=173$, rho=0.468, $P=0.014$).

3.3. Relative estrogenicity and nuclear receptor interactions

Estrogenic activity was above the quantitation limit in 21 of the 45 water samples tested. The maximum EEQ was 5.6 ng/L (Suppl. Fig. 2). On the basis of presence or absence, estrogenic activity was more commonly detected than glucocorticoid or androgenic activity in all samples tested (Suppl. Table 3). Estrogenic activity was clearly identified in all types of waterbodies and all effluent samples. Ranked in order of presence estrogenic activity was most commonly detected in WWTP effluent > reservoirs > rivers > ponds > lakes. Androgen receptor translocation activity was most commonly detected in WWTP effluent=river > reservoirs > ponds=lakes. Glucocorticoid receptor translocation activity was most commonly measured in WWTP effluent > rivers > reservoirs=ponds=lakes. Estrogenic activity was detected in 100% of WWTP effluents.

4. Discussion

Exposure to estrogens has been unequivocally demonstrated to induce intersex in some fish species. Such controlled laboratory experiments have identified critical windows of phenotypic sex-determination when juvenile fish are vulnerable to intersex induction (Kipfer et al., 2009; Koger et al., 2000; Krisfalusi and Nagler, 2000). In addition to estrogens, exposure to anti-estrogens, particularly from WWTP effluents, are associated with intersex (Jobling et al., 2009; Rempel and Schlenk, 2008). These emerging contaminants are known to affect resident fish populations (Kidd et al., 2007; Thorpe et al., 2009; Vos et al., 2000). Risk models have been developed that assign a risk ranking based on estrogen equivalents (Williams et al., 2009). Teleosts as a group exhibit many sexual reproductive strategies, thus it would not be surprising if even related species exhibited differential sensitivities to endogenous and exogenous cues that regulate the hypothalamic-pituitary-gonadal-axis. Long-term datasets are critical to better understand the effects of EDCs on fish and wildlife in the environment (Bernanke and Kohler, 2009). Here, we provide male bass intersex and other reproductive metric data to satisfy a data gap for such a national dataset.

Here the composite prevalence of testicular oocytes across all samples was 85% and 27% for male small- and largemouth bass, respectively. We identified that intersex in male small- and largemouth bass is a common observation in waters within and near the 19 Northeast Region NWRs. To the best of our knowledge, this work represents the most comprehensive analysis of intersex severity in these species in the Northeast Region. Previously (1995–2004) male smallmouth bass ($n=70$) and largemouth bass ($n=390$) were sampled in eight US River Basins from 1995 to 2004 that did not include the Northeast (Hinck et al., 2009). That sampling effort identified a 33% and 18% prevalence of testicular oocytes in male small- and largemouth bass respectively. Intersex severity was not determined in that study. Our composite intersex prevalence (85%) suggests that smallmouth bass inhabiting the Northeastern US may have a greater likelihood of developing intersex than those in other US regions previously sampled (results of Fisher's Exact Test; data not shown). It is possible that this reflects differences in sources and chemical mixtures present in the environment that lead to a higher risk exposure scenario. Holistic landscape analyses and comprehensive chemical analysis is necessary to support this assertion. The composite prevalence of intersex in smallmouth bass here is similar to that reported in the Susquehanna River, PA(> 90%) (Blazer et al., 2014). The prevalence of intersex in male largemouth bass was documented as 57% on the Delmarva Peninsula, MD, USA which is higher than our composite observation (Yonkos et al., 2014). Likewise, a 48% intersex prevalence in largemouth bass collected from impoundments in

Georgia, USA has been reported (Kellock et al., 2014).

Whether there is a natural baseline incidence of intersex in centrarchids is a point of discussion and there is no comprehensive dataset or controlled laboratory experiment that specifically addresses this question. Previous research has identified locations where intersex is observed at 10–14% in male smallmouth bass or as low as 0% (Blazer et al., 2014, 2007; Hinck et al., 2009). Interestingly the positive correlations of intersex prevalence and severity with anthropogenic land-uses, may suggest that scenarios exist where the absence or very low incidence of intersex is the natural physiological condition (Blazer et al., 2012). Comprehensive investigations of the roach (*Rutilus rutilus*) suggest that the phenomenon of intersex may be 0.5% under natural conditions (Geraudie et al., 2010). Of relevant consideration here, there are few aquatic habitats inhabited by smallmouth bass that are genuinely pristine or unimpacted. Observations of intersex prevalence in smallmouth bass seem to parallel that of the roach, which is commonly used as a sentinel of EEDC–associated disruption in European aquatic habitats (Tyler et al., 2007).

