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[Article]

Intersex (Testicular Oocytes) in Smallmouth Bass from the Potomac River and Selected Nearby Drainages

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Abstract.—Intersex, or the presence of characteristics of both sexes, in fishes that are normally gonochoristic has been used as an indicator of exposure to estrogenic compounds. In 2003, during health assessments conducted in response to kills and a high prevalence of skin lesions observed in smallmouth bass *Micropterus dolomieu* in the South Branch of the Potomac River, the presence of immature oocytes within testes was noted. To evaluate this condition, a severity index (0–4) was developed based on the distribution of oocytes within the testes. Using gonad samples collected from 2003 to 2005, the number of histologic sections needed to accurately detect the condition in mature smallmouth bass was statistically evaluated. The reliability of detection depended on the severity index and the number of sections examined. Examining five transverse sections taken along the length of the gonad resulted in a greater than 90% probability of detecting testicular oocytes when the severity index exceeded 0.5. Using the severity index we compared smallmouth bass collected at selected sites within the South Branch during three seasons in 2004. Seasonal differences in severity and prevalence were observed. The highest prevalence and severity were consistently noted during the prespawn–spawning season, when compared with the postspawn season. In 2005, smallmouth bass were collected at selected out-of-basin sites in West Virginia where fish kills and external skin lesions have not been reported, as well as at sites in the Shenandoah River, Virginia (part of the Potomac drainage), where kills and lesions occurred in 2004–2005. The prevalence of testicular oocytes is discussed in terms of human population and agricultural intensity.

Intersex, also referred to as ovotestis, testis–ova or testicular oocytes (Hecker et al. 2006), has received considerable attention recently in both the public and scientific press. While intersex is most commonly described as the presence of female germ cells, or oocytes, within a predominantly male gonad (Nolan et al. 2001), the term is nonspecific and has been used for a range of gonadal abnormalities in which both male and female characteristics are present. In smallmouth bass *Micropterus dolomieu* the abnormality observed was the presence of oocytes in the male gonad and will be referred to as testicular oocytes (TO). Occasionally these abnormalities are visible macroscopically but most often the gonad must be examined microscopically for detection. In fish that are normally gonochoristic, the presence of TO has been used as an indicator of exposure to estrogenic compounds and has been documented in a variety of wild fish species in numerous geographic areas. Male roach *Rutilus rutilis* populations in UK rivers downstream from sewage treatment works have a high frequency of TO and vitellogenin induction (Jobling et al. 1998). Intersex frequency in roach was also correlated with exposure to domestic sewage effluents in Danish streams (Bjerregaard et al. 2006). Other freshwater fishes that have been observed with TO include male spottail shiners

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Notropis hudsonius collected at sites in the St. Lawrence River with sewage contamination (Aravindakshan et al. 2004), sharptooth catfish *Clarias gariepinus* inhabiting a reservoir in South Africa with estrogenic water pollution (Barnhoorn et al. 2004), white suckers *Catostomus commersonii* from wastewater effluent-dominated Colorado streams (Woodling et al. 2006), gudgeon *Gobia gobia* in the UK (Van Aerle et al. 2001) and Switzerland (Faller et al. 2003), threespine stickleback *Gasterosteus aculeatus* in northeastern Germany (Gerken and Sordyl 2002), barbel *Barbus plejebus* in Italy (Viganò et al. 2001), shovelnose sturgeon *Scaphirhynchus platyorynchus* in the Mississippi River (Harshbarger et al. 2000), and white perch *Morone americana* from the Great Lakes (Kavanagh et al. 2004). In addition, the condition has occasionally been noted in estuarine or marine fishes including European flounder *Platichthys flesus* in the UK (Allen et al. 1999) and marbled flounder *Pleuronectes yokohamae* in Japan (Hashimoto et al. 2000), Mediterranean swordfish *Xiphias gladius* (De Metrio et al. 2003), and red mullet *Mullus barbatus* (Martin-Skilton et al. 2006).

Testicular oocytes have been induced experimentally by exposure of various fish species to estradiol, including medaka *Oryzias latipes* (Koger et al. 2000; Hirai et al. 2006), rainbow trout *Oncorhynchus mykiss* (Krisfalusi and Nagler 2000), zebrafish *Danio rerio* (Brion et al. 2004), and the estuarine fish, Javanese ricefish *Oryzias javanicus* (Imai et al. 2005). Exposure of carp *Cyprinus carpio* to 4-tert-pentylphenol (Gimeno et al. 1998); zebrafish to the fungicide prochloraz (Kinnberg et al. 2007); and medaka to octylphenol (Gray et al. 1999), o,p′-DDT (Metcalfe et al. 2000), nonylphenol (Balch and Metcalfe 2006), bisphenol A (Yokota et al. 2000), equol, an isoflavone detected in runoff from agricultural fields treated with hog manure, genistein, an isoflavone detected in effluents from sewage treatment plants and pulp mills (Kiparissis et al. 2003), or the synthetic estrogen 17α-ethinylestradiol (Seki et al. 2002) also induced TO.

In the summer and fall of 2002 fish kills and skin lesions on a number of fish species, including smallmouth bass, were observed in the South Branch of the Potomac River. Black bass species, including smallmouth and largemouth bass *M. salmoides*, are important sport fish throughout the United States and important top predators in many freshwater ecosystems. Histologic examination of the skin lesions indicated a number of causes including opportunistic bacteria, fungi, and parasites, which suggested immunomodulation. In 2003, a more comprehensive fish health assessment was conducted at a number of sites along the South Branch. During microscopic evaluation of gonadal tissues collected in these assessments a high prevalence of TO was noted in male smallmouth bass. Similar skin lesions and kills of smallmouth bass occurred in 2004–2005 in the Shenandoah River, Virginia, and in 2005 we had an opportunity to collect smallmouth bass from these sites and selected out-of-basin sites. The finding of a high prevalence of TO in the same areas as a high prevalence of skin lesions and significant kills of smallmouth bass has raised questions as to potential contaminant exposures and consequential effects on bass populations.

The objectives of this report are to describe the presence of TO in smallmouth bass, compare the prevalence of TO among seasons at selected sites in the South Branch of the Potomac River drainage, evaluate methodology for identification of the abnormality, and compare the results with observations from other sites within the Potomac drainage (including the Shenandoah River) and selected out-of-basin sites.

Methods

In 2003, in an attempt to determine the causes of skin lesions and fish kills observed in the South Branch of the Potomac River, smallmouth bass were collected at six sites along the South Branch by boat electrofishing (Figure 1). A complete necropsy-based health assessment (Schmitt et al. 1999) was conducted on 12 randomly collected bass and pieces of gonad were taken for histological evaluation. In 2004, we attempted to collect 20 smallmouth bass from selected sites during spring, summer, and fall. In spring 2005, we attempted to collect 20 bass from two sites in the South Branch and one site each in the Tygart and Greenbrier rivers. In fall 2005, 20 bass were collected from three sites of the Shenandoah River, and one site each in the Gauley, Greenbrier, and Elk rivers. In all studies bass over 200 mm were collected to ensure adult sexually mature fish were examined.

