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Widespread occurrence of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995–2004

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ABSTRACT

Intersex occurrence in freshwater fishes was evaluated for nine river basins in the United States. Testicular oocytes (predominantly male testes containing female germ cells) were the most pervasive form of intersex observed, even though similar numbers of male (n = 1477) and female (n = 1633) fish were examined. Intersex was found in 3% of the fish collected. The intersex condition was observed in four of the 16 species examined (25%) and in fish from 34 of 111 sites (31%). Intersex was not found in multiple species from the same site but was most prevalent in largemouth bass (*Micropterus salmoides*; 18% of males) and smallmouth bass (*M. dolomieu*; 33% of males). The percentage of intersex fish per site was 8–91% for largemouth bass and 14–73% for smallmouth bass. The incidence of intersex was greatest in the southeastern United States, with intersex largemouth bass present at all sites in the Apalachicola, Savannah, and Pee Dee River Basins. Total mercury, *trans*-nonachlor, *p*,*p*′-DDE, *p*,*p*′-DDD, and total PCBs were the most commonly detected chemical contaminants at all sites, regardless of whether intersex was observed. Although the genotype of the intersex fish was not determined, the microscopic appearance of the gonads, the presence of mature sperm, and the concentrations of sex steroid hormones and vitellogenin indicate the intersex bass were males. Few reproductive endpoints differed significantly among male and intersex bass; plasma vitellogenin concentration in males was not a good indicator of intersex presence. Hierarchical linkages of the intersex condition to reproductive function will require a more quantitative measure of intersex (e.g. severity index) rather than presence or absence of the condition. The baseline incidence of intersex gonadal tissue in black basses and other freshwater fishes is unknown, but intersex prevalence may be related to collection season, age, and endocrine active compounds in the environment. Intersex was not found in largemouth bass older than five years and was most common in 1–3-year-old male largemouth bass. The cause(s) of intersex in these species is also unknown, and it remains to be determined whether the intersex we observed in largemouth and smallmouth bass developed during sex differentiation in early life stages, during exposure to environmental factors during adult life stages, or both.

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1. Introduction

Recent reports of intersex fish in numerous water bodies have stimulated widespread interest. Intersex is a general term used to describe the presence of both male and female characteristics in an individual fish. Ovotestis (or testicular oocytes) and testis–ova are considered pathological conditions that commonly typifies intersex in normally gonochoristic species (Hecker et al., 2006). This condition is not routinely observed macroscopically in most fish species and generally requires microscopic examination of the gonad. Different terminology has been used to describe intersex gonads. Intersex is commonly described as the presence of female germ cells, or oocytes, within a predominantly male gonad (Nolan et al., 2001), but has also been used to describe male germ cells, or spermatocytes, within a predominantly female gonad (Vine et al., 2005). We will refer to gonads with mixed germ cells of both sexes as intersex.

The mechanism or mechanisms responsible for intersex is not known, but many factors including exogenous steroids, temperature, pH, behavioral cues, and pollutants can influence sex differentiation in fish (Devlin and Nagahama, 2002). Steroid hormones (e.g. 17β-estradiol, 11-ketotestosterone) are critical in early sex differentiation; they direct the development of the gonad into ovarian or testicular tissue during gonadogenesis and gametogenesis (Devlin and Nagahama, 2002; Piferrer, 2001). Fish are most responsive to the action of endogenous and exogenous steroids before sex differentiation (i.e. during the labile period; Piferrer, 2001); therefore, sex differentiation in fish can be influenced by acute exposure to sex steroids (estrogens or androgens)

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or aromatase inhibitors during the early stages of sex determination (Jobling et al., 1998). In general, estrogen exposure leads to feminization and androgen exposure leads to masculinization. Fish develop at different rates; therefore, the timing of the labile period is species specific, ranging from newly hatched larvae in salmonids to juveniles in seabass (Piferrer, 2001).

Exposure to a wide variety of chemicals or to mixtures of environmental pollutants can induce intersex (Nash et al., 2004; Zillioux et al., 2001). These varied chemical stressors all have the ability to cause primary or secondary effects on the endocrine system associated with the hypothalamus–pituitary–gonadal axis (Cooper and Kavlock, 1997) and as such are collectively referred to as endocrine active compounds (EACs). EACs include organochlorine pesticides, polychlorinated biphenyls (PCBs), heavy metals, pharmaceuticals, and surfactants and are diverse in terms of their production, use, distribution, and persistence in the environment. Fish exposed to EACs have exhibited a variety of reproductive problems such as morphological dysgenesis, reduced fecundity, abnormal gamete production, and sex reversal (Mills and Chichester, 2005). EACs are routinely found in surface waters and consequently may affect the reproductive health of fish populations (Jobling et al., 1998; Kime, 1999; Kolpin et al., 2002; Vajda et al., 2008).

The intersex condition in fish has been suggested as an indicator of exposure to EACs. Association between intersex occurrence and EAC presence in freshwater and marine environments have been reported by numerous studies over the last decade (Anderson et al., 2003; Baldigo et al., 2006; Blazer et al., 2007; Jobling et al., 1998; Lavado et al., 2004; Vajda et al., 2008; Woodling et al., 2006). For example, the increased prevalence of intersex in male roach (*Rutilus rutilus*) has been correlated positively with concentrations of wastewater treatment plant effluent (Bjerregaard et al., 2006; Jobling et al., 1998). Similarly, EACs from treated sewage and agriculture have been suggested as a potential cause of greater intersex prevalence in smallmouth bass (*Micropterus dolomieu*) (Baldigo et al., 2006; Blazer et al., 2007). The occurrence of intersex in white sucker (*Catostomus commersoni*) was also greater in streams dominated by wastewater effluent (Vajda et al., 2008; Woodling et al., 2006).

Surveys in which intersex in fish have been evaluated systematically across a large geographic range is an important component of aquatic biomonitoring efforts in Europe (Allen et al., 1999; Jobling et al., 1998; Minier et al., 2000; Stentiford et al., 2003). For example, intersex prevalence was 0–9% in male flounder (*Platichthys flesus*) from United Kingdom estuaries (Allen et al., 1999; Stentiford et al., 2003). Jobling et al. (1998) reported that incidence of intersex ranged from 4% to 100% in male roach from various rivers in the United Kingdom and suggested that wastewater effluent was involved with occurrence of the condition. In a subsequent study, wild intersex roach had decreased milt production, sperm motility, and fertilization success compared to histologically normal male roach (Jobling et al., 2002). Intersex prevalence ranged from 0% to 25% in roach and 0% to 3.6% in chub (*Leuciscus cephalus*) from rivers in England and France (Minier et al., 2000). Intersex prevalence differed in eelpout (*Zoarces viviparous*; 0–28%), three spined stickleback (*Gasterosteus aculeatus*; 0–13%), and perch (*Perca fluviatilis*; 0–33%) from coastal waters and small rivers in Germany (Gercken and Sordyl, 2002). Other regional studies have reported intersex in wild populations of common carp (*Cyprinus carpio*) in Spain (Solé et al., 2003), gudgeon (*Gobio gobio*) in the United Kingdom (van Aerle et al., 2001), bream (*Abramis brama*) in The Netherlands and Germany (Hecker et al., 2002; Vethaak et al., 2002), and flounder in Denmark (Bjerregaard et al., 2006).

