SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 36, No. 4, pp. 959–968, 2017
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Printed in the USA

ENDOCRINE ACTIVE CONTAMINANTS IN AQUATIC SYSTEMS AND INTERSEX IN COMMON SPORT FISHES

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(Submitted 26 May 2016; Returned for Revision 11 July 2016; Accepted 30 August 2016)

Abstract: Male fish are susceptible to developing intersex, a condition characterized by the presence of testicular oocytes. In the present study, the relationship between intersex and exposure to estrogenic endocrine active contaminants (EACs) was assessed for 2 genera of sport fish, *Micropterus* and *Lepomis*, at 20 riverine sites. Seasonal trends and relationships between EACs and intersex (prevalence and severity) were examined at varying putative sources of EACs throughout North Carolina, identified as point sources, nonpoint sources, and reference sites. Intersex was identified in both genera, which was documented for the first time in wild-caught *Lepomis*. Intersex was more prevalent (59.8%) and more severe (1.6 mean rank) in *Micropterus*, which was highly correlation to EACs in sediment. In contrast, intersex was less common (9.9%) and less severe (0.2 mean rank) in *Lepomis* and was highly correlated to EACs in the water column. The authors found that concentrations of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, industrial EACs, and estrogens were highest at point source sites; however, no source type variation was identified in the prevalence or severity of intersex, nor were there seasonal trends in intersex or EAC concentrations. The authors' results associate genus-specific prevalence of intersex with specific EAC classes in common sport fishes having biological, ecological, and conservation implications. *Environ Toxicol Chem* 2017;36:959–968. © 2016 SETAC

Keywords: Intersex Estrogens Endocrine disruption Black bass Sunfish

INTRODUCTION

An escalating global issue is the demasculinization (reduced expression of male-specific characteristics) and feminization (increased expression of female-specific characteristics) of male fish inhabiting waters contaminated with estrogenic endocrine active contaminants (EACs [1–4]). Estrogenic EACs are composed of several chemical classes, including natural estrogens (e.g., 17β-estradiol [E2] and isoflavones), synthetic estrogens (e.g., 17α-ethynylestradiol [EE2] and diethylstilbestrol), and estrogen mimics (e.g., polychlorinated biphenyls [PCBs], polycyclic aromatic hydrocarbons [PAHs], and bisphenol A [5,6]) These compounds originate from both point sources and nonpoint sources. Point sources (e.g., wastewater treatment plants [WWTPs] and industrial facilities) are permitted for direct discharge of effluent into water systems, whereas nonpoint sources (e.g., concentrated animal feeding operations [CAFOs] and agricultural waste sources) have the potential for indirect diffusion of EACs into water systems through runoff. Of great concern are the numerous findings of EACs in surface waters throughout the globe [2,7,8] and the issue of elevated estrogenic EACs downstream of WWTP effluent discharge points [2,9,10] and CAFOs [11,12]. Estrogenic EACs released into the environment can vary temporally as a result of seasonal output from agricultural pesticide application, land application of CAFO waste, and variability in the load and efficiency of WWTPs [13]. Seasonal variation in discharges may complicate the understanding of EAC influences on aquatic ecosystems and fish health.

Exposure to estrogenic contaminants has been linked to adverse effects in a variety of fish species [3,10,14–16]. For example, estrogenic EACs induce the expression of vitellogenin (VTG) in male fish; VTG is an egg yolk precursor protein normally found in gravid females [2,10,12,17]. Exposure to estrogenic contaminants can also reduce sperm [10,14] and egg [17] production in fish populations. Several studies have documented seasonal variation in hormone concentration, gonadal malformations and vitellogenin production in male fish [9,12]. Seasonal variation has also been documented in the intersex condition [12,18,19], which is characterized by the presence of both male and female gonad tissue within the testes of fixed-sex (gonochoristic) species [20]. Intersex has become a hallmark of estrogen exposure, a growing global concern [4,11,21,22], and has been documented in fish throughout North America [3,18,23,24] and Europe [4,14]. Prevalence of intersex in fish has been linked to proximity to WWTP effluent [4] and CAFOs [11,12,18]. United States Geological Survey (USGS) scientists recently examined the occurrence of intersex in 9 river basins across the United States [21]. They found the highest incidence of intersex (48–80%) in the Southeast, with the national maximum prevalence (80%) in the Pee Dee River basin of North Carolina and South Carolina.

Despite the widespread detection of intersex in fishes, few studies have investigated the direct relationship between intersex and the presence of EACs. Kolpin et al. documented links between intersex and several EACs in smallmouth bass (*Micropterus dolomieu*) [24]. However, EACs typically occur as complex mixtures in surface waters throughout the United States [2,7,8]. To better understand the relationship between

This article includes online-only Supplemental Data.

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Published online 1 September 2016 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3607

EACs and intersex, the objective of the present study was to conduct a statewide reconnaissance of the presence of EACs and intersex within fish populations inhabiting North Carolina water bodies. Two genera from the Centrarchidae family were targeted: *Micropterus* (black bass) and *Lepomis* (sunfish), both of which are widely distributed and commonly sought sport fishes. *Micropterus* are zooplanktivorous early in life and become piscivorous with growth, generally feeding on insects, fish, and crustaceans as adults [25]. *Micropterus* attain large sizes and usually occupy high trophic levels in aquatic systems. Similar to *Micropterus*, *Lepomis* are typically zooplanktivorous early in life. *Lepomis* diverge from *Micropterus* because they become omnivorous as they get larger [26], do not reach sizes as large as *Micropterus,* and are generally not piscivorous. *Lepomis* normally occupy a lower trophic level than the *Micropterus* species. The primary objective of the present study was to examine relationships between EACs detected in riverine sites throughout North Carolina and the prevalence and severity of intersex in *Micropterus* and *Lepomis* species, the latter a previously unstudied group in relation to intersex. A secondary objective was to examine variations in EAC contamination and fish intersex among 4 seasons and 3 EAC source types: point source, nonpoint source, and reference sites, with no known EAC sources.