The current study reinforces previously documented differences in the prevalence and severity of intersex between the two bass species. Namely, both the prevalence and severity of intersex in smallmouth bass is typically higher than in largemouth bass (Blazer et al., 2007). Given that these two species utilize different habitats, this observation may simply reflect differential endocrine disrupting potential in waters preferentially utilized by smallmouth bass. It is also possible that these species exhibit differential sensitivities in regards to estrogen receptor signaling networks. To date species differences have not been comprehensively evaluated. In the current study we only captured both species at a single site, ORI2, making inter-species comparisons across a larger geographical expanse impossible. That stated, the incidence of intersex in smallmouth bass at this location (72%) was statistically higher ($P=0.03$) than that observed in largemouth bass (10%). Moreover, this was also the case for intersex severity ($P=0.008$). No Vtg was detected in males of either species at this site. The fact that GSI was not statistically different suggests that these fish were at a similar stage of reproductive recrudescence.

There were two of five paired NWR sample locations that indicated significant differences in EEDC exposure. There were no differences in intersex prevalence in smallmouth bass at the 5 NWRs where this comparison was possible; however, there were differences in intersex severity at the Moosehorn and Sunkhaze NWRs (both of which are located in Maine). The Moosehorn sampling locations are separated by a dam, and intersex severity was statistically higher at the downriver location near a WWTP and pulp mill. The uppermost site in the Sunkhaze is also near a WWTP and a pulp mill. Intersex severity was significantly higher and the gonadosomatic index lower in male bass at this location compared to the on-NWR site located ~40 km downriver. This suggests biological effects associated with proximity to these point sources.

For largemouth bass, there were six NWRs where similar comparisons were possible. Differences in intersex prevalence were only noted at the Great Meadows NWR in Massachusetts. Intersex prevalence and severity were both greater in the Sudbury River compared to the adjacent Concord River. Interestingly, the Sudbury River was originally selected as the less impacted reference based on the contribution of WWTP discharge. The only other paired site with differences was observed at the Mason Neck NWR. Here, Burke Lake had a significantly higher intersex severity than Pohick Bay. These two locations are separated by a dam that prevents movement from the bay into the lake. Interestingly, Burke Lake has no known point source inputs and it was originally selected as a reference relative to the Pohick Bay site which is ~4 km downstream from the largest WWTP (~378.5 million liters per day) in Virginia. At both of these locations the intersex results were not associated with point-source contributions.

In the case of other NWRs where differences in intersex were not observed between paired sites, the uncontrolled variable of fish movement may be a factor. Depending on the aquatic system, largemouth bass is documented to range from a few to 21 km (Freund, 2003; Nack et al., 1993; Richardson-Heft et al., 2000). The homerange of smallmouth bass is reported to be hundreds of meters to 109 km (Beam, 1990; Langhurst and Schoenike, 1990). To the extent possible, we attempted to locate these sites where barriers such as dams were present that would restrict fish movement between the sites. In other cases, we selected collection sites at least 10 km apart to lessen the likelihood of site immigration/emigration.

In addition to possible movement of fish between sites, factors such as agricultural run-off originating above the upper most collection site may also explain a lack of differences between the paired sites. This was suggested as an explanation for the lack of an observed WWTP effect on smallmouth bass in a previous study of the Potomac River drainage (Iwanowicz et al., 2009). Observation of differences in the TO and Vtg endpoints at the upriver and downriver locations at the Great Meadows, Blackwater (Garrett Island Division), Mason Neck, Ohio River Islands, Moosehorn, and Sunkhaze NWRs suggests that point source contributions may be important. However, proximity to WWTP discharge was a poor predictor of intersex in other systems. For example, there was a 30% intersex prevalence in LMB from Triadelphia drinking water reservoir (PWR1) compared to the Western Branch (PWR2) site that is adjacent to a major WWTP where 0% intersex was observed (although this difference was not statistically significant; Fig. 4). Of course, mixture effects of agonists and antagonists could possibly explain this observation, but without chemical analysis this is simple conjecture. Establishment and application of species specific *in vitro* assays using liver or testes could facilitate screening of these environmental samples to assess cocktail effects. More comprehensive investigations are required to investigate site specific effects in regars to causation.

Observations of elevated plasma vitellogenin in smallmouth bass collected from the Missisquoi and Cherry Valley NWRs were indistinguishable from that of females (> 1 mg/ml) at the same locations (data not reported here). At the present time there are no diagnostic concentrations for vitellogenin in male plasma directly indicative of exposure to a specific concentration of estrogen. Conventional interpretation of this plasma vitellogenin data is that males should not express appreciable amounts of this egg precursor protein (Bahamonde et al., 2013). Thus, in many regards it can be used as a stand-alone indicator of exposure to estrogenic chemicals in male and juvenile fishes. There is evidence that vitellogenin has antimicrobial properties (Zhang et al., 2011) but it is unclear if microbes induce the synthesis of this protein. Temperature is also known to affect the response of fish to estrogens in regards to vitellogenin expression as it modulates cellular biochemical kinetics and may be an abiotic factor requiring consideration in this study given that water temperatures ranged from 9.3 to 29.0 °C (Anderson et al., 2012; Brian et al., 2008; Korner et al., 2008; Mackay and Lazier, 1993). A comprehensive re-evaluation that includes chemical analysis and seasonal snapshots of both sites is necessary to identify the likely cause(s) of elevated plasma vitellogenin in these male smallmouth bass.