The incidence of intersex in fish is unknown for most aquatic environments in North America. Intersex in freshwater fishes was reported in a national contaminant study in the United States (Hinck et al., 2006, 2007a,b, 2008a; Schmitt, 2002; Schmitt et al., 2005). In contrast to European studies, site selection for this study was not limited to sites with known EACs sources (e.g. wastewater treatment plants) but represented a range of contaminant sources. The national study measured the concentrations of "legacy contaminants" (e.g. mercury, PCBs, and dichlorodiphenyltrichloroethane (DDT) isomers), some of which have been associated with endocrine disruption in fish. More contemporary EACs such as synthetic and natural hormones, pharmaceuticals, water-soluble pesticides, and surfactants were not measured. Biological responses (including morphological and reproductive endpoints) were included as general indicators of exposure to a broader range of chemicals including those that tend not to bioaccumulate. Intersex was anticipated to be observed occasionally but was not expected to be widespread when the national survey was initiated in 1995. However, intersex was observed in more fish as sampling progressed, and the need to summarize this information on a larger geographic scale became apparent. The generally low occurrence of intersex fish in individual basins precluded statistical analysis of these data in the individual basin studies. Our objectives are therefore to summarize the occurrence of intersex in fish from nine large U.S. river basins by species, site, and basin; determine if intersex prevalence is associated with reproductive and morphological endpoints; and explore associations between intersex occurrence and legacy contaminants.

2. Methods

Details of the sampling, field, and laboratory procedures have been described previously (Hinck et al., 2006, 2007a,b, 2008a; Schmitt, 2002; Schmitt et al., 2005). A brief summary is presented here.

2.1. Sampling and field procedures

Fish were collected from 111 sites in nine U.S. river basins between 1995 and 2004 (Supplemental Table 1). Sites were located in the Apalachicola River Basin ($n = 3$), Colorado River Basin ($n = 14$), Columbia River Basin ($n = 16$), Mobile River Basin ($n = 4$), Mississippi River Basin ($n = 48$), Pee Dee River Basin ($n = 3$), Rio Grande Basin ($n = 10$), Savannah River Basin ($n = 3$), and Yukon River Basin ($n = 10$; Supplemental Table 1). Each site was sampled once, usually in the fall. Sampling sites were located on the mainstem and large tributaries in each basin without regard to contaminants; however, the sites did represent a range of contaminant sources. Specific contaminant sources were not identified at each site, but general land use was determined (e.g., urban areas, row crop, pastureland, industrial processing, etc.). Most fish were captured by electrofishing; hook-and-line and nets (gill, fyke, trammel, or hoop) were used in the Yukon River Basin, Red Bluff Lake (Site 65), Elephant Butte Reservoir (Site 63), and Arroyo Colorado (Site 511). One piscivorous and one benthivorous species were sought at each site. Target species were largemouth bass (*Micropterus salmoides*), smallmouth bass, and common carp, but these species were not collected at all sites (Supplemental Table 1). Alternate species included spotted bass (*Micropterus punctulatus*), white bass (*Morone chrysops*), striped bass (*Morone saxatilis*), channel catfish (*Ictalurus punctatus*), flathead catfish (*Pylodictus olivaris*), northern pike, burbot (*Lota lota*), brown trout (*Salmo trutta*), largescale sucker (*Catostomus macrocheilus*), longnose sucker (*C. catostomus*), and white sucker. The collection target at each site was 10 (each) adult male and female fish of similar length and weight for each species. Fish were held in aerated live-wells or net pens until processed (usually less than 3 h).

A field necropsy was performed on each fish. Briefly, blood samples, obtained from the posterior caudal artery and vein with a

heparinized needle and syringe, were centrifuged. The plasma was aspirated and frozen in dry ice for vitellogenin and sex steroid hormone analyses. The fish was then weighed, measured, and killed with a blow to the head. Scales, spines, or otoliths were collected for age determination. The abdominal cavity was dissected open to permit observation of the internal organs. The sex of the fish, along with observations of external and internal features, was recorded. The liver, spleen, and gonads were removed and weighed. Pieces of liver were collected and frozen in dry ice for analysis of ethoxyresorufin *O*-deethylase (EROD) activity. Pieces of liver, spleen, and gonad were preserved in 10% neutral buffered formalin for histopathological examination. Two to eight pieces of the gonad were collected from either the posterior tip of the gonads (Rio Grande and Columbia River Basins) or multiple positions along the entire length of the gonad (all other basins). Intersex was not observed macroscopically in any fish. All remaining tissues (those not frozen or fixed) were wrapped in aluminum foil and frozen for chemical analysis.

2.2. Laboratory analyses

Gonad samples from 16 species were examined microscopically for the intersex condition. Sections of each piece of gonad collected in the field were placed into cassettes and processed for microscopic evaluation. Samples were generally transverse sections of the gonad, but the whole gonad was embedded and sectioned longitudinally if the gonad was very small. Two to eight tissue pieces were dehydrated in alcohol, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin (H&E) (Luna, 1992), and examined microscopically. Fish were identified as intersex when oocytes were observed within testicular tissue or when spermatocytes were observed within ovarian tissue. Reproductive stage was also determined in ovary (0–5) and testes (0–4) sections (Blazer, 2002).

Several morphological and reproductive endpoints in addition to gonadal intersex were also determined. Condition factor, hepatosomatic index, splenosomatic index, gonadosomatic index, and health assessment index were calculated from field necropsy measurements (Hinck et al., 2008b). Hepatic EROD activity was determined on microsomal fractions (Whyte et al., 2000), and protein content was determined using the fluorescamine protein assay (Lorenzen and Kennedy, 1993). Histopathological analysis of splenic macrophage aggregates (MAs; MA-#, the number of aggregates in 2 mm^2 of tissue; MA-A, the mean size (area) of aggregates within those 2 mm^2; MA-%, the percentage of the tissue area occupied by aggregates) has been described by Blazer (2002). Plasma vitellogenin concentrations were determined by enzyme-linked immunosorbent assay (Denslow et al., 1999), and plasma 17β-estradiol and 11-ketotestosterone concentrations were measured by radioimmunoassay (Hinck et al., 2008a).

Fish carcasses were shipped to the laboratory frozen and stored at −20 C until prepared for chemical analysis. Individual carcasses were partly thawed, cut into pieces, and ground to a fine texture. The ground fish were then mixed together to create a single homogenous composite sample for each site, species, and sex combination. Details of the analytical methods and quality assurance/quality control procedures are described in the original studies (Hinck et al., 2006, 2007a,b, 2008a; Schmitt, 2002; Schmitt et al., 2005). Briefly, one sub-sample (10 g) of each composite sample was solvent-extracted and analyzed gravimetrically for lipid content and by high-resolution capillary gas chromatography with electron capture detection for organochlorine chemical residues (hexachlorobenzene, hexachlorocyclohexanes, dieldrin, endrin, chlordanes, DDT isomers, mirex, and toxaphene) and total PCBs after size exclusion and adsorption column cleanup procedures. A second composite sub-sample (100 g) was freeze-dried for elemental analysis, and percent moisture was determined as weight lost during lyophilization. Sub-samples were acid-digested and analyzed by atomic absorption spectroscopy for arsenic, lead (1995 and 1997 samples), and selenium and by inductively coupled plasma emission spectroscopy or inductively coupled plasma mass spectroscopy for other elements [barium, cadmium, chromium, copper, magnesium, manganese, molybdenum, nickel, lead (2002–2004 samples), strontium, vanadium, and zinc]. Thermal combustion, amalgamation, and atomic absorption spectroscopy were used to analyze directly for total mercury in 2002, 2003, and 2004 composite sub-samples. A third composite sub-sample (10 g) was solvent-extracted and subjected to reactive cleanup for use in the H4IIE bioassay (Whyte et al., 2004). Concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent doses (TCDD-EQ; pg/g wet weight (ww)) were determined by slope ratio assay (Ankley et al., 1991).