EXPERIMENTAL METHODS

Site selection

To allow a comprehensive analysis of intersex in fish throughout North Carolina, 20 lotic systems (rivers or streams) spanning the state were selected based on a previous geographical information system-based analysis of putative sources of EACs in North Carolina water bodies [27]. Briefly, a map of potential EAC contamination was generated using existing land use data collected from the National Pollutant Discharge Elimination System (NPDES), the US Environmental Protection Agency (USEPA), and the North Carolina Department of Environmental and Natural Resources (NCDENR). Previously measured estrogenic activity and site accessibility were among primary criteria for site selection. Three reference sites (1–3) were selected from the geographical information system analysis based on lack of identified upstream contaminant sources (Supplemental Data, Figure S1). Initially, we selected 4 reference sites; after initial sampling, however, we discovered a poultry farm upstream of site 20. Thus, this site was redesignated as a nonpoint source. To enable analysis of a broad range of contaminants, 8 sites (4–11) associated with potential point source discharge and 9 sites (12–20) associated with nonpoint source were selected from across the state. Four of these 20 sites (2 point source and 2 nonpoint source) were evaluated during 4 consecutive seasons to assess potential seasonal influences on the presence of EACs and occurrence of intersex. Statewide sampling occurred during daylight hours, under base-flow conditions, at 20 sites from March to June 2012 (spring). Additional seasonal sampling occurred at the 4 seasonal sites (sites 6, 10, 12, and 20) during August to September 2012 (summer), October 2012 (fall), and January 2013 (winter).

Water estrogenic bioactivity

To estimate total estrogenic activity of surface waters at each site, a single subsurface water sample was obtained by holding a certified-solvent (ethanol [EtOH] and acetone) rinsed and baked (300 °C for 18 h) amber glass bottle 0.5 m below the water surface with the opening held parallel to the surface. Surface samples were collected approximately 30 d after fish sampling. To prevent bacterial degradation, samples were acidified to a pH of 2 with hydrochloric acid. Samples were packed on ice and transported to North Carolina State University (Raleigh, NC, USA) where they were stored at 4 °C until filtration and solid phase extraction (SPE) within 72 h. Briefly, approximately 2 L of filtered samples were loaded onto 500-mg Oasis HLB Vac Cartridges (Waters). Cartridges were eluted using a successive series of solvents (12 mL each) including dichloromethane (DCM), methanol, and acetone. Samples were solvent exchanged into EtOH to a final volume of 1 mL. Details on filtration and SPE can be found in the Supplemental Data.

Estrogenic potency of water samples was assessed using the T47D-KBluc bioassay. This assay utilizes human breast adenocarcinoma cells that express both human estrogen receptors (alpha and beta) and are stably transfected with an estrogen-responsive luciferase reporter gene construct. The assay utilized a cell line developed by Wilson et al. [28] and was conducted as described in detail by Yost et al. [29]. Briefly, cells were dosed with cell media containing extract and incubated for 16 h to 24 h. Following treatment, cells were lysed and luciferase activity was quantified using a FluoStar Omega microtiter plate reader (BMG Labtech). Samples were run as 4 technical replicates per plate. Included on each plate were an E2 dose–response standard curve (Steraloids) and a solvent blank (0.1% EtOH). Sample responses were normalized to the solvent blank, and E2 dose-response standard curves were used to quantify E2 equivalent concentrations (ng/L). Mean E2 equivalent concentration was obtained from 3 to 4 experimental repeats. Details can be found in the Supplemental Data.

Water contaminants

Two universal passive sampling devices (PSDs) were used to sample organic contaminants and hormones from surface waters over an integrated time period. Cages containing PSDs were deployed approximately 1 m from the riverbank. Each cage was attached to a brick to minimize movement downstream and a small buoy to ensure that it remained suspended in the water column. Cages were deployed for approximately 30 d to obtain a time-integrated assessment of contaminants present. After completion of deployment, cages were retrieved and PSDs were removed, wrapped in combusted foil, transported on wet ice to North Carolina State University, and stored at –20 °C until extraction. Details on PSDs and extraction protocol are presented in the Supplemental Data. One PSD was used for organic chemicals' analysis and was extracted by shaking PSD in 20 mL of DCM at 180 rpm for 1 h; this was repeated once. The other PSD was used for analysis of hormones and industrial EACs and was extracted using 10 mL of ethyl acetate and shaking in 20-mL vials at 150 rpm for 1 h; this was repeated once. Extracts were concentrated in an autosampler vial to approximately 0.5 mL by nitrogen evaporation at 35 °C under 5 psi prior to analysis. Extracts for hormone and industrial EAC analysis were further filtered through a 0.45-μm polytetrafluoroethylene filter into a 1.5-mL microvial, evaporated at ambient temperature under a gentle nitrogen stream until dry, and cap-sealed under argon gas.

Sediment contaminants

Composite sediment samples were obtained using a Petite Ponar dredge; 4 grabs to 6 grabs of the top 5 cm of sediment were collected and placed in a 500-mL amber glass jar. Sediment samples were collected approximately 30 d after fish sampling. Sediment samples were stored at –20 °C until extraction. Sediments were Soxhlet extracted using USEPA method

3540C [30]. Fifteen grams of sediment were dried with anhydrous sodium sulfate and then extracted with 250 mL of a 1:1 solution of acetone:DCM for 18 h. Extracts were cleaned using gel permeation chromatography to remove nontarget co-extractables from extract prior to chemical analysis. Extracts were concentrated to 0.5 mL under a gentle stream of nitrogen.

Chemical analysis

To assess a wide range of EACs, 134 contaminants (Supplemental Data, Table S1) were selected for analysis in the present study. The EACs were classified into 6 groups: industrial compounds, hormones, PAHs, PCBs, organochlorine pesticides, and current-use pesticides. Contaminants were analyzed using an Agilent 6890 gas chromatograph connected to an Agilent 5973 mass selective detector and operated in select ion monitoring (SIM) mode for 48 current-use pesticides, 29 PAHs, 21 PCBs, and 28 organochlorine pesticides. Analyses were separated on a Restek Rtx-5MS column (30 m × 250 μm diameter × 0.25 μm film thickness) with a 5-m integrated guard column. Seven hormones and 2 industrial compounds were derivatized with N,O-bis (trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane in pyridine and then analyzed by gas chromatography–mass spectrometry SIM with backflushing after a blank injection with pyridine to condition the column. Concentrations in the water (ng/L) were determined by calibrating PSD concentrations by compound sampling rates [31,32]. Polycyclic aromatic hydrocarbons, PCBs, and organochlorine pesticides are only reported from the summer, fall, and winter from the 4 seasonal sites because of technical issues associated with spring sample analysis. Detection limits can be found in the Supplemental Data.

Fish sampling and health variables

Micropterus spp. and *Lepomis* spp. were collected by pulsed-DC, boat-mounted, and backpack electrofishing. Statewide sampling occurred at all 20 sites during spring 2012. Subsequently, during summer 2012, fall 2012, and winter 2013, fish were sampled at 4 sites (sites 6, 10, 12, and 20) for seasonal analysis. Fish were captured and processed according to a protocol approved by the North Carolina State University Institutional Animal Care and Use Committee. Males were selected by palpating ripe fish to induce milting. During the spawning season, in addition to palpating, external sexually dimorphic characteristics were used to identify male bluegill (*Lepomis macrochirus*), the only sexually dimorphic species collected. Fish presumed to be females were released at their capture site. Collected fish were euthanized using 300 mg/L of tricaine methanesulfonate (MS-222; Sigma-Aldrich) and buffered with sodium bicarbonate, and testicular tissues were extracted. All fish were weighed (g) and measured for total length (mm), and relative weight (a measure of condition) was calculated. Testes weight (g) was measured to calculate gonadosomatic index (GSI), an indicator of reproductive investment, and liver weight (g) was measured to determine hepatosomatic index (HSI), another indicator of health. For HSI, gonad weight was subtracted from body weight to minimize the effect of the reproductive cycle on this index. Equations can be found in the Supplemental Data.

