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Testicular oocytes in smallmouth bass in Northeastern Minnesota in relation to varying levels of human activity

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Abstract

Testicular oocytes (TOs) have been found in black basses (*Micropterus* spp.) from many locations in North America. The presence of TOs is often assumed to imply exposure to estrogenic endocrine disrupting compounds (EDCs), however, a definitive causal relationship has yet to be established, and TO prevalence is not consistently low in fish from areas lacking evident EDC sources. This might indicate any of a number of situations: (a) unknown or unidentified EDCs or EDC sources, (b) induction of TOs by other stressors, or (c) TOs occurring spontaneously during normal development. In the present study we analyzed TO occurrence in smallmouth bass (*M. dolomieu*) from eight populations in Northeastern Minnesota watersheds with differing degrees of human development and hence, presumed likelihood of exposure to anthropogenic chemicals. Three watersheds were categorized as moderately developed, based on the presence of municipal wastewater discharges and higher human population density (4-81 per km²), and five watersheds were minimally developed, with very low human population density (0-1 per km²) and minimal built environment. Testicular tissues from mature fish were evaluated using a semi-quantitative method that estimated TO density, normalized by cross-sectional area. TO prevalence and density among populations from moderately developed watersheds was higher than in populations from minimally developed watersheds. However, TO prevalence was unexpectedly high and variable (7-43%) in some populations from minimally developed watersheds, and only weak evidence was found for a relationship between TO density and watershed development, suggesting alternative or more complex explanations for TO presence in smallmouth bass from this region.

Keywords

histopathology; endocrine disruptors; estrogenic compounds; smallmouth bass; testicular oocytes

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INTRODUCTION

Endocrine disrupting compounds (EDCs), like estrogens and their mimics, are frequently detected in natural waters that receive inputs from wastewater treatment plants (WWTPs), industrial sites, and agricultural activities [1–3]. Estrogenic compounds have been associated with sexual disruption in many fish species in numerous laboratory and field studies [4]. Both juvenile and mature fish exposed to estrogenic compounds develop biomarkers of endocrine disruption, including altered steroid hormone levels, elevated vitellogenin (Vtg), and gonadal intersex (presence of both male and female gonadal phenotypes), as well as population-relevant effects like sex reversal and decreased reproduction [4]. Evidence that EDC exposure can cause reproductive pathology in wild populations exists for some species, notably white sucker (*Catostomus commersoni*), darters (*Etheostoma* spp.) and roach (*Rutilus rutilus*) exposed to WWTP effluent [5–8]. Wild black bass populations (*Micropterus* spp.) may also be subject to reproductive disruption, as evidenced by gonadal intersex rates as high as 100% of males per site in several major U.S. river systems [9–12], and according to a recent review, *M. dolomieu* and *M. salmoides*, combined, were the most commonly reported intersex fish species in North America [13]. Intersex is considered abnormal in most gonochoristic fish [14], and is recognized as a potential biomarker of exposure to EDCs from anthropogenic sources [15]. In *Micropterus* sp., intersex has been characterized as the presence of immature oocytes in the testes (testicular oocytes; TOs); however, the ontogeny of gonadal development and the degree to which TOs may occur spontaneously in these species is unknown. TOs have been induced in several other fish species during laboratory exposure to EDCs, including the natural estrogen 17- β -estradiol (E2), alkylphenols, and bisphenol-A, and the synthetic estrogen 17- α -ethynylestradiol (EE2) [16]. Lowest observed EE2 effect concentrations for altered gonadal development in juveniles range from 1 ng/L in fathead minnow (*Pimephales promelas*) and roach [17, 18], to 10 ng/L in medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*; [19, 20]). In comparison, some evidence exists that mummichog (*Fundulus heteroclitus*) is relatively insensitive to sexual disruption by EE2 concentrations up to 100 ng/L [21], indicating considerable differences in sensitivity among common model species. EE2 concentrations in most aquatic environments appear unlikely to exceed 1 ng/L, even in low-flow conditions [22], although the potential for aggregated effects with other EDCs (including other estrogens) is not well understood. Therefore, specific environmental conditions under which TOs could be induced in sensitive species are unknown.