2.3. Statistical analyses

All computations and statistical analyses were performed with SAS Version 9.1 (SAS Institute, Cary, NC). Arithmetic means and standard errors for biological endpoints were computed. Censored values (those less than the analytical limit of detection) for EROD and plasma vitellogenin concentrations were replaced by one-half the LOD in all statistical analyses. All data were $\log_{10}$-transformed to approximate normality for statistical analysis. Statistical analyses were only performed for species in which the intersex condition was observed in >10% of the fish (male smallmouth and largemouth bass). Most intersex fish were predominately male (gonads macroscopically appeared to be normal testes with a few oocytes found in the histological sections); therefore, intersex bass were compared to male bass. Analysis-of-variance (ANOVA) using PROC GLM followed by Fisher's restricted least significant difference (Saville, 1990) was used to test for differences in biological endpoints and chemical concentrations between intersex and male bass within and among basins. Species and basins were analyzed separately after biological endpoint differences between and among these factors were determined to be significant. Differences in geometric mean concentrations of only the most frequently detected chemical contaminants, mercury and *p*,*p*′-DDE, between sites with and without intersex bass were tested in order to alleviate limit of detection issues (Hinck et al., 2008b). Chemical contaminant concentrations were analyzed in whole body composite samples (by sex, species, and site) whereas biological endpoints were measured in individual fish; therefore, contaminant concentrations in individual intersex fish were not available.

3. Results

3.1. Occurrence of intersex fish

Overall, only 97 of 3110 fish (3%) were intersex; 96 of the 97 intersex fish were males with gonads comprising primarily testicular tissue with a few previtellogenic oocytes. Intersex occurrence differed among species and basin. The intersex condition was found in eight of the nine basins sampled and four of 16 species examined. Intersex was observed in fish from 34 of 111 sites (31%; Table 1, Fig. 1). At sites where intersex fish were found, the intersex condition was typically observed in a small number of fish (generally $n < 3$). The condition was not observed in multiple species from a single site. The intersex condition was observed in common carp, channel catfish, smallmouth bass, and largemouth bass, and was most prevalent in smallmouth bass and largemouth bass (Fig. 2A–D). Intersex was not observed in spotted bass ($n = 21$), hybrid largemouth/spotted bass ($n = 1$), striped bass ($n = 1$), white

Table 1
Occurrence of intersex by species[a].

Family, species	Female				Male			
	No. fish with intersex/*n*	%	No. sites with intersex/*n*	%	No. fish with intersex/*n*	%	No. sites with intersex/*n*	%
Centrarchidae								
Largemouth bass (*Micropterus salmoides*)	0/426	0	0/55	0	70/390	18	23/52	44
Smallmouth bass (*Micropterus dolomieu*)	0/90	0	0/15	0	23/70	33	7/16	44
Cyprinidae								
Common carp (*Cyprinus carpio*)	1/798	0.1	1/89	1	0/774	0	0/89	0
Ictaluridae								
Channel catfish (*Ictalurus punctatus*)	0/44	0	0/6	0	3/42	7	3/6	50

[a] Intersex was not observed in largescale sucker, longnose sucker, white sucker, spotted bass, northern pike, flathead catfish, burbot, striped bass, white bass, or brown trout.

bass ($n = 30$), hybrid striped/white bass ($n = 3$), burbot ($n = 13$), flathead catfish ($n = 10$), northern pike ($n = 156$), largescale sucker ($n = 157$), longnose sucker ($n = 45$), white sucker ($n = 19$), or brown trout ($n = 20$); however, sample sizes were notably small for some of these species.

Intersex was not observed in any of the 774 male common carp examined, but one of 798 female carp (0.1%) was intersex (Table 1). The gonads of the intersex female carp contained primarily ovarian tissue with some sperm (Fig. 2D). This fish was one of eight female carp collected from Site 320 (Colorado R. at Willow Beach, Arizona). Its ovaries were identified as abnormal during the field necropsy because both gonadal lobes were red and macroscopically devoid of eggs (i.e. similar to a spent ovary); normal carp ovaries typically contain yellow or brown eggs.

Intersex was observed in three of 42 male channel catfish (7%), but they represented three of six sites from which this species was collected and examined microscopically (Table 1, Fig. 1). At the three sites from which intersex fish were collected, the percentage of intersex channel catfish per site ranged from 13% to 33%. One intersex catfish was found at each of the following sites in the Colorado River Basin: Sites 312 (Green R. at Ouray National Wildlife Refuge, Utah), 317 (San Juan R. at