The sites in the South Branch of the Potomac were in four counties in West Virginia: Grant (SB1, SB2, SB8), Hardy (SB3), Pendleton (SB4), and Hampshire (SB5). The Tygart River (WV21) arises in Randolph County. The Gauley, Greenbrier, and Elk rivers arise in Pocahontas County. The Gauley (WV4) and the Elk (WV5) rivers flow into Webster County. We sampled two sites in the Greenbrier River, the first in the headwaters in Pocahontas County (WV6) and the second (WV20) in Greenbrier County. The sites on the Shenandoah River were all in Virginia and included sites on the South and North Forks and the main stem. The North Fork begins in Rockingham County, flows through Shenandoah County (North Fork site, SR3) into Warren county, where it joins the South Fork to form the Shenandoah (main stem site, SR4). The South

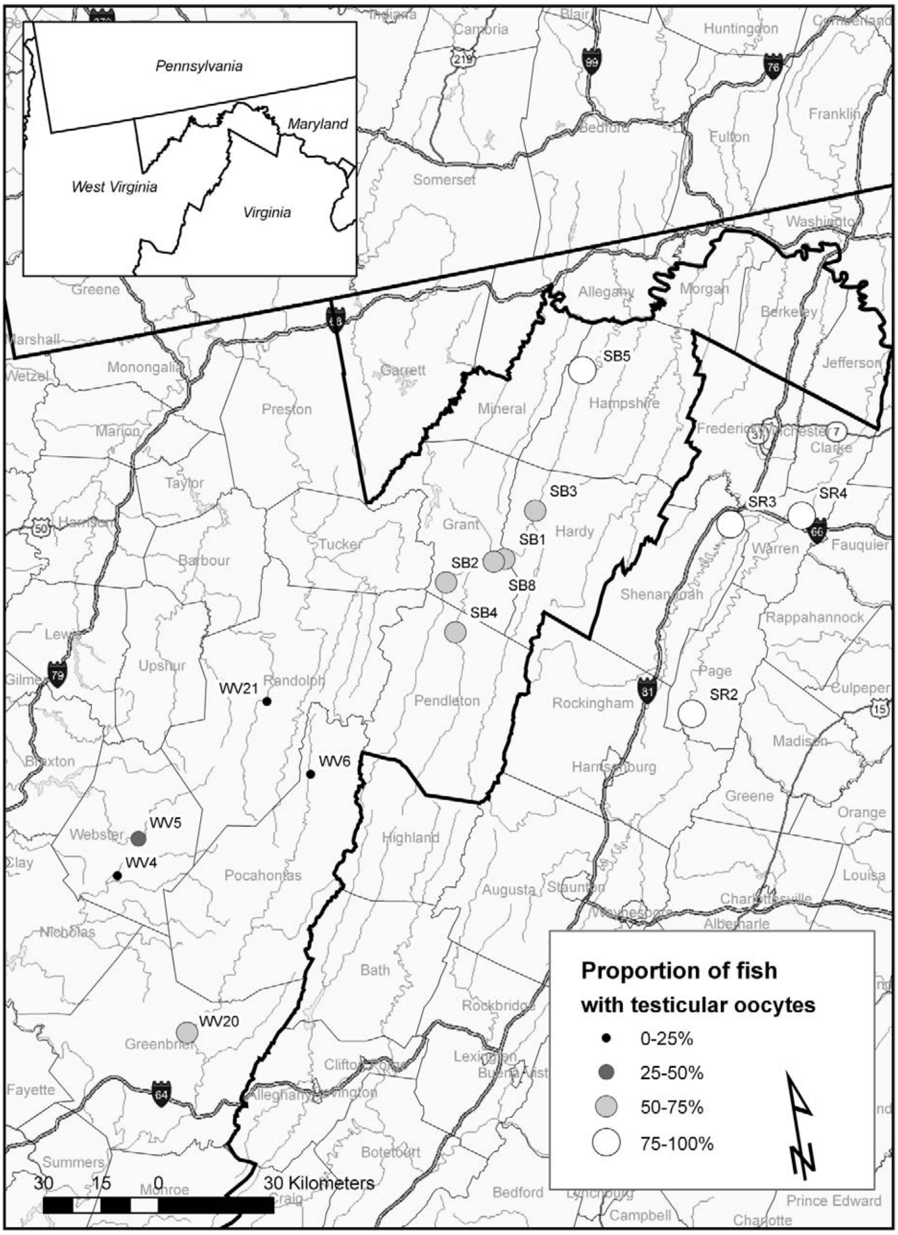

FIGURE 1.—Site locations and prevalence of testicular oocytes in smallmouth bass in the Potomac River drainage and adjacent rivers.

Fork begins in Augusta County, flows through Rockingham and Page (SR2) counties and into Warren County (Figure 1).

Fish were weighed, measured, observed for gross lesions, and bled with heparinized syringes from the caudal vein, and tissues were removed and fixed in Z-Fix (Anatech Ltd., Battle Creek, Michigan). In 2005, the gonads were removed and weighed to calculate the gonadosomatic index (GSI). At least five tissue cross-sections were taken along the length of each gonad and processed for histological evaluation. Tissue pieces were dehydrated in alcohol, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) (Luna 1992). Generally, one histologic section from each piece of cross-sectioned testicular tissue was examined. The diameter of these cross-

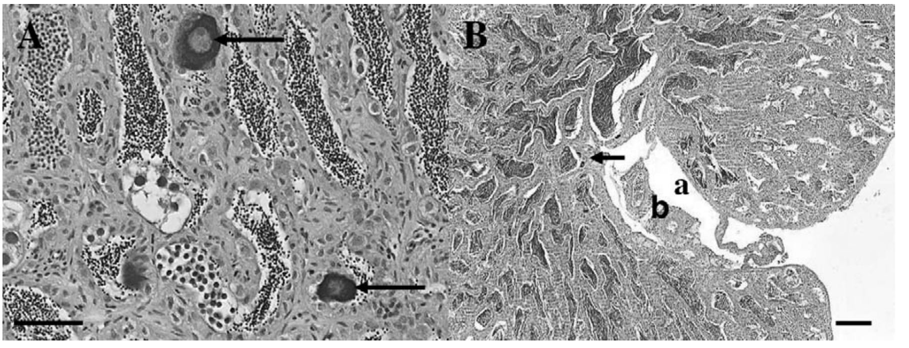

FIGURE 2.—Microscopic appearance of testicular oocytes in smallmouth bass. **(A)** Oocytes observed within testes of male smallmouth bass were primarily previtellogenic, chromatin nucleolus stage (arrow). Bar = 50 μm. **(B)** Immature oocytes (arrows) are most often observed around the central area (a) in close proximity to blood vessels and nerves (b). Bar = 100 μm. H&E stain used.

sections ranged from approximately 2 to 8 mm in most fish, with occasional larger cross-sections (10–14 mm) during the spring samples.