Several studies have reported high TO prevalence in *Micropterus* from regions with human activities like WWTPs, agriculture, and industry, which are known to release EDCs, but evidence for a causal relationship between EDC exposure and TOs in these species is weak and inconsistent. Many of these studies did not assess TO occurrence in bass near suspected EDC sources in comparison with reference sites [3, 10, 23], or reported that TO prevalence was not consistently or significantly lower at reference sites compared with sites near known EDC sources [9, 24–28]. For example, Hinck et al. [10] reported that high TO prevalence did not always correspond with a high number of detected contaminants, and that at least one site with a high TO prevalence had no obvious sources of EDCs. Blazer et al. [9] found 17–75% TO prevalence at sites described as having low human population and low

agricultural use, while other sites with low-to-moderate human population density and moderate-to-high agricultural use had 54-100% TO prevalence, suggesting a possible trend of increased TO occurrence with human activity, but the pattern was neither consistent nor strong. Stronger evidence was reported by another study [11] in which TO prevalence was 11% among male bass collected from a site with little human development compared with 55-100% at more developed sites. Blazer et al. [29] also found a significant correlation between TO prevalence and percent of agriculture in the watershed ($r^2 = 0.63$), although correlations between TO prevalence and other land-use characteristics, including number of animal feeding operations, number of WWTPs, and average permitted WWTP flow rate, were low. The same study also found evidence supporting a potential association between bass TOs and measurements of a known estrogenic compound, citing significant correlations between water concentrations of estrone and TO prevalence, TO severity, and the percentage of male bass detected with plasma Vtg [29]. A companion study conducted at the same sites also found significant correlations between TOs and sediment concentrations of two estrogenic sterols, but not estrone [30]. The potential contributions from non-point sources adds uncertainty to the likelihood that fish from any particular site might have been exposed to EDCs, and may be responsible for some of these incongruences. In addition, there is some evidence that agricultural sources like feedlot runoff and manure applications are more strongly associated with both TO occurrence [26] and estrogenic activity [1] than WWTP effluent. Even so, the current evidence supporting a causal relationship between non-point source EDCs and TOs in bass is no stronger than that for point source EDCs and TOs.

Relatively few studies of *Micropterus* intersex have included statistical comparisons of tissue collected from multiple reference sites where potential influences from both WWTP and agricultural activities are low. Documenting TO prevalence in areas where known sources of EDCs are minimal will provide important context for interpreting TO occurrence in areas of higher human activity and a correspondingly higher likelihood of exposure. Northeastern Minnesota supports smallmouth bass populations over a range of human activity and land use, including urban and heavily industrialized areas near the Duluth-Superior shipping harbor on the St. Louis River, as well as the Superior National Forest with largely undeveloped shorelines and extremely low human population densities. The central goal of the current study was to evaluate TO presence in smallmouth bass populations from watersheds across a range of development density. The prevalence of male fish with TOs and the estimated TO density per gonad (a semiquantitative estimate of severity) for each individual collection site was also evaluated with multiple regression analysis to identify potential predictors of TOs, including development category, human population density, collection season, fish weight, length, condition factor, age, gonadosomatic index, and hepatosomatic index.

METHODS

Collection sites

For the present study, it was presumed that in moderately developed watersheds, human activities, like WWTPs and urbanization, were more likely to result in EDC presence, compared with minimally developed watersheds. Therefore, we hypothesized that if the

occurrence of TOs in smallmouth bass was related to EDC exposure, TO prevalence and/or severity would be consistently higher among fish collected from moderately developed sites compared to those from minimally developed sites. Using two development categories (moderate v. minimal) was preferable to relying on a single metric such as population density, because the spatial scale at which human population is most strongly related to EDC presence is unclear. Two simple categories are also more useful than a multi-category ranking system that combines several indicators of EDC presence, because, at present, the relative importance of such indicators is unknown, and because many of these indicators are likely to be intercorrelated.