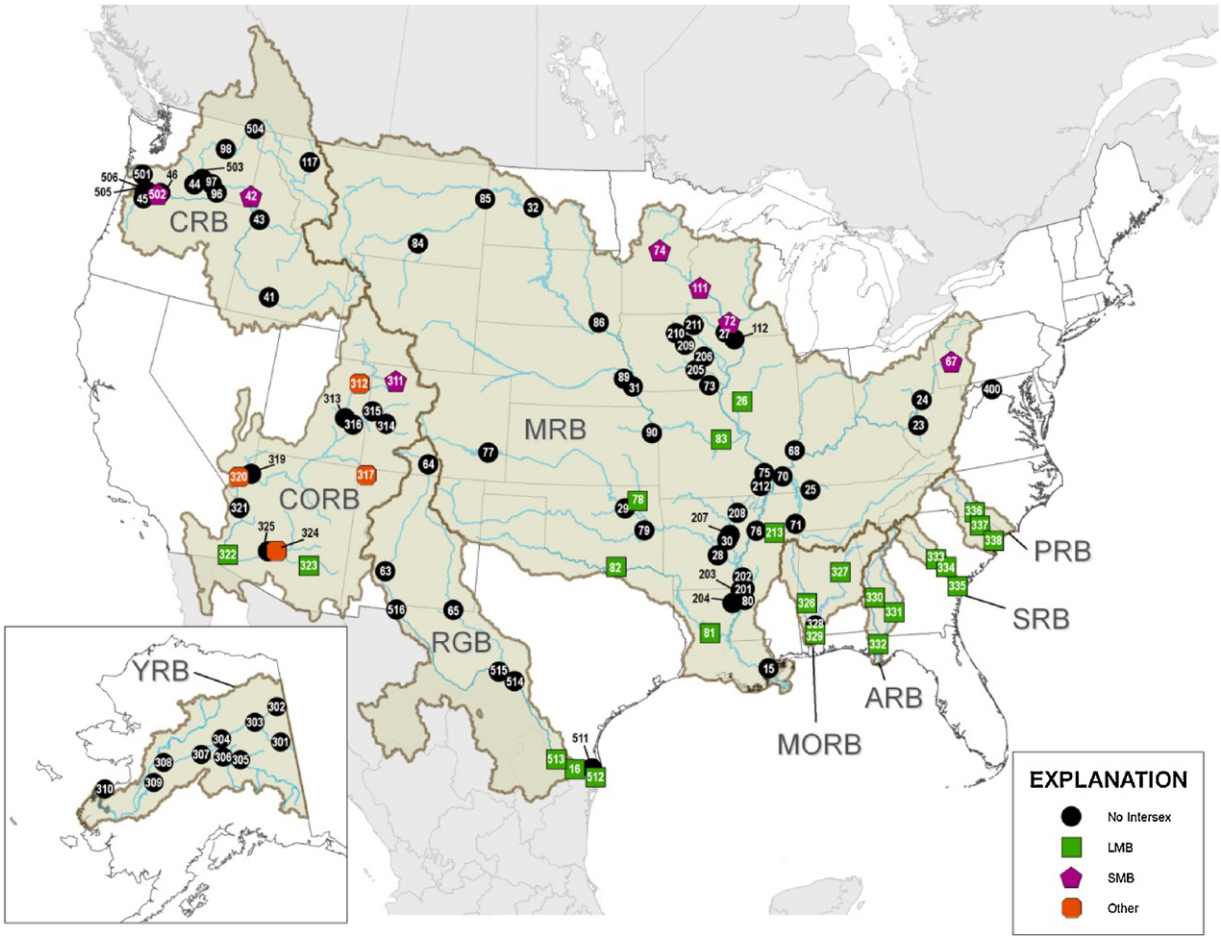

Fig. 1. Occurrence of intersex in fish. LMB, largemouth bass; SMB, smallmouth bass; Other, channel catfish (Sites 312, 317, and 324) and common carp (Site 320). Numbers inside symbols represent site numbers. ARB, Apalachicola River Basin; CORB, Colorado River Basin; CRB, Columbia River Basin; MORB; Mobile River Basin; MRB, Mississippi River Basin; PRB, Pee Dee River Basin; RGB, Rio Grande Basin; SRB, Savannah River Basin; YRB, Yukon River Basin. See Supplemental Table 1 for site descriptions.

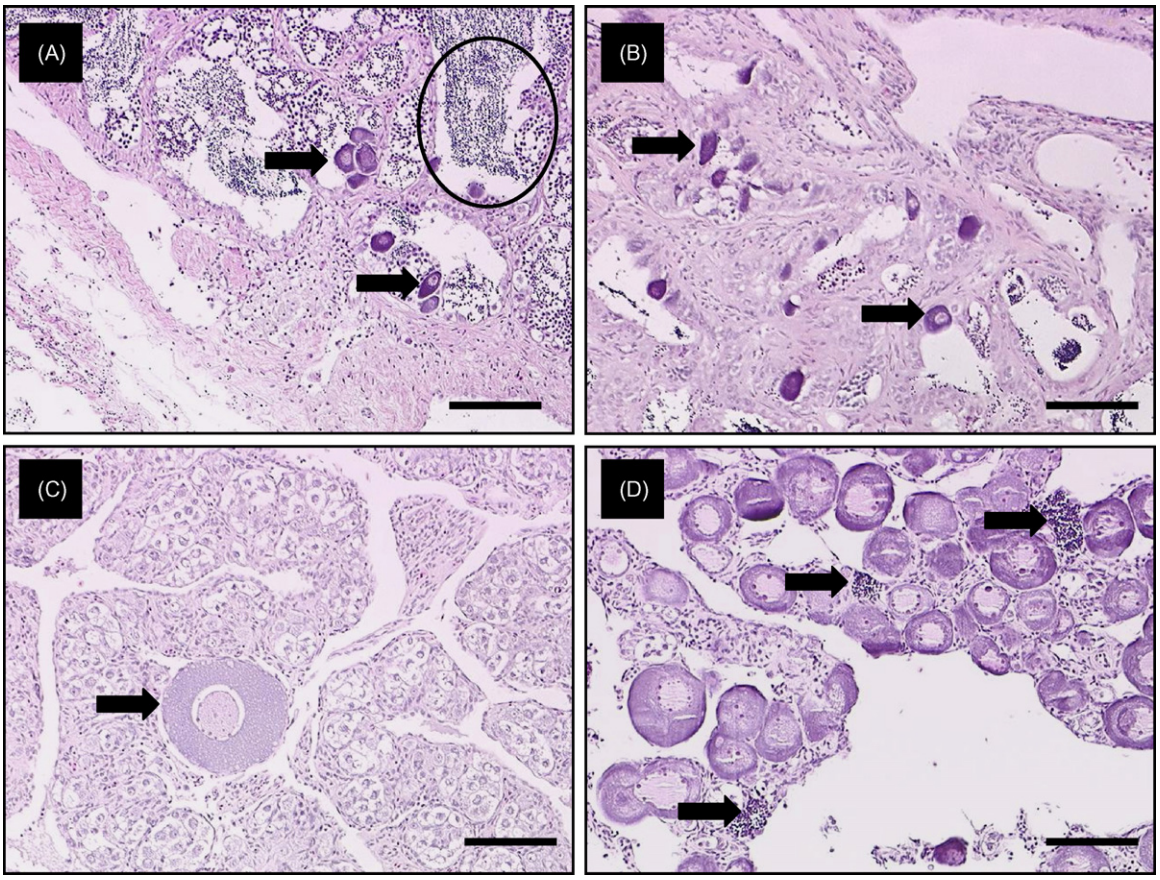

Fig. 2. Histological observations of intersex in fish. (A) Testicular oocytes (arrows) observed within testes of a largemouth bass from the Pee Dee River. Note mature sperm (circle) within the tubules. (B) Oocytes (arrows) within testes of a smallmouth bass from the Colorado River. (C) Oocyte (arrow) within an immature testes of a channel catfish from the Colorado River. (D) Foci of sperm (arrows) within the ovary of a common carp from the Colorado River. Scale bar = 100 μm. H&E stain.

Hogback Diversion, New Mexico), and 324 (Gila R. at Phoenix, Arizona). The gonads of channel catfish from sites in the Rio Grande Basin (Sites 511 and 516) were not examined microscopically.

Intersex smallmouth bass were found in the Columbia, Colorado, and Mississippi River Basins; the one male smallmouth bass from the Rio Grande Basin (Site 514) was not intersex. Intersex was observed in 23 of 70 male smallmouth bass (33%) from 7 of 16 sites (44%) (Table 1, Fig. 1). At sites where intersex smallmouth bass were collected, the percentage of intersex ranged from 14% to 73%. The occurrence of intersex was greatest in male smallmouth bass from Sites 111 (73%; Mississippi R. at Lake City, Minnesota), 311 (70%; Yampa R. at Lay, Colorado), 42 (43%; Salmon R. at Riggins, Idaho), and 502 (67%; Columbia R. at Warrendale, Oregon)