During microscopic examination of the slides it became evident that the severity of TO (or the number and distribution of oocytes) varied considerably among fish. Hence, a severity ranking system similar to that described by Bateman et al. (2004) was developed. The Bateman ranking system is based on both the developmental stage of the oocytes and the distribution of these oocytes within the testes. In smallmouth bass, generally only previtellogenic oocytes, primarily the chromatin nucleolus stage (Figure 2A) are observed. Hence, the testicular oocyte-severity index for bass was based on the distribution pattern only. Oocytes, when present, are generally located in the central portion of a cross-section of the testes (Figure 2B). Although the whole gonad section was scanned for presence of testicular oocytes at low magnification (4× objective or an approximately 24-mm^2 field of view), severity was rated by focusing on the central area of the section. Focal distribution (score 1) was a single oocyte within each microscopic field with 10× objective or approximately 4 mm^2 of tissue (Figure 3A), while a diffuse distribution (score 2) was defined as more than one oocyte in a field of view, but with no physical association with neighboring oocytes (Figure 3B). Cluster distribution (score 3) was more than one but less than five closely associated oocytes (Figure 3C), while the zonal distribution (score 4) was defined as more than five closely associated oocytes or numerous clusters in a field of view (Figure 3D). One histologic section was scored for severity from each of five pieces sampled approximately equidistant along the germinal portion of the testes. If multiple fields of view were examined and differed in severity, the most severe rating was used. The mean of the five individual section scores was used as the individual fish TO severity index.

To evaluate the number of sections required for confident diagnosis of TO, we analyzed testis sections from approximately 150 smallmouth bass collected during 2003–2005 that were detected as positive based on examination of five histological sections from an individual fish. Within each gonad, each section was considered to be an independent binomial trial for the detection outcome. Thus, we used logistic regression to estimate the probability of detecting TO from examination of a single section as a function of the fish's TO severity index. We then applied that result to calculate the probability of detecting TO from examination of multiple sections. We used PROC NLMIXED (SAS version 9.1) for the logistic regression because the procedure takes into account that multiple sections were taken from each gonad. The probability of detecting TO based on a sample size of n sections can be calculated as:

$$\text{Probability of detecting TO} = 1 - [1 - p(x)^n],$$

where $p(x)$ is the probability of detecting TO after examination of one section given that the fish's TO severity index $= x$. The logistic regression estimate for $p(x)$ was

$$p(x) = \frac{\exp(\beta_0 + \beta_1 x)}{1 + \exp(\beta_0 + \beta_1 x)},$$

where β_0 and β_1 are estimated regression parameters and x is the TO severity index. The regression parameter estimates were $\beta_0 = -1.7286$ (SE = 0.1834) and $\beta_1 = 2.3148$ (SE = 0.2019).

Site and seasonal differences in the prevalence and severity of TO were evaluated with one-way analysis

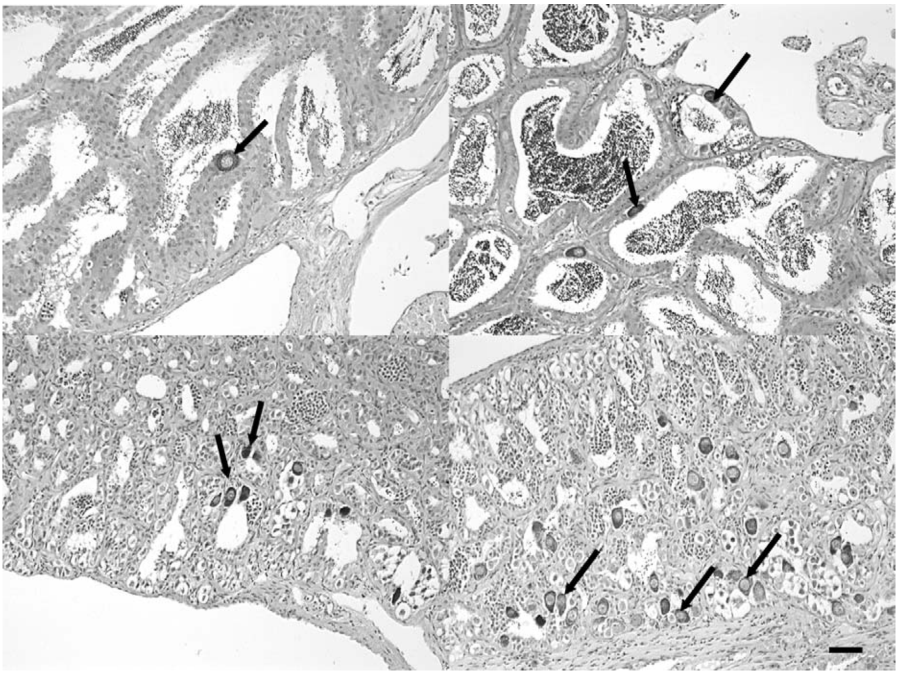

FIGURE 3.—Testicular oocyte severity index for evaluation of smallmouth bass gonads. **(upper left)** Severity rank 1, single oocyte. Individual oocyte (arrow) noted in field of view. **(upper right)** Severity rank 2, multifocal. More than one oocyte per field of view, but oocytes (arrows) are not closely associated. **(lower left)** Severity rank 3, cluster. Groups (2–5) of oocytes (arrows) closely associated with each other. **(lower right)** Severity rank 4, zonal. Multiple clusters or more than five closely associated oocytes. Bar = 50 μm. H&E stain used.

of variance (ANOVA) followed by Tukey's posthoc test. Additionally, given that sites with the highest prevalence of TO appeared to have the most severe cases, we used Pearson correlation statistics using Bonferroni adjustment to evaluate associations between these measures. Data for this analysis included site averages for all locations ($n = 29$) sampled between 2003 and 2005.

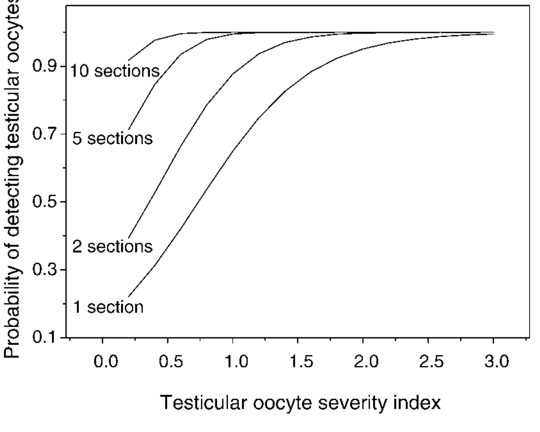

FIGURE 4.—Probability of detecting testicular oocytes in smallmouth bass as a function of the number of sections examined and severity.

Results

Evaluation of Required Sections

Reliability of detection of TO depended on the fish's severity index ($t = 11.61$, df $= 149$, $P < 0.0001$) and the number of sections examined (Figure 4). Based on one or two sections, TO would be unlikely to be detected in smallmouth bass unless the condition was severe. Based on five sections, the probability of detecting TO exceeded 0.9 for a severity index greater than 0.5, but was only around 0.7 for a severity index of 0.2. The probability of detecting TO even at a severity index of 0.2 was calculated to exceed 0.9 if 10 sections are examined. Prevalence and severity of TO were significantly positively correlated ($r = 0.81$, $p < 0.001$).