Relationships between plasma vitellogenin and intersex are not necessarily expected given temporal differences of biomarker induction. Intersex is presumed to be induced during early developmental stages while the induction of vitellogenin in male fishes can be the result of transient or prolonged exposure to an estrogen, and is less dependent on life stage. Interestingly we observed a statistically significant negative correlation (albeit weak)

between vitellogenin and intersex severity in smallmouth bass based on 118 observations. To our knowledge this is the first time such an observation has been published. Conversely a positive correlation between these endpoints was observed in largemouth bass based on 291 observations. At the scale of this reconnaissance study it is not possible to make inferences regarding the mechanisms associated with these observations. It should also be noted that while these relationships were statistically significant, the strength of the relationships given the correlation coefficients ($r=0.61$ and 0.47) are modest at best.

The predicted no-effects concentration (PNOEC) for 17β-estradiol has been derived by others and ranges from 1 to 10 ng/L (Caldwell et al., 2012; Young et al., 2004). A more recent PNOEC of 0.73 ng E2/L has been recommended for protecting organisms from chronic and full-life cycle exposure to E2 (Wu et al., 2014). A PNOEC for E2 has not been experimentally derived for large or smallmouth bass, and differential species sensitivity to estrogens is well accepted (Miyagawa et al., 2014). Here, sites above the 1 ng/L PNOEC were the Cherry Valley site CHV2, Assabet River site ASR1 and Rappahannock River Valley site RPP4. Water samples collected from 79% of the sites were above the 0.73 ng/L PNOEC. The CHV2 site is 0.2 and 1.3 km from two major WWTPs which likely explains the estimated EEQ of 0.99 ng/L. Elevated vitellogenin concentrations observed in male smallmouth bass from this location were indistinguishable from those observed in females. Mean plasma vitellogenin in males at this location was one of the highest recorded during the course of this study. The ASR1 site had the highest non-effluent EEQ (2.2 ng/L) measured during the study. Interestingly while largemouth bass had a significantly higher prevalence of intersex than those at the uppermost reservoir (ASR2), plasma vitellogenin was not detected in the males sampled. The EEQ estimated in Wilna Pond (RPP4) was 1.3 ng/L. This water body has no known contaminant sources suggesting that estrogenicity is either associated with non-point source run-off, or possibly phytoestrogens. Similar to the observation at ASR1 there was no measurable plasma vitellogenin in males. Intersex was not observed in largemouth bass from this site. These observations highlight the temporal disconnect and complexities encountered when comparing data obtained from discrete water samples and biomarkers in resident fish. All of these measures have a kinetic component that cannot always be easily captured with snapshot sampling. Of note, nuclear receptor agonist activity (glucocorticoid and androgen) other than estrogenicity was measured at these sites, thus highlighting the need for a holistic evaluation of exposure to other endocrine active chemicals in addition to estrogens. Here, we should note that analysis of single grab water sample at the time of fish collection only provides a snap-shot of potential organismal exposure. More frequent sampling or passive sampling methods would likely provide a better estimation of exposure.

The prevalence of intersex was high at all 12 locations where smallmouth bass were collected (Fig. 2). Currently the specific chemicals and other factors specifically associated with intersex in smallmouth bass are unknown; however, agricultural land-use (livestock density) and WWTP flow have been statistically associated with this observation (Blazer et al., 2012, 2007). Based on the observations of elevated vitellogenin, intersex prevalence and severity further investigations of the Missisquoi, Moosehorn, Sunkaze Meadows, Assabet River and Great Meadows NWRs that include a biological effects monitoring strategy over several seasons and comprehensive chemical analysis of contaminants of emerging concern is warranted. Results from such work should better inform management decisions on lands on or near National Wildlife Refuges.

5. Conclusions

Here we identify that prevalence and severity of intersex in smallmouth bass inhabiting aquatic habitats in and proximate to NWRs is higher in the Northeast region of the United States compared others previously sampled. Intersex prevalence and severity in male smallmouth bass was higher than that observed in largemouth bass, but these fish were also observed in different habitats. Biomarkers indicative of male fish exposure to estrogens were evident in fish from a number of NWRs. In short, NWRs in the Northeast region of the United States are impacted by EEDCs.

Acknowledgements

This study was funded by the U.S. Fish and Wildlife Service Division of Environmental Quality. The USGS Priority Ecosystems, Contaminant Biology and Fisheries Programs also contributed to this work. Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the U. S. Government. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. We thank the 50 or so U.S. Fish and Wildlife Service biologists, NWR managers and State fisheries biologists who assisted with logistics and field work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.09.035.

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