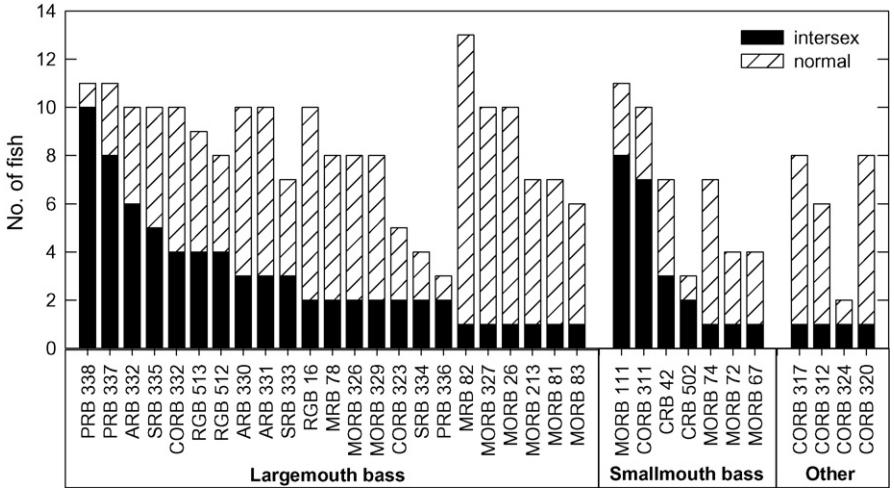

Fig. 3. Intersex condition in male fish by site. The number of intersex in female fish is presented for Site CORB 320. Other, channel catfish (Sites CORB 312, CORB 317, and CORB 324) and common carp (Site CORB 320). ARB, Apalachicola River Basin; CORB, Colorado River Basin; CRB, Columbia River Basin; MORB; Mobile River Basin; MRB, Mississippi River Basin; PRB, Pee Dee River Basin; RGB, Rio Grande Basin; SRB, Savannah River Basin. See Supplemental Table 1 for site descriptions.

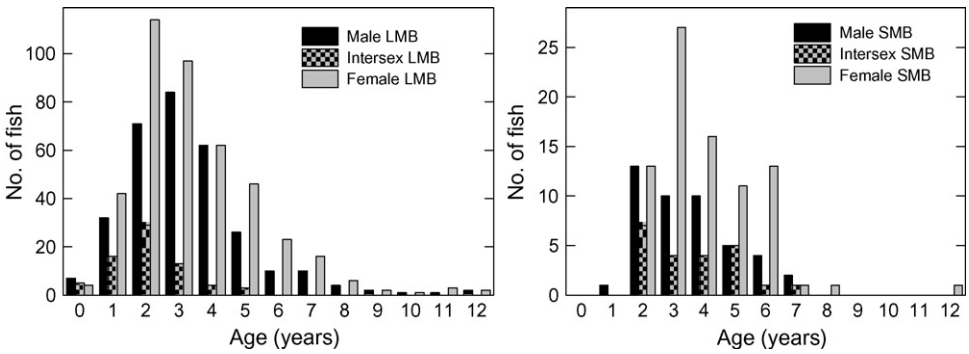

Fig. 4. Intersex condition in largemouth bass (LMB) and smallmouth bass (SMB) by age.

(Fig. 3). Percent intersex was lower at Sites 67 (25%; Allegheny R. at Natrona, Pennsylvania), 72 (25%; Wisconsin R. at Woodman, Wisconsin), and 74 (14%; Mississippi R. at Little Falls, Minnesota), where only one intersex smallmouth bass per site was found (Fig. 3).

Intersex largemouth bass were found in the Colorado, Rio Grande, Mississippi, Mobile, Apalachicola, Savannah, and Pee Dee River Basins; male largemouth bass from the Columbia River Basin were not intersex. Intersex was observed in 70 of 390 male largemouth bass (18%) from 23 of 52 sites (44%) (Table 1, Fig. 1). At sites where intersex fish were observed, the percentage of intersex largemouth bass per site ranged from 8 to 91% and was most prevalent in the southeastern United States. Intersex occurrence was greatest in male largemouth bass from Sites 338 (91%; Pee Dee R. at Bucksport, South Carolina), 336 (67%; Pee Dee R. at Rockingham, North Carolina), 337 (64%; Pee Dee R. at Pee Dee, South Carolina), 332 (60%; Apalachicola R at Blountstown, Florida), 335 (50%; Savannah R. at Port Wentworth, Georgia), 334 (50%; Savannah R. at Sylvania, Georgia), 333 (43%; Savannah R. at Augusta, Georgia), 330 (30%; Chattahoochee R. at Omaha, Georgia), and 331 (30%; Flint R. at Albany, Georgia) (Fig. 3). A lower occurrence of intersex was also observed in male largemouth bass from the Mobile River Basin at Sites 326 (25%; Tombigbee R. at Lavaca, Alabama), 329 (25%; Mobile R. at Bucks, Alabama), and 327 (10%; Coosa R. at Childersburg, Alabama) (Fig. 3). Relatively high proportions of intersex largemouth bass were observed at three sites in the lower Rio Grande Basin including Sites 512 (50%; Rio Grande at Brownsville, Texas), 513 (44%; Rio Grande at Falcon Dam, Texas), and 16 (20%; Rio Grande at Mission, Texas) (Fig. 3). In addition, 40% of male largemouth bass from Sites 322 (Colorado R. at Imperial Dam, Arizona) and 323 (Gila R. at Hayden, Arizona) in the Colorado River Basin were intersex (Fig. 3). Intersex occurrence was lower in males at Sites 78 (22%; Verdigris R. at Oolagah, Oklahoma), 213 (14%; Wolf R. at LaGrange, Tennessee); 83 (11%; Missouri R. at Hermann, Missouri), 26 (10%; Illinois R. at Beardstown, Illinois), 81 (8%; Red R. at Alexandria, Louisiana), and 82 (8%; Red R. at Lake Texoma, Texas/Oklahoma) in the Mississippi River Basin, typically being observed in only one fish per site (Fig. 3).

There was no significant difference in the mean age of intersex and male smallmouth bass ($F_{1,66}$ = <0.01, P = 0.98; Fig. 4). In contrast, intersex largemouth bass were significantly younger than male largemouth bass ($F_{1,368}$ = 29.57, P < 0.01; Fig. 4). Age ranged

Table 2
Occurrence of intersex in male largemouth bass (LMB) and smallmouth bass (SMB) by basin, and associations of biological endpoints between male bass with and without the condition.

Basin, species	No. of fish	% Intersex fish	No. of sites	% Sites with intersex fish	Significant differences in endpoint means
Mississippi River Basin (1995)					
LMB	174	4	22	27	Length (−), MA-# (−)
SMB	27	41	5	80	MA-% (+), 17β-estradiol (+)
Columbia River Basin (1997)					
LMB	34	0	7	0	NA
SMB	24	21	8	25	MA-A (+), HAI (−)
Rio Grande Basin (1997)					
LMB	29	35	4	75	None
SMB	1	0	1	0	NA
Colorado River Basin (2003)					
LMB	42	14	6	33	EROD (−)
SMB	18	39	2	50	Age (+), EROD (−), HSI (+), SSI (+), HAI (+)
Apalachicola River Basin (2004)					
LMB	30	67	3	100	None
Mobile River Basin (2004)					
LMB	36	14	4	75	HSI (+)
Pee Dee River Basin (2004)					
LMB	25	80	3	100	Age (−), stage (−)
Savannah River Basin (2004)					
LMB	21	48	3	100	None

Biological endpoints for which mean values were significantly different (P < 0.05, one-way ANOVA) between intersex and male bass are noted; +, mean for intersex fish greater than male fish; −, mean for intersex fish less than male fish. Sampling year is noted in parentheses.
NA, not applicable.

from 0 to 5 years for intersex largemouth bass and 0 to 12 years for male largemouth bass (Fig. 4).