Testicular Oocytes in Smallmouth Bass Collected in the South Branch Potomac River

Prevalence of TO in smallmouth bass collected in the South Branch in the summer of 2003 ranged from

TABLE 1.—Prevalence (%) of testicular oocytes in smallmouth bass at selected sites in the South Branch of the Potomac River, 2003–2004. Number in parentheses is the total number of males collected. NS = no samples.

	Percent of fish with testicular oocytes observed			
Site	Summer 2003	Spring 2004	Summer 2004	Fall 2004
SB1	33% (6)	100% (7)	50% (4)	NS
SB2	NS	75% (12)	25% (8)	NS
SB3	0% (4)	85% (13)	40% (10)	100% (3)
SB4	NS	69% (13)	36% (11)	NS
SB5	60% (5)	90% (10)	67% (12)	88% (8)
SB8	80% (10)	73% (11)	33% (6)	NS

0% to 80% (Table 1); however, sample sizes of male fish were small. In 2004 we attempted to acquire larger sample sizes and collected during three seasons. At every station the prevalence was significantly higher ($P = 0.001$) in the spring during the prespawn–spawning period (69–100%) than in the summer postspawn period (25–67%). Prevalence of TO was also significantly higher in the fall than in the previous summer ($P = 0.018$); however, smallmouth bass were only collected at two sites and sample sizes were small. Data combined from all sites showed a similar seasonal pattern in prevalence (spring versus summer, $P < 0.001$; fall versus summer, $P = 0.018$) and severity (spring versus summer, $P = 0.005$; fall versus summer, $P = 0.005$) of TO (Figure 5). There was a significant correlation between prevalence and severity of TO ($r = 0.866$, $P < 0.001$).

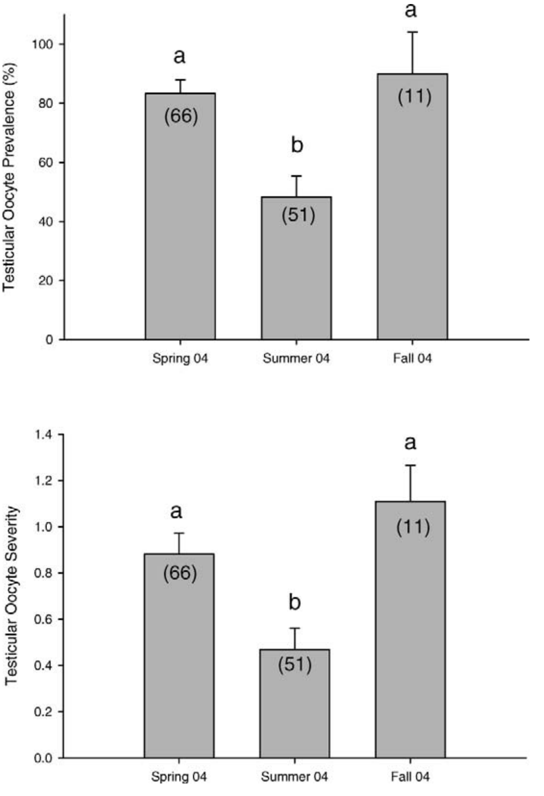

FIGURE 5.—Seasonal prevalence and severity of testicular oocytes in male smallmouth bass from the South Branch of the Potomac River. Bars labeled with the same letter are not significantly different. Numbers in parentheses indicate the number of males collected each season.

Testicular Oocytes in Smallmouth Bass Collected in the Tygart, Shenandoah, Gauley, Greenbrier and Elk River Drainages

In spring 2005, we compared smallmouth bass collected at two sites on the South Branch, one site on the Greenbrier River and one on the Tygart River (Table 2). The prevalence of TO in bass collected in the Greenbrier River was 75%, within the range (50–100%) of those collected in the South Branch, while only 14% of the male fish collected in the Tygart River contained TO. In fall 2005, we collected smallmouth bass farther upstream on the Greenbrier and on the Elk, Gauley and Shenandoah rivers. The prevalence of TO in fish at the three out-of-basin sites ranged from 17 to 36%, while in fish collected from the Shenandoah River it was 80–100% (Table 3).

Discussion

Microscopic gonadal abnormalities, including TO, have been used as indicators of exposure to chemicals causing modulation of the reproductive endocrine system. Many of the studies have been done with small fish species, such as medaka and fathead minnows *Pimephales promelas*, for which gonads can be processed whole. For larger fish species, standard methodology does not exist to evaluate potential gonadal abnormalities. The number of tissue pieces fixed for histology and the number of sections processed and evaluated, particularly for monitoring programs in which large numbers of fish are collected, is sometimes determined by time and cost. However, it is important to understand the effect of these decisions on the probability of observing TO and other pathological changes. In mature smallmouth bass (gonad weights ranging from 0.044 to 8.742 g), we determined that evaluating only one or two sections would result in a 50% or more chance of not detecting low severity intersex. Observing five sections would result in approximately 90% detection if the severity index was 0.5 or greater, while examining 10 sections should result in greater than 90% detection even at severity levels of 0.2. Hence, particularly when multiple cross-sections can be placed on one slide, at

TABLE 2.—Morphometric comparisons, prevalence and severity of testicular oocytes in smallmouth bass collected at sites within and outside of the Potomac River drainage, spring 2005. Values are presented as means ± SE. Those followed by the same letter are not significantly different from each other ($P < 0.05$).

River, site	n[a]	Mean total length (cm)	Mean weight (gm)	KTL[b]	GSI[c]	% with testicular oocytes	Testicular oocyte severity
South Branch, SB1	10	26.8 ± 2.2 z	265 ± 72 z	1.18 ± 0.03 z	0.36 ± 0.04 z	50% zy	0.6 ± 0.3 z
South Branch, SB3	5	25.7 ± 2.1 z	234 ± 59 z	1.30 ± 0.04 z	0.46 ± 0.22 z	100% z	1.4 ± 0.4 y
Greenbrier River, WV20	12	23.1 ± 1.9 z	187 ± 65 z	1.20 ± 0.07 z	0.78 ± 0.18 z	75% zy	0.9 ± 0.2 z
Tygart River, WV21	7	22.3 ± 1.3 z	153 ± 28 z	1.38 ± 0.11 z	0.67 ± 0.10 z	14% y	0.1 ± 0.1 z

[a] Total number of male smallmouth bass collected.
[b] KTL = Condition factor, calculated by (body weight/total length^3) × 100.
[c] GSI = Gonadosomatic index calculated by (gonad weight/body weight) × 100.

least five sections should be examined, and it would be prudent to examine 10 sections. For fish with larger gonad cross-sections this number will probably increase. In addition, the location examined within the testes may be important and may vary among species. Testicular oocytes were most apparent in the central region of the smallmouth bass testes, similar to that described for Japanese medaka exposed to 17β-estradiol (Hirai et al. 2006). This may not be the case for all fish species.