Histological assessment

Testes were fixed in Modified Davidson Fixative (35.15% distilled water, 31.35% of 100% EtOH, 22% of 37% formaldehyde, and 11.5% glacial acetic acid) for 24 h. Fixative was replaced with 70% EtOH and samples were held at room temperature until processing. Following specific chain-of-custody procedures, tissue samples were processed, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) by the North Carolina State University College of Veterinary Medicine Histopathology Laboratory, and analyzed with light microscopy by a board-certified American College of Veterinary Pathologist (ACVP). To quantify intersex, at least 5 testicular sections were examined for incidence and severity of intersex based on a ranking by Blazer et al. [23]. Intersex severity was ranked from 1 through 4, with 4 being the most severe. Absence of intersex was assigned a rank of 0 for analysis. To screen fish for background diseases (e.g., microbial pathogens), in parallel with the sections of testes, H&E-stained sections of the liver, spleen, kidney, and gill were examined.

Statistical analysis

Statistical analysis was conducted using JMP Pro 11 statistical software (SAS). All continuous variables were examined for normality using the Shapiro-Wilk normality test prior to statistical analysis. Because data did not conform to a normal distribution, nonparametric procedures were applied. Spearman correlations were used to investigate colinearity among EACs. Highly correlated compounds within EAC classes were combined to minimize colinearity and redundancy in statistical analysis, as described in the Supplemental Data. Spearman correlations were also used to assess the relationship between fish health variables and severity ranking. Kruskal-Wallis analysis was conducted to evaluate the effects of season and source type (point source, nonpoint source, or reference) on water bioactivity, EAC concentrations, fish health variables, and severity rank. Kruskal-Wallis analysis was also conducted to investigate the relationship between intersex condition and health variables, as well as the relationships of species or genus to severity ranking. Logistic regression was used to ascertain associations among species and genera to intersex condition. Occurrence of intersex was converted to percentage of individual fish per site to assess the effect of season and source type on site-specific prevalence of intersex using Kruskal-Wallis analysis. Pairwise comparisons were conducted using Wilcoxon pairwise analysis. To assess the relationship between EAC mixtures and intersex, principal component analysis was conducted with percent intersex, average severity rank, and EAC concentrations for each site as variables. Analysis was weighted by the number of fish collected per site.

RESULTS

Water estrogenic bioactivity and contaminants

Mean E2 equivalent concentrations ranged between 0.4 ng/L and 8.8 ng/L throughout the present study (Supplemental Data, Table S3). There was no significant difference ($p > 0.05$) in E2 equivalent concentrations among sites of the 3 source types (reference, nonpoint source, and point source; Table 1), nor was there a significant difference ($p > 0.05$) among the 4 seasons in E2 equivalent concentration (Table 2). Of the 134 chemicals analyzed, 44 were detected in the water column (Supplemental Data, Table S4). In the water, current-use pesticides were the most frequently detected EAC group; 4 current-use pesticides detected in the water were found at 14 sites. Four hormones were detected at 11 sites, and no androgen hormones were detected in the water ("hormones" will hereafter be referred to as "estrogens"). The only 2 industrial EACs analyzed were detected at 8 sites. Seven organochlorine pesticides and 22 PAHs were detected at the 4 seasonal sites. The least abundant EACs in the water were PCBs; 4 PCBs were detected at 2 of the

Table 1. Mean estimated 17β-estradiol equivalent concentration (E2 Eq) and concentration of endocrine active contaminants (EACs) detected in water and sediment among source types[a]

	Source type		
EACs	REFs	NPSs	PSs
Water (ng/L)			
E2 Eq	1.07 (0.45) A	1.23 (1.20) A	1.91 (2.07) A
Metolachlor	0.01 (0.01) A	5.20 (8.82) A	4.57 (8.29) A
Triazines	0.00 (0.00) A	3.24 (6.36) A	9.51 (22.50) A
Industrials	0.00 (0.00) A	0.00 (0.00) A	1.19 (1.05) B
Estrogens	0.12 (0.16) A,B	0.17 (0.43) A	1.46 (1.41) B
OCPs	NA	0.15 (0.33) A	0.13 (0.21) A
PAHs	NA	3.33 (5.39) A	5.82 (8.28) A
PCBs	NA	0.00 (0.00) A	0.02 (0.06) A
Sediment (ng/g)			
CUPs	0.00 (0.00) A	2.18 (5.67) A	0.00 (0.00) A
Chlordanes	0.00 (0.00) A	0.14 (0.21) A	0.55 (0.71) A
DDTs	1.59 (2.25) A	0.75 (1.01) A	1.04 (2.57) A
Hexachlorobenzene	0.00 (0.00) A	0.00 (0.00) A	0.01 (0.06) A
PAHs	41.29 (29.11) A	414.23 (417.79) A	1201.48 (978.72) B
PCBs	0.15 (0.21) A,B	0.06 (0.11) A	2.35 (3.25) B

[a]Values are expressed as means (standard deviation in parentheses). Letters denote significant differences ($p < 0.05$) among source types.
REFs = reference sources; NPSs = nonpoint sources; PSs = point sources; triazines = total triazine pesticides; industrial = bisphenol A and nonylphenol; estrogens = total synthetic and natural estrogens; OCPs = organochlorine pesticides; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; CUPs = current use pesticides; chlordanes = total chlordane; DDTs = total DDTs and DDT metabolites; NA = not applicable because of processing error.

seasonal sites. Comparison among source types revealed that industrial EACs were only detected at point source sites (Table 1). Mean concentration of total estrogens was 1.3 ng/L higher ($p = 0.002$) at point source sites compared with nonpoint source sites. There were no other differences detected ($p > 0.05$) in EACs in water samples among the 3 source types (Table 1), nor were there any differences in water EACs among the 4 seasons (Table 2).