Collection sites from watersheds with moderate human development were selected from among lakes and rivers with direct input from municipal WWTP effluent. Sites classified as minimally developed were selected from among lakes with lower levels of human activity; specifically, no direct WWTP inputs, no agricultural activities in the surrounding area, low human population density, and relatively little evidence of recreational amenities or use. Other site selection criteria included an abundance of smallmouth bass and direct access to the lakeshore via road. The extent of development in each watershed was evaluated with GIS spatial analysis by quantifying human population density, WWTPs, registered feedlots, and percent of agricultural land (crop land and pasture) within the immediate watersheds (the portions draining directly into the sample sites) and the extended watersheds (i.e. subbasins; areas that drain into the sample site through tributaries). Watershed delineations and land use data were obtained from publicly available datasets. Human population data were derived from the US Census Bureau's 2010 census, and apportioned by watershed area.

Fish were collected from water bodies in three moderately developed watersheds and five minimally developed watersheds (Fig. 1, Table 1). The sites within moderately developed watersheds included Lake Vermilion (near Tower, MN, St. Louis County), Lake Shagawa (near Ely, MN, St. Louis County), and the lower St. Louis River (near Duluth, MN, St. Louis County). At Lake Vermilion, fish were collected from Pike Bay, located near the small town of Tower (St. Louis County, MN), which has a municipal WWTP that employs stabilization ponds serving roughly 500 households, an immediate watershed human population density of 3.9 per km², and 0.11% agricultural land. The extended watershed of Lake Vermilion is adjacent to the St. Louis River watershed but flows north into the Rainy River basin (Fig. 1), and includes several third order streams and a single fourth order stream (Vermilion River). It has a human population density of 4.0 per km², with 0.71% agriculture.

At Lake Shagawa (St. Louis County, MN), fish were collected from points both upstream and downstream of the wastewater treatment plant that serves the town of Ely (1.5 million permitted gallons per day treated with activated sludge, extended aeration, and sand filters [3]). The human population density of the immediate watershed of Lake Shagawa is 80.7 per km² and there is 0.41% agricultural land, compared with 12.3 per km² and 0.18% agriculture in the extended watershed. The extended watershed includes headwaters that enter the west end of Lake Shagawa through a third order stream. The wastewater treatment plant effluent enters the lake on the south shore, and the outflow (Shagawa River) is on the east end of the lake and flows north into the Rainy River basin.

The extended St. Louis River watershed contains 25 minor WWTPs and one major plant, the Western Lake Superior Sanitary District (WLSSD), which has a design flow of 48 million gallons per day, consisting of roughly 50% municipal and 50% industrial waste (mostly pulp and paper mill wastewater [31]), and uses activated sludge, pure oxygen, and sand filters [3]. It has a human population density of 10.9 per km², and receives input from municipalities, agriculture, including 3.21% farmed land and 44 feedlots, and current and legacy mining activities associated with the Mesabi Iron Range, including active and restored pit mines, tailings ponds, and residual lean ore piles. The lower St. Louis River watershed, which includes our sampling site, has a human population density of 32.5 per km², and 6.80% agricultural land, and has been designated by the International Joint Commission as a Great Lakes Areas of Concern because of multiple impairments. The majority of the fish collected from the lower St. Louis River were taken from an area just below the Fond du Lac Dam, located 34 (river) km upstream of the Duluth-Superior Harbor and WLSSD outflow, and 14 km downstream of the city of Cloquet, which conveys its wastewater to WLSSD. Substantial efforts were made to collect fish from a segment of the watershed with more potential influence of WLSSD effluent, but an adequate sample size was not obtained due to poor smallmouth bass habitat.

Sites representing minimal watershed development included Crescent Lake (12 mi N of Tofte, Cook County, MN), Two Island Lake (19.3 km NW of Grand Marais, Cook County, MN), Dumbbell Lake (6.4 km E of Isabella, Lake County, MN), Pike Lake (14.5 km W of Grand Marais, Cook County, MN), and Devilfish Lake (19.3 km NW of Hovland, Cook County, MN), which all have immediate and extended watersheds with very low human population densities (< 1 person per square km) and no WWTP effluent inputs, industrial activities, agricultural land, or feedlots. Two Island, Dumbbell, Pike, and Devilfish Lakes all have immediate watersheds with only first order headwater streams. The Crescent Lake headwaters originate in a small extended watershed that has several second order streams and a single third order stream.