Our results indicate collection time may also be important in detection of TO in wild fishes, although more studies are needed to verify this observation. At all six sites sampled in the South Branch of the Potomac River in both spring and summer, prevalence was higher in the spring. During evaluation of gonadal sections from fish collected in summer and fall (postspawning), oocytes were occasionally observed in the lumen of the ducts, suggesting these cells were sloughed from the epithelium and possibly released with the sperm (Figure 6). Regression or degeneration of TO was also reported in Japanese medaka several months after exposure to estradiol (Okada 1964) and octylphenol (Gray et al. 1999).

A low level of gonadal intersex may be a natural phenomenon in some gonochoristic fishes. Jobling et al. (1998) reported that intersex occurs "naturally" in approximately 4% of male roach, while 4.5–5% prevalence was observed at reference sites by Bjerregaard et al. (2006). However, it must be questioned whether this is "natural" or whether some fish species are very sensitive to the contaminants that may cause TO because of spawning times or environments, food habits, or other life history traits. We are aware of only two other studies in which smallmouth bass reproductive health was evaluated. Anderson et al. (2003) examined smallmouth bass at two sites on the Kalamazoo River, Michigan, in the fall (September), specifically targeting polychlorinated biphenyl (PCB) contamination from pulp and paper mills. All male bass collected (seven upstream and eight downstream) had TO, with the downstream fish having slightly higher scores. However, the upstream site was near an urban area and hence, not a "pristine" area. Baldigo et al. (2006) collected smallmouth bass from six sites on the Hudson River, New York, in late spring–early summer (May–June); two of these sites were considered "reference" based on PCB concentrations in sediment and fish tissue. Testicular oocytes were observed in none out of nine and three of nine smallmouth bass collected at these two sites. The prevalence of TO at the other four sites ranged from 0% to 50% (Baldigo et

TABLE 3.—Morphometric comparisons, prevalence and severity of testicular oocytes in smallmouth bass collected at sites within and outside of the Potomac drainage, fall 2005. Values are presented as mean ± SE. Those followed by the same letter are not significantly different from each other ($P < 0.05$).

River, site	n[a]	Mean length (cm)	Mean weight (gm)	KTL[b]	GSI[c]	% with testicular oocytes	Testicular oocyte severity
Gauley River, WV4	6	23.8 ± 1.1 z	165 ± 20 z	1.20 ± 0.04 z	0.55 ± 0.04 z	17% z	0.2 ± 0.2 z
Elk River, WV5	11	27.2 ± 1.5 z	271 ± 43 z	1.23 ± 0.02 z	0.59 ± 0.05 z	36% zx	0.2 ± 0.1 z
Greenbrier River, WV6	18	23.1 ± 1.1 z	166 ± 26 z	1.20 ± 0.03 z	0.60 ± 0.04 z	22% z	0.1 ± 0.05 z
Shenandoah River							
South Fork, SR2	10	24.0 ± 1.3 z	195 ± 34 z	1.31 ± 0.03 z	0.64 ± 0.03 z	80% yx	1.2 ± 0.3 y
North Fork, SR3	8	26.0 ± 1.8 z	250 ± 63 z	1.26 ± 0.05 z	0.83 ± 0.03 z	100% y	1.2 ± 0.3 y
Mainstem, SR4	13	26.0 ± 0.6 z	249 ± 17 z	1.40 ± 0.03 y	0.55 ± 0.04 z	100% y	1.7 ± 0.2 y

[a] Total number of male smallmouth bass collected.
[b] KTL = Condition factor calculated by (body weight/total length^3) × 100.
[c] GSI = Gonadosomatic index calculated by (gonad weight/body weight) × 100

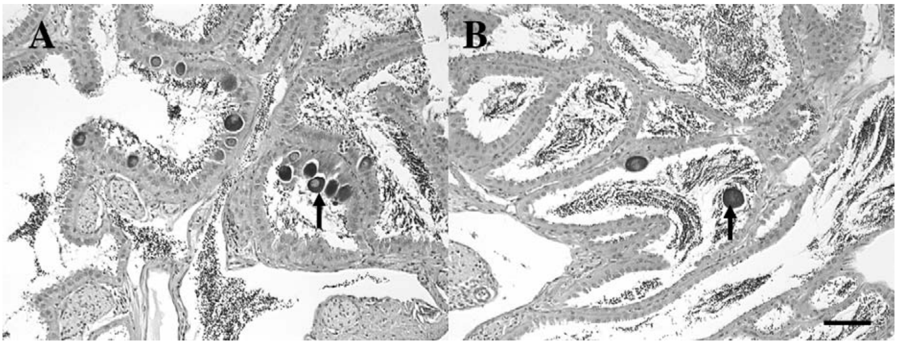

FIGURE 6.—Testicular oocytes in smallmouth bass. Two sections (**A**) and (**B**) illustrating oocytes (arrows) could be observed free in the lumen of the seminiferous tubules during and after spawning. Bar = 50 μm. H&E stain used.

al. 2006). Again, while these were reference sites in terms of PCB contamination, they were not free of anthropogenic influence, such as wastewater effluent and agricultural runoff. To better characterize whether a certain level of TO is a "natural" occurrence or due to a species sensitivity to the widespread occurrence of endocrine-disrupting compounds, further research is needed for individual species.

The most sensitive periods for the development of TO in bass have not been documented. Primordial germ cells are present in gonads before gonadal sex differentiation and have the ability to become oogonia or spermatogonia. Studies with other fish species indicate that the period during sexual differentiation may be the most sensitive exposure time to induce intersex (Koger et al. 2000; Krisfalusi and Nagler 2000), but TO can be induced in older fish as well (Gray et al. 1999; Kang et al. 2002). This may indicate that the bipotentiality of germ cells decreases with time but is not entirely ablated (Shibata and Hamaguchi 1988; Gray et al. 1999); hence, induction in older fish may be the result of testicular germ cell plasticity (Okutsu et al. 2006).

Roach, a fish species used extensively in England for monitoring, appears to have a similar sensitivity as smallmouth bass. In some English rivers, 100% of male roach were found to have TO (Jobling et al. 1998), which was linked to exposure to sewage treatment plant effluents containing estrogenic substances (Purdom et al. 1994). Experimental studies with roach indicate that in adult fish with a previous exposure, the degree of intersex increased during subsequent exposure. Hence, it was suggested that intersex in roach occurs as a consequence of long-term exposure to estrogenic compounds or as a consequence of programming during early life that manifests later as the fish goes through sexual maturation (Liney et al. 2005). Levels of estrogenic activity have been found to be much higher (532–748-fold) in sediments than in the overlying water, indicating these chemicals accumulate in the bed-sediment phase (Peck et al. 2004). Hence, species such as smallmouth bass, whose eggs and fry are associated with sediment, are more likely to be exposed to higher levels of estrogenic compounds during these sensitive stages.