3.2. Trends among intersex fish by basin

Few mean biological endpoints differed significantly among male and intersex smallmouth and largemouth bass within a basin (Table 2; Supplemental Tables 2 and 3). Intersex largemouth bass were significantly shorter and had fewer numbers of MAs than male largemouth bass from the Mississippi River Basin. Mean MA-% and 17β-estradiol concentrations were significantly greater in intersex smallmouth bass than male smallmouth bass from the Mississippi River Basin. In the Columbia River Basin, mean MA-A was greater but mean HAI was lower in intersex smallmouth bass compared to male smallmouth bass. Mean EROD activity was lower and mean age, HSI, SSI, and HAI were greater in intersex smallmouth bass than in male smallmouth bass in the Colorado River Basin. Hepatic EROD activity was also significantly lower in intersex largemouth bass compared to male largemouth bass. No biological endpoints were significant between intersex and male largemouth bass from the Rio Grande, Apalachicola, and Savannah River Basins. Mean HSI was significantly greater in intersex largemouth bass than in male largemouth bass from the Mobile River Basin. Mean age and stage were lower in intersex largemouth bass compared to male largemouth bass in Pee Dee River Basin. Differences in mean condition factor, gonadosomatic index, vitellogenin, 11-ketotestosterone, and E/KT were not significant in any ANOVA model (Supplemental Tables 2 and 3).

3.3. Associations between intersex bass and contaminant concentrations

Twenty-four chemical contaminants including organochlorine pesticides, total PCBs, TCDD-EQs, and total mercury were measured in largemouth and smallmouth bass samples ($n = 60$) across all basins (Supplemental Table 4). The most commonly detected contaminants were mercury ($n = 60$), *p*,*p*′-DDE ($n = 57$), *p*,*p*′-DDD ($n = 39$), TCDD-EQ ($n = 39$), PCBs ($n = 37$), *trans*-nonachlor ($n = 32$), dieldrin ($n = 26$), *cis*-nonachlor ($n = 25$), *cis*-chlordane ($n = 24$), and *p*,*p*′-DDT ($n = 21$). Sites with the greatest number of detected contaminants were in the Colorado, Mobile, Apalachicola, Savannah, and Pee Dee River Basins. However, the limits of detection and measured concentrations were two orders of magnitude lower for some organochlorine pesticides in these samples compared to those from the Mississippi, Rio Grande, and Columbia River Basins (0.0001 μg/g ww vs. 0.01 μg/g ww). The intersex condition was not always found in bass from sites with a high number of detected contaminants. For example, multiple contaminants were detected in largemouth bass from Sites 24 and 76 in the Mississippi River Basin and Site 328 in the Mobile River Basin, although no intersex fish were observed at these sites. Conversely, some sites such as Sites 512 and 513 in the lower Rio Grande had relatively high occurrence of intersex with fewer contaminants detected. Although the mean concentration of mercury was greater at sites with intersex largemouth bass (0.24 μg/g ww) compared to sites with only male largemouth bass (0.16 μg/g ww), these differences were not significant ($F_{1,47} = 3.44$, $P = 0.07$). Mean mercury concentrations at sites with (0.16 μg/g ww) and without (0.14 μg/g ww) intersex smallmouth bass also did not differ significantly ($F_{1,9} = 0.17$, $P = 0.69$) nor did mean concentrations of *p*,*p*′-DDE differ between sites with (0.04 μg/g ww) and without (0.06 μg/g ww) intersex largemouth bass ($F_{1,47} = 0.30$, $P = 0.59$). In contrast, the mean concentration of *p*,*p*′-DDE at sites without intersex smallmouth bass (0.16 μg/g ww) was greater than sites where the condition was found (0.03 μg/g ww). However, these differences were also not significant ($F_{1,9} = 3.08$, $P = 0.11$), which was likely because of the small number of sites with intersex smallmouth bass.

4. Discussion

4.1. Prevalence of intersex in freshwater fish

Among the species we investigated, intersex was most prevalent in male largemouth and smallmouth bass. Predominantly male testes containing female germ cells were the most pervasive form of intersex observed, even though equal numbers of male and female fish were examined. However, the proportion of tissue examined in individual male bass was likely greater than that in female bass because the testes are typically smaller than ovaries. Previous field studies have also reported intersex in largemouth and smallmouth bass, some at comparable percentages to what we observed (Anderson et al., 2003; Baldigo et al., 2006; Blazer et al., 2007; James, 1946). James (1946) was among the first to report intersex in largemouth bass based on macroscopic observation (one ovary and one testis) from lakes in Illinois, whereas other studies have reported microscopic observations of intersex in black basses (*Micropterus* spp.). Intersex (testicular oocytes) was reported in male largemouth (4 of 15) and male smallmouth bass (12 of 33) from contaminated and reference sites in the Hudson River, New York (Baldigo et al., 2006). In addition, and in contrast to our results, Baldigo et al. (2006) observed intersex in males of multiple species at some sites; intersex largemouth and smallmouth bass were collected at one site and intersex largemouth bass and carp were collected at another. Intersex (testicular oocytes) was also observed in all of the male smallmouth bass ($n = 15$) collected from a reference site and a PCB-contaminated site on the Kalamazoo River, Michigan (Anderson et al., 2003). Intersex (testicular oocytes) occurrence in smallmouth bass in the Potomac River Basin was 69–100% in the spring, 25–67% in the summer, and up to 100% in the fall but was only 14–36% in adjacent basins with smaller human populations and less agriculture (Blazer et al., 2007).

To the best of our knowledge, intersex as defined in our study has not been previously reported in channel catfish. Female channel catfish with male secondary sexual characteristics have been observed in the Red River of the North, North Dakota (Hegrenes, 1999). For common carp, other field studies have occasionally reported a low occurrence of intersex. For example, Lavado et al. (2004) reported intersex (testicular oocytes) in one of six male common carp from the Ebro River in Spain, and Baldigo et al. (2006) reported one intersex male common carp (out of nine) from the Hudson River, New York. An intersex male common carp with half testicular tissue and half ovarian tissue in one gonad was reported in a caged fish study conducted in the Las Vegas Wash, Nevada (Snyder et al., 2004). Although Snyder et al. (2004) concluded that intersex occurred spontaneously in the farm-raised male carp (from California) and was not related to EACs in the Las Vegas Wash, we note that our only intersex carp was found in the same region (Site 320), downstream of Lake Mead. Nevertheless, the cause or causes of intersex in the carp from this area remains unknown.

Of the other species we examined, intersex has been reported in striped bass (Moser et al., 1983), northern pike (June, 1977; Vine et al., 2005), white sucker (Sikstrom et al., 1975; Vajda et al., 2008; Woodling et al., 2006), and brown trout (Körner et al., 2005; O'Farrell and Peirce, 1989). To the best of our knowledge, intersex has not been documented in the scientific literature for largescale sucker, longnose sucker, flathead catfish, burbot, spotted bass, white bass, or hybrid bass.