There are no standards for grouping watersheds relative to development, and our criteria separating moderately and minimally developed are somewhat arbitrary. However, our groups are discrete in that they do not overlap ranges of population density, agricultural activity, or the presence of WWTP. As indicated above, the lower St. Louis River watershed contains urban, industrial, and commercial development, and is served by a large WWTP. Lake Shagawa also receives direct inputs from the WWTP serving the town of Ely, and is a popular tourist destination, with many year-round homes and seasonal cabins present on the lakeshore. Lake Vermilion has a lower watershed human population density than the lower St. Louis River and Lake Shagawa, but was characterized as moderately developed because the collection site, Pike Bay, receives direct input from a minor WWTP and the area is heavily-used for recreation. In contrast, the five lakes categorized as minimally developed have limited road access, few residences or other amenities, and little evidence of recreational use.

Fish collection

Adult male fish greater than 200 mm long were collected just before or during the spring spawning season (surface water temperature 15-21° C) using hook and line or electroshocking methods. In June 2012, fish were collected from the lower St. Louis River, Lake Shagawa, Pike Lake, Two Island Lake, and Crescent Lake. In June 2013, additional fish were collected from the lower St. Louis River and Lake Shagawa, as well as from Lake Vermillion, Dumbbell Lake, and Devilfish Lake. Additional fish were collected during early October 2012 and late September 2013 (surface water temperature 15-20° C) from the lower St. Louis River and Lake Shagawa to examine potential seasonal differences in TO occurrence. We aimed for pooled sample sizes of 100 in each development category, which provided a 95% likelihood of collecting 10 fish with TOs in a population with 15% prevalence overall [32]. A minimum of 20 adult male fish were collected from each site during the spring, resulting in a total of 100 fish from moderately developed watersheds and 116 fish from minimally developed watersheds. We collected 24 fish from the St. Louis River and Lake Shagawa in the fall and an additional 80 fish from those sites in the spring (Table 2).

Fish were held in aerated or refreshed water containers before being euthanized with an overdose of neutral buffered MS-222 (3 g/L, Western Chemical, Ferndale, WA). Phenotypic sex was determined by gross morphology of the gonads and confirmed by histological evaluation [14]. Total length (to the nearest mm), total weight (to the nearest g), liver weight (to the nearest 0.01 g), and testis weight (to the nearest 0.01 g) were measured. Sagittal otoliths were collected for age determination. Each testis was cut into three, roughly equal sized, pieces. Representative transverse sections were taken from the center of each piece and color-coded with tissue marking dye (Cancer Diagnostics, Inc., Durham, NC) to identify them as anterior, middle, or posterior regions. Tissue samples were placed in Bouin's fixative (Thermo Fisher Scientific, Waltham, MA) for 72 hours, then stored in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) until histological processing.

Both sagittal otoliths from each fish were prepared for age determination by fracturing them into two rough halves and stabilizing each piece in modeling clay with the cross section facing up. Annuli were counted in cross sections of three of the four pieces using a light microscope with small diameter fiber optic light placed directly on the broken edge of the otolith, which was covered with immersion oil [33]. The age of each fish was determined independently by two different researchers, and if readings differed by more than one year, a reading by a third researcher was performed. Age ($\pm$ one year) was recorded as the reading agreed upon by two out of three researchers, or by the more experienced researcher when only two readings were made.

Histological methods

Testicular tissue was prepared for evaluation using standard histological methods. Briefly, tissues were processed by successive transfers into ethanol and paraffin, and embedded in paraffin blocks. Two, 4 μ m transverse sections from the anterior, middle, and posterior regions of one testis were collected (6 sections per fish). Sections were spaced 200 μ m apart

to avoid capturing the same oocyte in more than one section. Sections were collected onto glass slides, dried, stained with hematoxylin and eosin, and cover-slipped [34].