The finding of a high prevalence of male smallmouth bass with TO in the South Branch drainage was unexpected. The South Branch drains an area of low human population density in the eastern panhandle of West Virginia. The area is primarily rural and land use is dominated by agriculture, with poultry rearing and poultry processing as main sources of income. Other agriculture includes low-density cattle and sheep rearing. Potential sources of contaminants in this area include agricultural runoff, municipal and industrial wastewater, and treated and untreated domestic wastewater (Chambers and Leiker 2006). To date, a high prevalence of intersex in wild fishes has primarily been associated with exposure to human wastewater effluent (Purdom et al. 1994; Jobling et al. 1998). Estrogens found in these effluents include estrone, 17β-estradiol, and the synthetic estrogen 17α-ethinylestradiol used in birth control and hormone replacement medications (Desbrow et al. 1998). Agricultural runoff has also been associated with endocrine disruption or reproductive abnormalities due to the presence of natural and synthetic hormones, pesticides, and herbicides (Zhan et al. 2000; Hanselman et al. 2003; Kiparissis et al. 2003; Kojima et al. 2004; Orlando et al. 2004).

Passive samplers, semipermeable-membrane devices (SPMDs), and polar organic compound integrative samplers (POCIS), were deployed for approximately 6 weeks in spring 2004 at sites close to those where smallmouth bass were collected in the South Branch (Chambers and Leiker 2006). These samplers are designed to accumulate and sequester hydrophobic (SPMD) and hydrophilic (POCIS) compounds over

TABLE 4.—Comparison of human population and agricultural use in the counties containing collection sites for smallmouth bass. ND = no data available.

Site and county	% testicular oocytes (n)	Human population density[a] (per km^2)	% land farmed[b]	Livestock[b] Poultry inventory	Broilers sold	Cattle	Sheep
			Out of basin sites				
WV6, Pocahontas	22 (18)	4	20.5	1,597	13	14,096	4,341
WV21, Randolph	14 (7)	10	15.2	1,410	ND	10,892	2,602
WV4,	17 (6)						
WV5, Webster	36 (11)	7	3.2	484	ND	723	277
WV20, Greenbrier	75 (12)	13	29.5	54,838	ND	35,144	2,495
			South Branch Potomac sites[c]				
SB4, Pendleton	54 (24)	5	38.2	3,919,946	18,946,253	20,821	6,761
SB3, Hardy	65 (35)	8	34.4	6,388,038	41,650,710	21,535	2,332
SB1,2,8, Grant	47 (74)	9	35.2	2,860,699	16,823,102	12,481	1,190
SB5, Hampshire	77 (35)	12	33.8	903,436	5,155,147	18,553	589
			Shenandoah sites				
Rockingham		31	44.7	20,835,812	104,985,748	119,938	7,165
SR2, Page	100 (8)	28	32.2	8,365,320	42,916,313	23,418	ND
SR3, Shenandoah	80 (10)	26	40.6	3,858,637	19,608,058	38,317	2,910
SR4, Warren	100 (13)	57	35.8	ND	ND	8,788	330

[a] Based on U.S. Census Bureau (2002).
[b] Based on U.S. Department of Agriculture (2004).
[c] Includes data from all male smallmouth bass collected in 2004–2005.

time that may be present at concentrations below the detection level of grab-sample methods for water analysis (Petty et al. 2004). Unfortunately, the compounds previously shown to induce TO in other fish species (natural and synthetic estrogens, atrazine, nonylphenol, octylphenol, bisphenol A, equol, and genistein) were not included in the compounds analyzed in the sampler extracts. However, a number of chemicals known to be endocrine disruptors and having estrogenic activity were detected. These included the herbicide trifluralin, the fungicide hexachlorobenzene, pentachloroanisole (degradation product of industrial phenols), the pesticides gamma hexachlorohexane, chlorpyrifos, *cis*- and *trans*-chlordane and *cis*-nonachlor, and the flame retardant bromodiphenyl ethers, BDE 47 and 99 (Chambers and Leiker 2006). It is important to identify possible causes for the observed TO; however, it is unlikely that any one contaminant or source is responsible for inducing TO in smallmouth bass. Agricultural runoff, human wastewater effluent, and industrial sources can all introduce estrogenic compounds into the aquatic environment (Nichols et al. 1997; Kolpin et al. 2002; Orlando et al. 2004). In addition, additive effects of many of these chemicals have been demonstrated even when each compound is present below the threshold of detectable effects (Payne et al. 2000; Rajapakse et al. 2002; Brian et al. 2007). Hence, evaluating the overall estrogenicity of water and sediment extracts at various sites during multiple seasons will be important in determining sources and potential mitigation.

A comparison of the prevalence and severity of TO in smallmouth bass from the Shenandoah River and South Branch of the Potomac River with the out-of-basin sites indicated a possible pattern of higher prevalence and severity as anthropogenic stressors increased. Hence, as a preliminary assessment, recognizing that point sources may also be very important local influences, we compared human population density and agricultural intensity in the counties where fish were collected (Table 4). In general, the counties in which our out-of-basin sites were located had low human population, low intensity agriculture, and a lower prevalence of TO. The South Branch sites, with an intermediate prevalence of TO (between 47 and 77%), were in counties that also had generally low human population density, but an increased agricultural intensity compared with most of the out-of -basin sites. Although none of our Shenandoah River sites were in Rockingham County, both the South Fork and North Fork flow through that county, which has one of the highest human population densities and agricultural intensities of the areas we sampled. However, the counties that contained our sampling sites in Virginia all had higher human population and agricultural use when compared with the West Virginia sites. The Shenandoah River sites also have the highest prevalence of TO (Figure 1; Table 4). Hence, this crude analysis indicates that land use may significantly influence the prevalence of TO and other reproductive abnormalities in fishes. A recent study of roach from 39 rivers throughout the UK tested risk assessment models to predict the location and severity of endocrine disruption in river systems to identify areas where regulation of sewage discharges to remove these contaminants is necessary. Both incidence and severity of intersex (testicular oocytes) was significantly correlated with predicted concentrations of natural (17β-estradiol and estrone) and synthetic (17α-ethinylestradiol) estrogens in sewage effluent (Jobling et al. 2007).

There are still many questions that must be addressed concerning TO in smallmouth bass. We believe it is an important indicator of potential endocrine disruption, although recognize the current lack of evidence for population-level or ecosystem effects. Roach with intersex have reduced sperm production and the quality of the sperm produced is also reduced (Jobling et al. 2002a, 2002b); hence, the possibility for population effects certainly exists. However, it should be recognized that TO and other responses such as vitellogenin production in male fish are only one type of indicator of exposure to endocrine disruptors. Although potential effects on reproductive capacity are certainly important on a population level, there may be other effects such as immunomodulation and associated increased susceptibility to infectious and neoplastic diseases that are equally important to population health. It is interesting to note that the South Branch of the Potomac and the Shenandoah River have experienced significant kills of smallmouth bass and selected other species since 2002. In the North Fork and South Fork of the Shenandoah River it is estimated that 80% of the adult smallmouth bass population was lost during the kills of 2004 and 2005.