Sediment contaminants

Of the 134 chemicals analyzed, 53 were detected in sediment (Supplemental Data, Table S4). In sediment, 29 PAHs were found throughout the 20 sites, making PAHs the most frequently detected EAC group. Six organochlorine pesticides were detected at 14 sites, and 15 PCBs were detected in sediment at 11 sites. The least abundant group of EACs in the sediment was current-use pesticides; 2 of 49 analyzed current-use pesticides were detected at 1 site. Hormones and industrial EACs were not detected in the sediment at any site. Comparison among source types revealed that total PAHs were 787.3 ng/g to 1160.2 ng/g higher ($p = 0.006$) at point source sites compared with nonpoint source and reference sites (Table 1). Mean concentration of total PCBs was 1.8 ng/g higher ($p = 0.001$) at point source sites compared with nonpoint source sites. There were no other differences detected ($p > 0.05$) in EACs among the 3 source types. There was no seasonal trend in sediment EAC detection (Table 2).

Fish health variables

Lepomis spp. ($n = 302$) were collected at all sites, and *Micropterus* spp. ($n = 122$) at 16 sites (Table 3). Collections included 106 bluegill (*L. macrochirus*), 1 dollar sunfish

Table 2. Mean estimated17β-estradiol equivalent concentration (E2 Eq) and concentration of endocrine active contaminants (EACs) detected in water and sediment among seasons[a]

EACs	Spring 2012	Summer 2012	Fall 2012	Winter 2013
Water (ng/L)				
E2 Eq	1.58 (0.42) A	1.04 (0.25) A	2.90 (1.97) A	1.46 (0.50) A
Metolachlor	6.57 (8.09) A	0.00 (0.00) A	0.01 (0.01) A	0.01 (0.01) A
Triazines	27.31 (39.02) A	3.03 (6.06) A	0.03 (0.04) A	0.01 (0.02) A
Estrogens	1.53 (2.59) A	0.93 (1.08) A	0.56 (0.65) A	0.94 (1.04) A
Industrials	0.82 (1.46) A	1.17 (1.65) A	0.65 (0.76) A	0.85 (0.97) A
OCPs	NA	0.62 (0.45) A	0.25 (0.17) A	0.15 (0.11) A
PAHs	NA	12.58 (9.50) A	12.72 (5.27) A	6.72 (5.26) A
PCBs	NA	0.08 (0.10) A	0.00 (0.00) A	0.00 (0.00) A
Sediment (ng/g)				
CUPs	0.50 (1.01) A	5.40 (10.79) A	1.70 (3.40) A	0.57 (1.13) A
Chlordanes	0.14 (0.20) A	0.33 (0.42) A	0.17 (0.29) A	0.25 (0.41) A
DDTs	0.36 (0.53) A	0.69 (1.11) A	0.85 (1.43) A	0.37 (0.64) A
Hexachlorobenzene	0.00 (0.00) A	0.00 (0.00) A	0.00 (0.00) A	0.00 (0.00) A
PAHs	548.91 (209.71) A	663.59 (627.33) A	617.38 (448.38) A	542.63 (736.67) A
PCBs	0.50 (0.51) A	1.65 (3.18) A	0.64 (0.68) A	2.28 (3.91) A

[a]Values are expressed as means (standard deviation in parentheses). Letters denote significant differences ($p < 0.05$) among the four seasons.
Triazines = total triazine pesticides; estrogens = total synthetic and natural estrogens; industrial = bisphenol A and nonylphenol; OCPs = organochlorine pesticides; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; CUPs = current use pesticides; chlordanes = total chlordane; DDTs = total DDTs and DDT metabolites; NA = not applicable because of processing error.

(*Lepomis marginatus*), 16 green sunfish (*Lepomis cyanellus*), 98 redbreast sunfish (*Lepomis auritus*), 77 redear sunfish (*Lepomis microlophus*), 1 spotted sunfish (*Lepomis punctatus*) and 3 warmouth (*Lepomis gulosus*). As a result of the low detection frequency (collections at $\leq$ 2 sites) of dollar sunfish, green sunfish, spotted sunfish, and warmouth, these species were excluded from statistical analysis. *Micropterus* spp. collections included 93 largemouth bass (*Micropterus salmoides*) and 29 smallmouth bass. No bluegill, redear sunfish, or largemouth bass were sampled at reference sites. Analysis of source type variation revealed that the weight, GSI, HSI, and relative weight were greater ($p < 0.05$) for bluegill at nonpoint source sites than for those at point source sites (Figure 1). The weight, HSI, and relative weight of redbreast sunfish sampled at nonpoint source and point source sites were greater ($p < 0.05$) compared with those of redbreast sunfish sampled at reference sites. The GSI of redbreast sunfish sampled at point source sites was higher ($p = 0.015$) than the GSI of redbreast sunfish sampled at nonpoint source sites (Figure 1C). The HSI of redear sunfish sampled at point source sites was higher ($p = 0.032$) than the HSI of redear sunfish sampled at nonpoint source sites (Figure 1D). There were no other differences detected ($p > 0.05$) in health variables among the 3 source types for the 5 species. Ancillary analysis of background pathogens revealed that many of the *Micropterus* spp. and *Lepomis* spp. had small numbers of encysted, intermediate stages of digenean trematodes within their livers and spleens; no remarkable lesions were found to indicate bacterial or viral infection in fish from any of the sites. Gill samples had only scattered, small numbers of trematodes and remarkably few protozoans. No clear evidence of disease was found in any of the treatment site fish in comparison with the reference site fish.

Intersex occurrence and severity

Intersex prevalence and severity were uniform among source types, seasons, and species within genera. Although dollar sunfish, green sunfish, spotted sunfish, and warmouth were not included in statistical analysis, the absence of intersex in those species is noteworthy. There was no interspecies difference ($p > 0.05$) in prevalence of intersex among bluegill (10.4% of 106 individuals), redbreast sunfish (13.3% of 98 individuals), and redear sunfish (7.8% of 77 individuals). Intersex among *Lepomis* was detected in 30 of the 302 (9.9%) collected individuals (Table 3) and ranged from 10.0% to 37.5% of collected individuals across 13 sites (or 23 of the 32 sampling events including seasonal samples; Supplemental Data, Table S5). Within *Lepomis*, only 1 fish had a severity rank of 4, a bluegill at site 17 (a nonpoint source site; Table 3). There was no difference detected ($p > 0.05$) in severity among the 3 *Lepomis* spp. Low severity rankings (1 or 2) were found in 9 (81.8%) of the 11 intersex bluegill. Similarly, more than 65% of intersex redear sunfish and redbreast sunfish were assigned a severity rank of 1 or 2. The average severity rank for *Lepomis* (including intersex and normal fish) ranged from 0.1 at site 19 (a nonpoint source site) to 0.75 at site 4 (a point source site; Supplemental Data, Table S5). Intersex prevalence and severity rankings in bluegill, redbreast sunfish, and redear sunfish were not different ($p > 0.05$) among the 3 source types or the 4 seasons (Figure 2).