The occurrence of intersex in fish relative to EACs has been documented in field studies, many of which have focused on wastewater treatment facilities (Jobling et al., 1998; Lavado et al., 2004; Liney et

al., 2005; Sumpter, 2002; Vajda et al., 2008; Woodling et al., 2006). However, intersex has also been documented in fish from rural areas (Blazer et al., 2007), where agricultural runoff can be a significant source of EACs (Hanselman et al., 2003; Kolodiei and Sedlak, 2007; McDaniel et al., 2008; Orlando et al., 2004). At least one of our sites (Yampa R. at Lay, Colorado) with a high prevalence of intersex did not have obvious EAC point sources. Other sites with high occurrence of intersex (e.g. Mississippi R. at Lake City, Minnesota; Pee Dee R. at Pee Dee and Bucksport, South Carolina) were located on rivers with dense human populations or industrial and agricultural activities. The presence of intersex bass at all sites in the Pee Dee, Savannah, and Apalachicola River Basins indicate that the condition may be more prevalent in the southeastern U.S. river basins than elsewhere and warrants further investigation. We might also expect high occurrence of intersex largemouth bass in similar agricultural regions (e.g. row crop, livestock) of the lower Mississippi River (e.g. Mississippi R. at Luling, Louisiana; Big Sunflower R. at Anguilla, Mississippi; Bogue Phalia R. at Leland, Mississippi; Steele Bayou R. at Rolling Fork, Mississippi; Tensas R. at Tendal, Louisiana), but where bass were not collected. Intersex male largemouth bass were observed at Sites 81 (Red R. at Alexandria, Louisiana) and 82 (Red R. at Lake Texoma, Texas/Oklahoma), where agriculture is a primary land use. It is also important to note that exposure to EACs is not the only possible explanation for intersex in fish given that baseline occurrence of the condition is unknown among most species of feral fishes.

4.2. Factors to consider when evaluating intersex occurrence

Neither sex determination, which is the genetic and environmental variables and processes that influence phenotypic sex, nor sex differentiation, which is the manifestation of these processes into the development of an ovary or testis (Piferrer, 2001; Devlin and Nagahama, 2002), have been extensively studied in black bass. Sex-specific genes have been identified in only a few fish species. More research is needed at the molecular level to understand how autosomes and sex chromosomes of gonochoristic species, such as largemouth and smallmouth bass, interact with environmental variables to influence sexual development (Devlin and Nagahama, 2002; Orlando and Guillette, 2007). Largemouth bass are believed to exhibit differentiated gonochorism (i.e. primordial germ cells directly differentiate into an oogonia or spermatogonia) and seemingly do not have hermaphroditic tendencies (Johnston, 1951). All of the data we have presented on intersex relates to the phenotypic sex of the fish. The genotypic sex of our fish is unknown because sex-specific chromosomal markers have not been identified for bass. Although we were unable to make a definitive determination whether the intersex condition observed in bass was the result of feminized males or masculinized females, the microscopic appearance of the gonads, the presence of mature sperm, and the steroid hormone and vitellogenin concentrations indicate that the intersex bass were males which had been feminized. Additionally, we could not definitively determine whether reproductive function was inhibited by the intersex condition in these fish. Oocytes found within the testes were previtellogenic; mature oocytes were not observed. It remains to be determined whether the intersex we observed in bass developed during sexual differentiation in early life stages, during exposure to environmental factors in adult life stages, or some continuum between these life stages.

The natural incidence of intersex gonadal tissue among fish species is unknown, but the prevalence of intersex is potentially related to collection season and age of the fish as well as to EAC exposure. Blazer et al. (2007) reported that the highest prevalence and severity of intersex in male smallmouth bass consistently occurred during the pre-spawn–spawning season and was lower during the post-spawn season. The study also concluded that oocytes may be released with the sperm because oocytes were observed in the lumen of the ducts of male smallmouth bass during summer and fall (post-spawn; Blazer et al., 2007). We sampled our sites once; therefore, the seasonal influence on intersex prevalence could not be determined. All bass in our study were collected in the fall (post-spawn season), except for one male smallmouth bass from Site 506 (pre-spawn season; Supplemental Table 1). Given these data and the results of Blazer et al. (2007), the occurrence of intersex in freshwater fish may have been further underestimated because our study did not include a seasonal component. Stage of reproductive development (i.e. pre- and post-spawn) should also be considered when interpreting intersex data.

Fish age may also be an important determinant of intersex occurrence in largemouth bass, which can live up to 15 years and reach sexual maturity by age 1 in the southern United States (Stuber et al., 1982). Intersex was not found in largemouth bass older than five years and was most common in 1–3-year-old male largemouth bass. However, the low incidence of intersex in older fish may be low due to small sample size of older fish (>6 years old) in our collections. We also note that the intersex condition was observed in the few fish <1-year-old at a relatively high frequency compared to normal male largemouth bass. Alternatively, the greater occurrence of intersex in younger largemouth bass may be a result of smaller gonads in younger fish and hence, a larger proportion of the gonad being examined. An apparent greater frequency of intersex in younger individuals was not observed in smallmouth bass (Fig. 4b); therefore, the evidence for an age-related frequency difference is not conclusive from our dataset. Also, the hypothesis that the intersex male largemouth bass do not survive past 5 years old seems unlikely since the age distribution curves of male and intersex largemouth bass are similar, with ages 1–4 predominant in both groups (Fig. 4).

Intersex evaluations were conducted under criteria of simple presence/absence. Therefore, quantitative assessment of background rates of intersex prevalence is limited by our approach. A more quantitative measure of intersex, such as a severity index ranking system (Bateman et al., 2004; Blazer et al., 2007), is needed to evaluate background rates of intersex in fish and to define hierarchical linkages to endocrine function. A more quantitative assessment of intersex would also provide a stronger basis for evaluation of factors associated with the intersex condition. The presence of testicular oocytes in gonads and circulating vitellogenin in plasma of male fish have both been used as indicators of exposure to estrogenic EACs (e.g. Jobling et al., 1998; Vajda et al., 2008); however, we did not observe significant correlations between the presence/absence of intersex and the other reproductive endpoints measured in this study (gonadosomatic index, vitellogenin, 17β-estradiol, and 11-ketotestosterone). Plasma concentration of vitellogenin in males was not a good indicator of the presence of intersex in our study. This lack of a relationship may not be unexpected, as the complexity of the vitellogenic response is not as great as the complexities involved with tissue restructuring that occurs with intersex. Estradiol regulates growth and maturation of oocytes in the ovary and induction of vitellogenin in the liver, but a more complex regulatory mechanism is most certainly involved for the development of intersex in male bass. Alternatively, the lack of a relationship between male vitellogenin and intersex may indicate that vitellogenin only represents recent exposure to EACs. Vitellogenin in male fish is indicative of a recent and/or ongoing exposure to an estrogen or estrogenic compound. Induction of the protein occurs within days, and the protein can persist in the plasma for weeks to months after exposure because male fish have no specific mechanisms to excrete vitellogenin (Schmid et al., 2002; Thorpe et al., 2007). Induction of vitellogenin

is a receptor-mediated response, largely controlled through estrogen receptors in the liver (Sumpter and Jobling, 1995). In contrast, the biochemical and cellular mechanisms responsible for the development of intersex in gonadal tissues are not known, and they may represent a more chronic response in these fishes. The period of sexual differentiation has been identified as the most sensitive period for the induction of intersex in some species including medaka (*Oryzias latipes*; Koger et al., 2000), roach (Liney et al., 2005), and rainbow trout (*Oncorhynchus mykiss*; Krisfalusi and Nagler, 2000), but intersex can also be induced in later life stages (Gray et al., 1999; Liney et al., 2005). Interestingly, exposure to EACs later in life exacerbated the severity of intersex in roach when the initial exposure occurred at critical stages of sexual differentiation (Liney et al., 2005). The high prevalence of intersex in adult largemouth bass and smallmouth bass indicates that exposures during both early life stages and later life stages may be important; therefore, multi-generational studies should be conducted to determine the most sensitive life stage (egg to adult) for black basses. Last, we must consider other environmental factors that may be important in the induction of intersex in black bass. The intersex condition may not be entirely dependent on exposure to xenoestrogens in black basses, and some level or baseline occurrence of intersex in smallmouth and largemouth bass may be natural. Understanding the baseline occurrence of intersex in black basses will help to interpret these observations and determine the utility of this condition as an indicator of endocrine disruption in bass.