Studies are continuing to (1) assess the extent of TO in bass throughout the Potomac River drainage, (2) examine bass collected at reference sites within and outside of the drainage basin to attempt to determine a "background" prevalence for both smallmouth and largemouth bass, (3) identify potential causes and assess both reproductive health (sperm quantity and quality, reproductive hormones, vitellogenin production) and general health (parasite loads, other microscopic changes, immune cell function) at sites with high and low prevalences of TO, and (4) better understand population effects of exposure to endocrine-disrupting chemicals. In addition, attempts will be made to further evaluate land use in risk assessment models.

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References

Allen, Y., A. P. Scott, P. Matthiessen, S. Haworth, J. E. Thain, and S. Feist. 1999. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. Environmental Toxicology and Chemistry 18:1791–1800.

Anderson, M. J., D. Cacela, D. Beltman, S. J. Teh, M. S. Okihiro, D. E. Hinton, N. Denslow, and J. T. Zelikoff. 2003. Biochemical and toxicopathic biomarkers assessed in smallmouth bass recovered from a polychlorinated biphenyl-contaminated river. Biomarkers 8:371–393.

Aravindakshan, J., V. Paquet, M. Gregory, J. Dufresne, M. Fournier, D. J. Marcogliese, and D. G. Cyr. 2004. Consequences of xenoestrogen exposure on male reproductive function in spottail shiners (*Notropis hudsonius*). Toxicological Sciences 78:156–165.

Balch, G., and C. Metcalfe. 2006. Developmental effects in Japanese medaka (*Oryzias latipes*) exposed to nonylphenol ethoxylates and their degradation products. Chemosphere 62:1214–1223.

Baldigo, B. P., R. J. Sloan, S. B. Smith, N. D. Denslow, V. S. Blazer, and T. S. Gross. 2006. Polychlorinated biphenyls, mercury, and potential endocrine disruption in fish from the Hudson River, New York, USA. Aquatic Sciences 68:206–228.

Barnhoorn, I. E. J., M. S. Bornman, G. M. Pieterse, and J. H. J. van Vuren. 2004. Histological evidence of intersex in feral sharptooth catfish (*Clarias gariepinus*) from an estrogen-polluted water source in Gauteng, South Africa. Environmental Toxicology 19:603–608.

Bateman, K. S., G. D. Stentiford, and S. W. Feist. 2004. A ranking system for the evaluation of intersex condition in European flounder (*Platichthys flesus*). Environmental Toxicology and Chemistry 23:2831–2836.

Bjerregaard, L., B. Korsgaard, and P. Bjerregaard. 2006. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotoxicology and Environmental Safety 64:321–328.

Brian, J. V., C. A. Harris, M. Scholze, A. Kortenkamp, P. Booy, M. Lamoree, G. Pojana, N. Jonkers, A. Marcomini, and J. P. Sumpter. 2007. Evidence of estrogenic mixture effects on the reproductive performance of fish. Environmental Science and Technology 41:337–344.

Brion, F., C. R. Tyler, X. Palazzi, B. Laillet, J. M. Porcher, J. Garrie, and P. Flammarion. 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryolarval, juvenile- and adult-life stages in zebrafish (*Danio rerio*). Aquatic Toxicology 68:193–217.

Chambers, D. B., and T. J. Leiker. 2006. A reconnaissance for emerging contaminants in the South Branch Potomac River, Cacapon River and Williams River basins, West

Virginia, April–October 2004. U.S. Geological Survey, Open File Report 2006–1393. Available: pubs.usgs.gov/of/2006/1393. (March 2007).

De Metrio, G., A. Corriero, S. Desantis, D. Zubani, F. Cirillo, M. Deflorio, C. R. Bridges, J. Ericker, J. M. de la Serna, P. Megalofonou, and D. E. Kime. 2003. Evidence of a high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius* L.). Marine Pollution Bulletin 46:358–361.

Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. 1998. Identification of estrogenic chemicals in STW effluents. Chemical fractionation and in vitro biological screening. Environmental Science and Technology 32:1549–1558.

Faller, P., B. Kober, A. Peter, J. P. Sumpter, and P. Burkhardt-Holm. 2003. Stress status of gudgeon (*Gobio gobia*) from rivers in Switzerland with and without input of sewage treatment plant effluent. Environmental Toxicology and Chemistry 22:2063–2072.

Gerken, J., and H. Sordyl. 2002. Intersex in feral marine and freshwater fish from northeastern Germany. Marine Environmental Research 54:651–655.

Gimeno, S., H. Komen, S. Jobling, J. Sumptor, and T. Bowmer. 1998. Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during spermatogenesis. Aquatic Toxicology 43:93–109.

Gray, M. A., A. J. Niimi, and C. D. Metcalfe. 1999. Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. Environmental Toxicology and Chemistry 18:1835–1842.

Hanselman, T. A., D. A. Graetz, and A. C. Wilkie. 2003. Manure-borne estrogens as potential environmental contaminants: a review. Environmental Science and Technology 37:5471–5478.

Harshbarger, J. C., M. J. Coffey, and M. Y. Young. 2000. Intersexes in Mississippi River shovelnose sturgeon sampled below Saint Louis, Missouri, USA. Marine Environmental Research 50:247–250.

Hashimoto, S., H. Bessho, A. Hara, M. Nakamura, T. Iguchi, and K. Fujita. 2000. Elevated serum vitellogenin levels and gonadal abnormalities in wild flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. Marine Environmental Research 49:37–53.

Hecker, M., M. B. Murphy, K. K. Coady, D. L. Villeneuve, P. D. Jones, J. A. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, T. Gross, L. Du Preez, R. J. Kendall, and J. P. Giesy. 2006. Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. Reviews in Environmental Contamination and Toxicology 187:103–131.

Hirai, N., A. Nanba, M. Koshio, T. Kondo, M. Morita, and N. Tatarazako. 2006. Feminization of Japanese medaka (*Oryzias latipes*) exposed to 17 beta-estradiol: formation of testis-ova and sex-transformation during early ontogeny. Aquatic Toxicology 77:78–86.

Imai, S., J. Koyama, and K. Fujii. 2005. Effects of 17β-estradiol on the reproduction of Java-medaka (*Oryzias javanicus*), a new test fish species. Marine Pollution Bulletin 51:708–714.

Jobling, S., N. Beresford, M. Nolan, T. Rodgers-Gray, G. C. Brighty, J. P. Sumpter, and C. R. Tyler. 2002a. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biology of Reproduction 66:272–281.

Jobling, S., S. Coey, J. G. Whitmore, D. E. Kime, K. J. W. van Look, B. G. McAllister, N. Beresford, A. C. Henshaw, G. Brighty, C. R. Tyler, and J. P. Sumpter. 2002b. Wild intersex roach (*Rutilus rutilis*) have reduced fertility. Biology of Reproduction 67:515–524.

Jobling, S., M. Nolan, C. R. Tyler, G. Brighty, and J. Sumpter. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology 32:2498–2506.

Jobling, S., R. Williams, A. Johnson, A. Taylor, M. Gross-Sorokin, M. Nolan, C. R. Tyler, R. van Aerle, E. Santos, and G. Brighty. 2007. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environmental Health Perspectives 114:32–39.