There were also clear intraspecies variations for *Lepomis* in health variables between intersex and normal fishes. Comparison of health variables between intersex and normal *Lepomis* fish revealed that normal redbreast sunfish had greater weight and total length ($p < 0.05$) than redbreast sunfish with intersex (Figure 3A, B) and these metrics were inversely correlated (Spearman's coefficient [ρ] = –0.25 to –0.27, $p < 0.05$) with

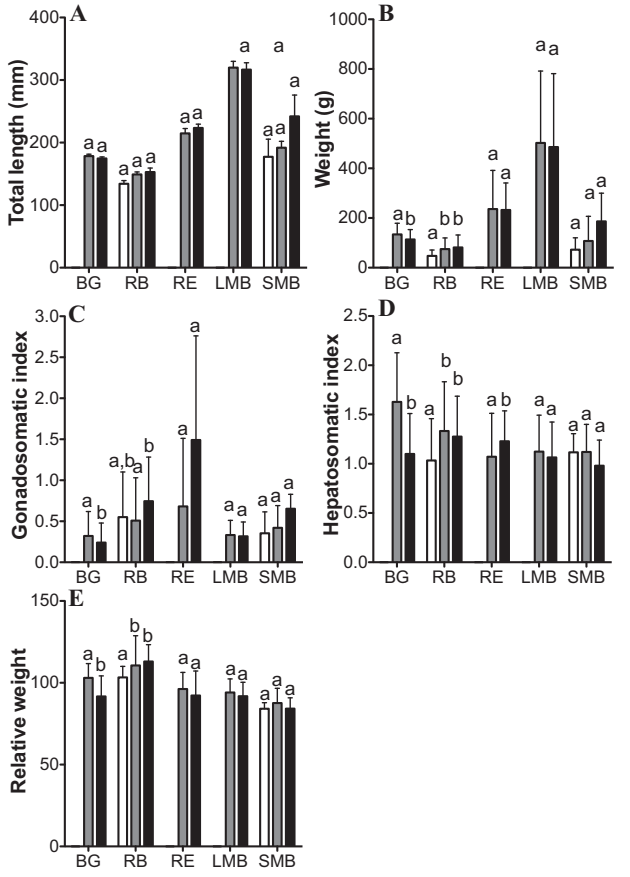

Figure 1. Mean (± standard error) fish (**A**) total length, (**B**) weight, (**C**) gonadosomatic index, (**D**) hepatosomatic index, and (**E**) relative weight, according to species at different source types. Different letters denote significant differences (Wilcoxon, $p < 0.05$) among reference sites (white bars), nonpoint sources (gray bars), and point sources (black bars) for each species: Bluegill (BG; $n = 106$), redbreast sunfish (RB; $n = 98$), redear sunfish (RE; $n = 77$), largemouth bass (LMB; $n = 93$), and smallmouth bass (SMB; $n = 29$).

Table 3. Incidence of intersex (number of fish) and severity rank for *Lepomis* spp. and *Micropterus* spp.

		Intersex and severity rank					
Genus	Species	Incidence	1	2	3	4	*n*
Lepomis							
	BG	11	5	4	1	1	106
	DS	0	0	0	0	0	1
	GS	0	0	0	0	0	16
	RB	13	4	5	4	0	98
	RE	6	3	1	2	0	77
	SS	0	0	0	0	0	1
	WM	0	0	0	0	0	3
	All[a]	30	12	10	7	1	302
Micropterus							
	LMB	59	11	14	17	17	93
	SMB	15	3	5	4	3	29
	All	74	14	19	21	20	122

[a]Includes only BG, RB, and RE (DS, GS, SS, and WM were not detected frequently).
BG = bluegill; DS = dollar sunfish; GS = green sunfish; RB = redbreast sunfish; RE = redear sunfish; SS = spotted sunfish; WM = warmouth; LMB = largemouth bass; SMB = smallmouth bass.

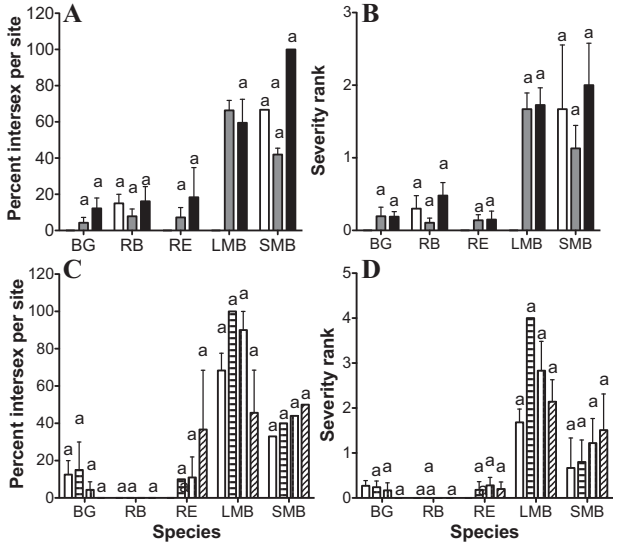

Figure 2. Mean (± standard error) (**A, C**) percent intersex per site, and (**B, D**) severity ranking according to species and season. (**A, B**) Distribution among reference sources (white bars), nonpoint sources (gray bars), and point sources (black bars). (**C, D**) Distribution among the 4 seasons within each species: spring 2012 (white bars), summer 2012 (white bars with horizontal stripes), fall 2012 (white bars with vertical stripes), and winter 2013 (white bars with diagonal stripes). Different letters denote significant differences (Wilcoxon, $p < 0.05$) within species for source type and season. BG = bluebill ($n = 106$); RB = redbreast sunfish ($n = 98$); RE = redear sunfish ($n = 77$); LMB = largemouth bass ($n = 93$); SMB = smallmouth bass ($n = 29$).

severity ranking (Figure 3F and G). The GSI of normal redear sunfish was about 5 times greater ($p = 0.017$) than the GSI of redear sunfish with intersex (Figure 3C) and was inversely related ($\rho = -0.28$, $p = 0.014$) to severity ranking (Figure 3H). There was no difference detected in health variables between intersex and normal bluegill sunfish. There were no other significant relationships ($p > 0.05$) between severity ranking and health variables in *Lepomis* spp.