We present the observed occurrence of intersex in a variety of freshwater fish species in the United States but not information on the potential causes. The causes for intersex may vary by location; therefore, one anthropogenic activity or class of contaminants will not explain the occurrence of intersex in all species or locations. Each site was sampled once in our study, so it is unknown whether the intersex occurrence has become more severe at sites where the condition was observed. If the condition is related to EACs, increased intersex occurrence may be expected as industrial and municipals waste discharges increase. Studies to evaluate the severity of intersex and potential local stressors are recommended for sites where the majority of male bass were found to be intersex. In addition, future studies should determine why intersex is more prevalent in certain species; the mechanisms through which intersex is induced in fish; whether intersex has a hierarchical linkage to endocrine function; and whether certain compounds (estrogens, anti-estrogens, androgens, and anti-androgens) in the environment increase the severity of intersex in fish. Ultimately, we also need to know if individuals with the intersex condition relate to reduced reproductive output for a given populations. A better understanding of these questions is required if we are to truly understand the significance and the cause of intersex in wild fish populations. Results from our study provide fundamental information on the occurrence and distribution of intersex in freshwater fishes throughout the United States that will be necessary to begin to address these questions.

4.3. Sampling design limitations for defining background occurrence of intersex

Intersex has been reported in freshwater and marine fishes for decades (Sumpter, 2002), but in studies prior to 1980, it was typically observed macroscopically in one or two fish at a site (e.g. James, 1946; Sikstrom et al., 1975). As concerns about EACs in aquatic ecosystems have increased, recent studies have included microscopic examination of the gonads for intersex. Consequently, the condition has been reported with greater frequency. In our study, gonadal tissues were collected for histological examination primarily to document stage of reproductive development and percent of atretic oocytes. However, all other abnormalities, including intersex, were noted. Remarkably, intersex was detected despite relatively small sample sizes at some sites (e.g. 2 of 3 male bass in Columbia R. at Warrendale, Oregon), which may indicate an even greater prevalence of intersex in the population at certain locations. Our sampling methodology did not allow us to determine the true prevalence or severity of intersex in the fish species collected and is therefore not adequate to estimate or quantify a baseline occurrence in these species. The true occurrence of intersex in our samples and these populations of fishes are probably underestimated. Nevertheless, and as noted by Devlin and Nagahama (2002), field studies that examine gonad developmental stage or sex ratio provide an unbiased minimum approximation for the spontaneous frequency of intersex in wild populations.

We positively identified a fish as intersex when a mixture of oocytes and sperm was observed histologically within the gonadal tissue. However, the lack of evidence of oocytes within the testicular sections (or spermatocytes within the ovarian sections) examined does not preclude the possibility of intersex in other sections or elsewhere in the gonad. Blazer et al. (2007) estimated that examining five transverse sections taken along the length of each testis would allow greater than 90% detection of intersex in smallmouth bass. The low occurrence of intersex in common carp may be influenced by the large gonad size of this species; the proportion of tissue examined microscopically is much smaller for carp, which decreases the likelihood of finding the condition with our sampling protocol. We observed that testicular oocyte density was greatest in the center of a transverse section near blood vessels and nerves of both smallmouth and largemouth bass testes. The intersex condition may be underestimated in species with larger gonads if the central region is not collected; however, the optimal location for estimating intersex occurrence may vary by fish species. For black basses, we recommend examining five to ten transverse sections (based on the size of the gonad), sampled along the entire length of each gonadal lobe, to determine the occurrence of intersex.

Our sampling design precluded rigorous statistical testing of associations between the intersex condition and chemical contaminants because contaminant concentrations were measured in whole-body composite samples comprising multiple individuals. Changes in analytical limits of detection for contaminants also restricted the interpretation of these data; limits of detection decreased by two orders of magnitude for some organochlorine pesticides (e.g. 1995 vs 2004 samples) (Hinck et al., 2008b). As a result, our finding of the greatest number of detected contaminants at the same locations as the greatest occurrence of intersex (i.e. southeastern United States) may not reflect the actual associations between these endpoints. The mere detection of a chemical contaminant also does not imply causation of intersex. Nevertheless, the most commonly detected contaminants (total mercury, *p*,*p*′-DDE, and PCBs) have all been identified as EACs that are associated with reproductive impairment in fish, albeit not necessarily at the concentrations measured in our samples (Drevnick and Sandheinrich, 2003; Garcia-Reyero et al., 2006). Our results also highlight that contaminants tend to co-occur in fish. Other more contemporary pesticides that do not bioaccumulate and which we did not measure are also likely to be present at these sites because of their current use on row crops and in urban landscapes (e.g. Gillom et al., 2006). For example, numerous herbicides, insecticides, and fungicides are applied to crops in the southeastern United States, and application rates are generally greater in the Apalachicola, Savannah, and Pee Dee River Basins than the Mobile River Basin (U.S. Geological Survey, 2003). It is unknown if these higher pesticide application rates are associated with the greater incidences of intersex we observed in

fish from these basins. We also did not measure other chemical groups such as surfactants, personal care products, and pharmaceuticals that have the potential to cause endocrine disruption in fish.

4.4. Conclusions

The occurrence of intersex differed among species collected from large U.S. rivers. Largemouth and smallmouth bass would be good candidates for monitoring changes in and severity of intersex in waters of the United States because of their broad distribution and apparent sensitivity or susceptibility to this condition. Intersex was likely underestimated in the species examined given our sample sizes and small percent of gonad tissue examined per fish. Despite these study design limitations, this work is significant in that it is the first to document the widespread occurrence of intersex in black basses in the United States. Intersex was widely observed in male largemouth bass (18% of all individuals; 44% of sites) and smallmouth bass (33% of all individuals; 44% of sites) and was found in most of the largemouth bass we examined from the southeastern United States. Intersex occurrence in largemouth and smallmouth bass was not correlated with differences in reproductive endpoints such as gonad size, vitellogenin concentrations, or sex steroid hormone concentrations. Intersex in fish did not appear to be limited to locations with suspected sources of EACs. Therefore, the intersex condition in black basses may not be the result of exposure to xenoestrogens; we simply need to know more about the factors (hormonal and environmental) which contribute to this condition in fish. We know EACs can cause intersex in fish, but we lack information on species sensitivities, pathological mechanisms, and the importance of environmental factors., Further studies should determine the mechanisms responsible for and environmental factors contributing to intersex in these species and the implications of this condition for fish populations. Recommendations for designing future monitoring studies would include considering black bass as the target species, obtaining fish of varying ages, collecting fish during different stages of reproductive development and over multiple years, using a severity index for intersex occurrence, and measuring EACs in the water. Our study highlights sites where intersex was more common and may be of concern for certain species. The cause or causes for the widespread occurrence of intersex in largemouth bass from the southeastern United States and elsewhere remains unknown and warrants further investigation. Proper diagnosis of this condition in feral fish is important because if the primary causes are EACs, then the widespread occurrence of intersex in fish would be a critical environmental concern.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.08.001.

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