Kang, I. J., H. Yokota, Y. Oshima, Y. Tsuruda, T. Yamaguchi, M. Maeda, N. Imada, H. Tadokoro, and T. Honjo. 2002. Effect of 17β-estradiol on reproduction of Japanese medaka (*Oryias latipes*). Chemosphere 47:71–80.

Kavanagh, R. J., G. C. Balch, Y. Kiparissis, A. J. Niimi, J. Sherry, C. Tinson, and C. D. Metcalfe. 2004. Endocrine disruption and altered gonadal development in white perch (*Morone americana*) from the lower Great Lakes region. Environmental Health Perspectives 112:898–902.

Kinnberg, K., H. Holbech, G. I. Petersen, and P. Bjerregaard. 2007. Effects of the fungicide prochloraz on the sexual development of zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology 145C:165–170.

Kiparissis, Y., G. C. Balch, T. L. Metcalfe, and C. D. Metcalfe. 2003. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*). Environmental Health Perspectives 111:1158–1163.

Koger, C. S., S. J. Teh, and D. E. Hinton. 2000. Determining the sensitive stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17 β-estradiol or testosterone. Marine Environmental Research 50:201–206.

Kojima, H., E. Katsura, S. Takeuchi, K. Niiyama, and K. Kobayashi. 2004. Screening for estrogen and androgen receptor activities in 200 pesticides in in vitro reporter gene assays using Chinese hamster ovary cells. Environmental Health Perspectives 112:524–531.

Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams 1999–2000: a national reconnaissance. Environmental Science and Technology 36:1202–1211.

Krisfalusi, M., and J. J. Nagler. 2000. Induction of intersex in genotypic male rainbow trout (*Oncorhynchus mykiss*) embryos following immersion in estradiol-17β. Molecular Reproduction and Development 56:495–501.

Liney, K. E., S. Jobling, J. A. Shears, P. Simpson, and C. R. Tyler. 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. Environmental Health Perspectives 113:1299–1307.

Luna, L. G. 1992. Histopathologic methods and color atlas of special stains and tissue artifacts. American Histolabs, Inc., Gaithersburg, Maryland.

Martin-Skilton, R., R. Lavado, R. Thibaut, C. Minier, and C. Porte. 2006. Evidence of endocrine alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. Environmental Pollution 141:60–68.

Metcalfe, T. L., C. D. Metcalfe, Y. Kiparissis, A. J. Niimi, C. M. Foran, and W. H. Benson. 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o,p'*-DDT in water and through maternal transfer. Environmental Toxicology and Chemistry 19:1893–1900.

Nichols, D. J., T. C. Daniel, P. A. Moore, Jr., D. R. Edwards, and D. H. Pote. 1997. Runoff of estrogen hormone 17β-estradiol from poultry litter to pasture. Journal of Environmental Quality 26:1002–1006.

Nolan, M., S. Jobling, G. Brighty, J. P. Sumpter, and C. R. Tyler. 2001. A histological description of intersexuality in the roach. Journal of Fish Biology 58:160–176.

Okada, Y. K. 1964. A further note on testis-ova in the teleost, *Oryzias latipes*. Proceedings of the Japan Academy 40:753–756.

Okutsu, T., K. Suzuki, Y. Takeuchi, T. Takeuchi, and G. Yoshizaki. 2006. Testicular germ cell can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proceedings of the National Academy of Sciences of the USA 103:2725–2729.

Orlando, E. E., A. S. Kolok, G. A. Binzcik, J. L. Gate, M. K. Horton, C. S. Lambright, L. E. Gray, Jr., A. M. Soto, and J. L. Guillette, Jr. 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environmental Health Perspectives 112:353–358.

Payne, J., N. Rajapakse, M. Wilkins, and A. Kortenkamp. 2000. Prediction and assessment of the effects of mixtures of four xenoestrogens. Environmental Health Perspectives 108:983–987.

Peck, M., R. W. Gibson, A. Kortenkamp, and E. M. Hill. 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environmental Toxicology and Chemistry 23:945–952.

Petty, J. D., J. N. Huckins, D. A. Alvarez, W. G. Brumbaugh, W. L. Cranor, R. W. Gale, A. C. Rastell, T. L. Jones-Lepp, T. J. Leiker, C. E. Rostad, and E. T. Furlong. 2004. A holistic passive integrative sampling approach for assessing the presence and potential impacts of waterborne environmental contaminants. Chemosphere 54:695–705.

Purdom, C. E., P. A. Hardiman, V. J. Bye, N. C. Eno, C. R. Tyler, and J. P. Sumpter. 1994. Estrogenic effects of effluents from sewage treatment works. Chemical Ecology 8:275–285.

Rajapakse, N., E. Silva, and A. Kortenkamp. 2002. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environmental Health Perspectives 110:917–921.

Schmitt, C. J., V. S. Blazer, G. M. Dethloff, D. E. Tillitt, T. S. Gross, W. L. Bryant, Jr., L. R. DeWeese, S. B. Smith, R. W. Goede, T. M. Bartish, and T. J. Kubiak. 1999. Biomonitoring of Environmental Status and Trends (BEST) program: field procedures for assessing the exposure of fish to environmental contaminants. U.S. Geological Survey, Biological Resources Division, Information and Technology Report USGS/BRD-1999-000-7, Columbia, Missouri.

Seki, M., Y. Hirofumi, M. Haruki, T. Yukinari, M. Masanobu, T. Hiroshi, and K. Kunio. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 21:1692–1699.

Shibata, N., and S. Hamaguchi. 1988. Evidence for the sexual bipotentiality of spermatagonia in the fish, *Oryzias latipes*. Journal of Experimental Zoology 245:71–77.

U.S. Census Bureau. 2002. United States census 2000. Summary file 1, geographic comparison tables. Available: www.census.gov/Press-Release/www/2001/sumfile1.html. (March 2007).

U.S. Department of Agriculture. 2004. 2002 census of agriculture. State and county reports. Available: www.nass.usds.gov/Census_of_Agriculture/index.asp. (March 2007).

Van Aerle, R., M. Nolan, S. Jobling, L. B. Christiansen, J. P. Sumpter, and C. R. Tyler. 2001. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. Environmental Toxicology and Chemistry 20:2841–2847.

Viganò, L., A. Arillo, S. Bottero, A. Massari, and A. Mandich. 2001. First observation of intersex cyprinids in the Po River (Italy). Science for the Total Environment 269:189–194.

Woodling, J. D., E. M. Lopez, T. A. Maldonado, D. O. Norris, and A. M. Vajda. 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comparative Biochemistry and Physiology 144C:10–15.

Yokota, H., Y. Tsuruda, M. Maeda, Y. Oshima, H. Tadokoro, A. Nakazono, T. Honjo, and K. Kobayashi. 2000. Effect of bisphenol A on early life stage in Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 19:1925–1930.

Zhan, W., Y. Xu, A. H. Li, J. Zhang, K.-W. Schramm, and A. Kettrup. 2000. Endocrine disruption by hexachlorobenzene in Crucian carp (*Carassius auratus gibelio*). Bulletin of Environmental Contamination and Toxicology 65:560–566.