Similar to *Lepomis* spp. there was no interspecies difference ($p > 0.05$) in intersex prevalence or severity ranking among *Micropterus* spp.; 63.4% of 93 largemouth bass and 51.7% of 29 smallmouth bass collected exhibited intersex (Table 3). Of the 122 *Micropterus* males collected, 73 (59.8%) were intersex. The prevalence of intersex ranged from 14.0% to 100.0% of *Micropterus* spp. collected per site at 13 sites (or 21 of the 23 sampling events including seasonal samples; Supplemental Data, Table S5). High severity rankings (3 or 4) were found in 58% of the intersex largemouth bass and 47% of the intersex smallmouth bass (Table 3). Average severity rank ranged from 0.29 at site 5 (a point source site) to 4 at sites 6 and 10 (both point source sites; Supplemental Data, Table S5). For both largemouth bass and smallmouth bass, there was no significant difference ($p > 0.05$) in intersex prevalence or severity among source type or season (Figure 2). Comparison of health variables between intersex and normal fish revealed that total length, weight, and relative weight of smallmouth bass with intersex were greater ($p < 0.05$) than those of normal smallmouth bass (Figure 3). In addition, the weight and GSI of smallmouth bass were positively related ($\rho = 0.47$ to 0.52, $p < 0.05$) to severity ranking (Figure 3G, H). There were no other significant relationships ($p > 0.05$) between intersex and health variables in *Micropterus* spp.

Genus-specific variations occurred in intersex prevalence and severity ranking among the species collected. Intersex was

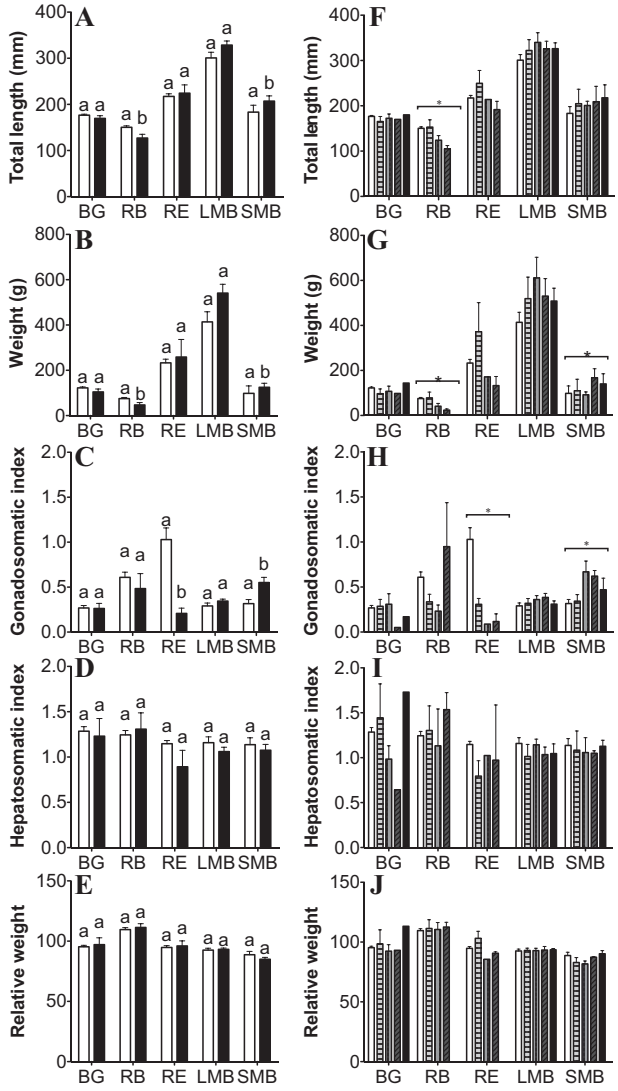

Figure 3. Mean (± standard error) fish (**A, F**) total length, (**B, G**) weight, (**C, H**) gonadosomatic index, (**D, I**) hepatosomatic index, and (**E, J**) relative weight, of normal or severity rank 0 (white bars), intersex males (black bars), rank 1 (light gray bars), rank 2 (gray bars), rank 3 (dark gray bars), and rank 4 (black bars) for each species: bluegill (BG; $n = 106$); redbreast sunfish (RB; $n = 98$); redear sunfish (RE; $n = 77$); largemouth bass (LMB; $n = 93$); and smallmouth bass (SMB; $n = 29$). Different letters denote significant differences (Kruskal-Wallis, $p < 0.05$) within each species. Asterisks indicate significant differences (Spearman correlation, $p < 0.05$) among severity ranks.

12.9 times (95% confidence interval: 7.6–21.8 times) more likely to be present in *Micropterus* than in *Lepomis*. In addition, severity ranking was greater in *Micropterus* compared with *Lepomis*; the mean severity ranking for *Lepomis* (mean, 0.20; standard error, 0.04) was significantly lower ($p < 0.0001$) than the mean severity ranking for *Micropterus* spp. (mean: 1.60; standard error: 0.14).

Relationship of EACs to intersex

The relationship of EACs to intersex prevalence and severity was analyzed by genus (combining species within each genus). Two retained principal components of *Lepomis* occurrence and severity ranking accounted for 51.2% of the variability in the data (Figure 4A; loadings and variance can be found in Supplemental Data, Table S6). For *Lepomis*, there was a distinct clustering of sites with low incidence of both

intersex and severity ranking, as well as low concentrations of EACs (Table 4). Prevalence and severity of intersex clustered closely with the hydrophilic contaminants (industrial EACs, estrogens, and triazines) in ambient water and farther from the hydrophobic compounds (chlordanes, DDTs, PAHs, and PCBs) in sediment. In the case of *Micropterus* (Figure 4B), 2 principal components accounted for 45.8% of the variability in the data (Supplemental Data, Table S6). Similar to the *Lepomis* principal component analysis, the *Micropterus* principal component analysis produced a cluster of sites; however, these sites ranged from low to high incidence of intersex and low to moderate severity rankings while still having a lower concentration of EACs (Table 4). Occurrence of intersex in *Micropterus* clustered with the hydrophobic compounds in the sediment and water and farther from hydrophilic compounds in the water.

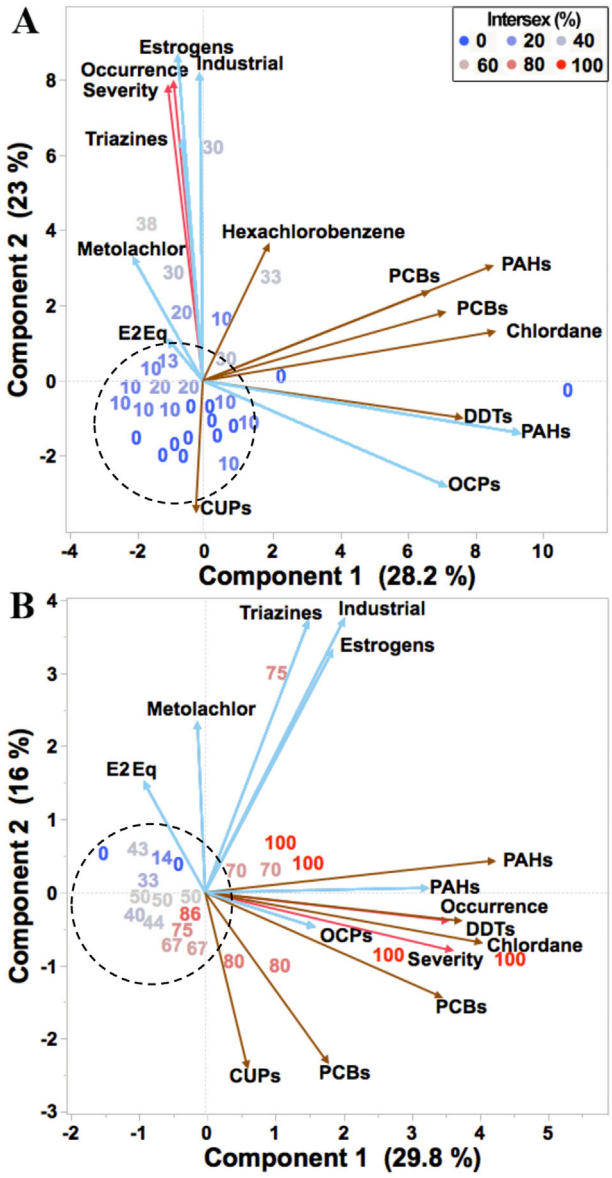

Figure 4. Principal component analysis of sediment (brown lines) and water (light blue lines) endocrine active contaminants, E2 Eq (light blue line) with incidence and severity of intersex (red lines) within (**A**) *Lepomis* spp. ($n = 281$) and (**B**) *Micropterus* spp. ($n = 122$). Numeric labels are percent intersex per site (blue to red, see legend). Variance explained (%) appears after each component in parentheses. Dashed black circles denote distinct clusters. PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; E2 Eq = 17β-estradiol equivalent; OCPs = organochlorine pesticides; CUPs = current use pesticides.

DISCUSSION

A distinct difference in prevalence and severity of intersex was observed between 2 genera of common and widespread sport fishes. We found a high incidence and severity of intersex in largemouth bass (63.4%) and smallmouth bass (51.7%), which corroborates previous studies that have documented high prevalence of intersex in black basses [21,24]. In comparison, we found a lower incidence and severity in bluegill (10.4%), redbreast sunfish (13.3%), and redear sunfish (7.8%), similar to prevalence of intersex (13.3%) in bluegill populations fed 50 mg/kg of an E2-spiked diet in a previous controlled investigation [33]. In another more recent study, no intersex was detected in bluegill inhabiting PCB-contaminated systems [34]. We are not aware of other studies documenting intersex in wild-caught *Lepomis,* and to our knowledge the present study is the first to do so. Our results suggest that prevalence of intersex is similar among species of the same genera. This is unique to current literature that typically documents higher prevalence of intersex in smallmouth bass relative to largemouth bass found within the same system [16,21,35,36]. However, smallmouth bass and largemouth bass were never sampled at the same location within our present study.

The present study illustrates correlations between intersex (occurrence and severity) and several classes of EACs in our analysis of North Carolina surface waters. This finding is similar to documented associations between atrazine, sitosterol, trans-nonachlor, and stigmastanol and intersex in smallmouth bass from the Potomac River of the mid-Atlantic US coast [24]. These results along with our findings imply that intersex is not driven solely by single compounds but rather by mixtures of compounds that may act in an additive or synergistic manner. Our results suggest that, within the 5 most abundant species collected, there are genus-specific associations between intersex and EAC classes. Intersex in *Lepomis* was strongly associated with EACs predominant in the water (industrial EACs, estrogens, and current-use pesticides). In contrast, PAHs, organochlorine pesticides, and PCBs prevalent in the sediment were important correlates with intersex occurrence and severity in *Micropterus*.

The divergence in relationships between the 2 Centrarchidae genera may be driven by differences in their ecological niches. *Micropterus* typically receive their nourishment by consumption of fish [25] and are more susceptible to bioaccumulation/magnification of hydrophobic contaminants relative to *Lepomis* spp. that are not apex predators [26,37]. Studies have documented elevated concentrations of organochlorine pesticides and PCBs in the tissues of *Micropterus* spp. [1,11,16]. Biomagnification of hydrophobic EACs could also account for the high prevalence and severity rank in *Micropterus*. Comparing tissue concentrations of contaminants between the 2 genera could clarify the differences we observed. Movement behavior could also account for the differences observed between the 2 genera. *Micropterus* generally have a larger home range than *Lepomis* [38] and, as a result, the elevated intersex observed in *Micropterus* may be associated with chemicals far from collection sites. Intersex in *Micropterus* may also be associated with aquatic contaminants not investigated in the present study. For example, phytoestrogens (plant-derived estrogens), which are known to activate estrogen receptors [39] and cause feminization in male fish [40],

Table 4. Intersex occurrence and mean severity rank and endocrine active contaminant (EAC) group concentrations from PCA clustering[a]

	Lepomis PCA		*Micropterus* PCA	
Variable	Clustered sites ($n = 12$)	Other sites ($n = 18$)	Clustered sites ($n = 15$)	Other sites ($n = 8$)
Intersex				
Occurrence (%)	7.5/0.0	13.0/0.0	45.9/0.0	88.1/70.0
Severity rank (0–4)	0.17 (0.16)	0.23 (13.61)	1.18 (0.78)	2.76 (0.88)
Water EACs (ng/L)				
E2 Eq	1.75 (2.56)	1.40 (0.25)	1.48 (1.26)	1.23 (0.62)
Metolachlor	4.25 (9.08)	4.77 (0.55)	4.58 (9.13)	6.21 (9.32)
Triazines	0.95 (2.41)	9.29 (7.82)	2.45 (5.42)	13.27 (29.40)
Industrials	0.12 (0.42)	0.84 (0.05)	0.16 (0.43)	1.09 (0.99)
Estrogens	0.29 (0.49)	1.09 (889.62)	0.32 (0.56)	1.71 (1.80)
OCPs	8.93 (4.86)	13.20 (3.04)	0.15 (0.11)	0.36 (0.27)
PAHs	0.24 (0.12)	0.45 (0.66)	5.13 (3.11)	17.04 (6.94)
PCBs	0.00 (0.00)	0.05 (2.35)	0.00 (0.00)	0.02 (0.05)
Sediment EACs (ng/g)				
CUPs	0.36 (0.83)	1.58 (5.24)	0.15 (0.58)	1.10 (2.41)
Chlordanes	0.11 (0.16)	0.47 (1.41)	0.11 (0.26)	0.77 (0.80)
DDTs	0.81 (1.00)	1.03 (20.14)	0.28 (0.47)	2.01 (3.30)
Hexachlorobenzene	0.00 (0.00)	0.01 (6.95)	0.00 (0.00)	0.00 (0.00)
PAHs	195.27 (190.30)	1131.07 (0.40)	410.19 (398.84)	1461.23 (1189.28)
PCBs	0.20 (0.40)	1.76 (0.08)	0.71 (2.07)	2.44 (3.65)

[a]Intersex per site is expressed as mean/minimum. All other values are expressed as means (standard deviation in parentheses).
PCA = principal component analysis; E2 Eq = 17β-estradiol equivalent concentration; triazine pesticides; industrial = bisphenol A and nonylphenol; estrogens = total synthetic and natural estrogens; OCPs = organochlorine pesticides; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; CUPs = current use pesticides; chlordanes = total chlordane; DDTs = total DDTs and DDT metabolites.

were not analyzed because of the current capability of the analytical lab; however, they have been detected in surface waters throughout the United States [24].

In addition to relationships between intersex and EACs, we observed species- and genus-specific differences between intersex and fish health variables. For both bluegill and largemouth bass, we identified no relationship between intersex and health variables. This is unlike the pattern observed in largemouth bass by Kellock et al. [41], who documented reduced length in intersex males. Similarly, we observed reduced weight, total length, and GSI in redbreast sunfish and redear sunfish in association with intersex prevalence and severity. This pattern has been previously observed in the roach (*Rutilus rutilus* [14]). In contrast, total length, weight, and GSI in smallmouth bass had a positive relationship with severity ranking and were greater in intersex fish. Factors influencing these differences in health variables among the 5 species analyzed are unclear. Filby et al. [42] illustrated that EE2 exposure can alter expression of growth factors in the liver and testes of fathead minnows (*Pimephales promelas*). Research focused on connecting growth factor increase or suppression with the intersex condition could provide valuable insight into mechanisms underlying these associations.

We also found a relationship between fish health variables and source type within several species; whereas there was no source-specific variation in *Micropterus* spp. health variables, those differences were detected among *Lepomis* spp. The GSI and HSI of both redear sunfish and redbreast sunfish, as well as the weight and relative weight of redbreast sunfish, were greater at point source sites. These findings are similar to those documented in male rainbow darters and fathead minnows, where exposure to WWTP effluent caused an increase in HSI and GSI [22]. Both studies suggest that increase in health variables may be a result of extra nutrients and productivity derived from WWTP effluent. During our sampling, we noted an abundance of *Lepomis* spp. aggregating near effluent pipes, possibly in response to elevated nutrients and associated forage that might promote the larger sizes of redbreast sunfish and redear sunfish at point source sites. Conversely, we found that the weight, GSI, HSI, and relative weight of bluegill were greater at nonpoint source sites compared with point source sites. The variation in bluegill health variables may be driven by altered expression of growth factors, which can be attenuated by estrogenic EACs predominant at point source sites; however, the conflicting patterns seen among *Lepomis* spp. make this result difficult to interpret. Differences in life history, behavioral patterns, and energy allocation strategies could be important drivers of source-specific differences in the health variables that we measured.

Industrial EACs and estrogens in water, which typically enter aquatic systems through WWTP effluent [2,13], were more prevalent at point source sites than nonpoint source and reference sites. Similarly, PAHs and PCBs in the sediment were more abundant at point source sites, consistent with previous work in the Potomac River drainage, USA [7]. Despite the source-specific variations observed in EAC concentrations, there was no similar variation in intersex within the species assessed. There was also no seasonal variation in EACs or intersex. This lack of seasonal and source type variation in intersex may be a result of the lack of seasonal and site variation of E2 equivalent concentration in the present study. Estrogenic EACs mediate their toxicity in an additive manner [5]. However, estimated total E2 concentrations in the water were relatively low throughout the present study and did not vary with source type or season, possibly accounting for the minimal variation in intersex.

Although we observed no seasonal variation in intersex or relationships between intersex and source type among the species sampled, our results suggest that *Micropterus* have a higher predisposition for intersex and higher-ranking severity than *Lepomis.* There are a number of potential explanations for this pattern, including differences in responsiveness of estrogen receptors among the species [43]. The underlying mechanism of action for estrogenic compounds is activation of estrogen receptors, a class of highly conserved ligand-dependent

transcription factors [44]. Despite the evolutionary conservation of estrogen receptors, researchers have documented differential activation of estrogen receptors among fish species [43]. Another possible explanation for the variation of intersex prevalence between the 2 genera relates to baseline level of intersex in fish populations (i.e., the expected prevalence in the absence of contaminant influence). Our results indicated that at low concentrations of EACs, intersex in *Lepomis* was 0% to 7.5%. In contrast, at similarly low EAC concentrations, intersex in *Micropterus* was 0% to 45.9%. Similarly, Bahamonde et al. [20] reviewed 15 studies of 11 fish species and documented variation in baseline levels of intersex. Without a clear understanding of baseline levels of intersex in *Lepomis* and *Micropterus* species, ruling out baseline differences as a contributing mechanism driving our genus-specific results is not possible. Nonetheless, although baseline levels of intersex may vary between *Micropterus* and *Lepomis*, the levels of intersex observed in the present study are abnormal and likely environmentally influenced. Previous studies suggest that natural levels of intersex may be approximately 8% in largemouth bass and 10% to 14% in smallmouth bass [21]. Levels of intersex detected in our survey far exceeded these levels.

Overall, our findings suggest that anthropogenic-derived EACs may influence the levels of intersex observed in the present study. This, coupled with the global incidence of fish mortalities [11], immune suppression [1], and sexual disruption [7,45], is foreboding for freshwater fish populations and fisheries. Our research informs and begins to elucidate the complex spatial, temporal, and causal dynamics of the intersex condition of fishes in inland surface waters, and we await future findings to further enhance inference and understanding toward mitigating the condition.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3607.

Acknowledgment—This research was funded by the North Carolina Wildlife Resources Commission through Federal Aid in Sport Fish Restoration grant NC-F-99-R. The North Carolina Cooperative Fish and Wildlife Research Unit is jointly supported by North Carolina State University, the North Carolina Wildlife Resources Commission, the US Geological Survey, the US Fish and Wildlife Service, and Wildlife Management Institute. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government. We thank S. Buczek, B. Dooley, D. Owensby, and J. Wehbie for their assistance with field sampling and laboratory processing and analyses. A. Glazie and R. Njagu provided laboratory assistance, and D. Shea, P. Lazaro, A. McEachran, and X. Xia conducted chemical analysis of PSDs and sediment. We thank S. Gadi for her assistance in processing fish tissues for the histological section and C. Jennings for a constructive manuscript review.

Disclaimer—There is no conflict of interest.

Data availability—Data are available on request from C. Lee Pow (cleepow8@gmail.com).

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