Histological sections were evaluated by light microscopy (Zeiss Axiovert 35) and digital images were obtained using a Spot scanner (Diagnostic Instruments) with Spot software. All TOs were counted in each of the six transverse sections collected per fish, and the area of each section (cm^2) was estimated with the formula $A = \pi ab$, where a is equal to half of the longest aspect of the section and b is equal to half of the shortest aspect of the section, measured to the nearest 0.1 cm. TOs were counted by scanning with the 10 $\times$ objective lens, increasing to 20 $\times$ if needed to confirm potential TOs. Average TO density (TOs/ cm^2) was estimated for each fish by dividing the total number of TOs by the sum of the estimated areas of the six sections evaluated.

Blazer et al. [9] employed a severity ranking system of 0 to 4, where 1 corresponded to the presence of no more than one TO in any field of view (approximately 4 mm^2). Given an average severity rank of 0.5 for all sections, examination of five sections resulted in a 90% probability of detecting TOs, while severity ranks of > 0.5 required examination of 10 sections to achieve the 90% level.

Data evaluation

All statistical analyses were conducted using R version 3.2.2 [35]. Scatterplots of pairs of biometric variables were visually inspected for differences among collection sites. Two-sided t-tests with Hedges g corrections for clustered samples (package ‘fishmethods’, R-project, [36]) were used to compare the means of age, weight, length, condition factor, gonado-somatic index, and hepato-somatic index between spring-collected fish from moderately and minimally developed watersheds and between fall- and spring-collected fish collected from two moderately developed watersheds [37]. Differences between groups were considered to be significant at a level of $\alpha = 0.05$.

TO prevalence (the percent of males with at least one TO) was evaluated for spring-collected fish from sites characterized as either moderately or minimally developed, for spring-collected fish from each site individually, and for spring- and fall-collected fish from two moderately developed sites (lower St. Louis River, STL and Lake Shagawa, SHG). Differences in overall TO prevalence between pooled groups were tested with the Rao-Scott Cochran-Armitage by Slices (RSCABS) analysis for categorical data [38]. Multiple comparisons of TO prevalence at each individual site were conducted using Tukey’s HSD with Holm-adjusted p-values (package ‘multcomp’, R-project [39]).

Mean TO density, calculated by including fish in which TOs were either present or absent, was evaluated for spring-collected fish from moderately or minimally developed sites, for spring-collected fish from each site individually, and for spring- and fall-collected fish from sites STL and SHG. Differences in overall mean TO density between pooled groups were evaluated with two-sided t-tests with Hedges g corrections for clustered samples [36]. Mann-Whitney-U tests for multiple pairwise comparisons between individual sites were conducted with Holm-adjusted p-values (Wilcox.test, R-project [35]).

In addition to categorical analyses, TO density and probability were evaluated as a function of several characteristics of both landscape and spring-collected fish: watershed development, human population density (both within the immediate and extended watersheds); and fish age, weight, length, condition factor, hepato-somatic index (HSI), and gonado-somatic index (GSI). Pearson's correlation coefficient (r) was calculated for each pair of characteristics to identify variables that were strongly related to each other. Residual plots of linear regressions of TO density vs fish characteristics showed a bimodal pattern such that the samples in which TOs were not detected were grouped separately from the samples in which TOs were detected, suggesting that the data were "zero-inflated" [40], and that simple linear regressions would not be appropriate. Therefore, trends were fitted using two-part multiple regression models with the likelihood function identified by Duan et al. [41], where the probability of TO occurrence was modeled using logistic regression and, simultaneously, the magnitude of TO density (among fish with TOs only) was modeled using linear regression. This method allowed us to explicitly model potential sources of both the presence/absence of TOs and of the range of non-zero TO density in the sample set. TO density among fish with TOs was log transformed for the linear regressions to meet the assumption of normality. Models were developed with a forward selection protocol [42] in which potential predictors of TOs were added in one at a time, and only retained if they resulted in a significant improvement to the null model, as indicated by Akaike Information Criteria (AIC) at least 3.8 greater than that of the null model [43]. All significant models with AICs that differed by less than 3.8 were also considered statistically equivalent. The R squared values for the two-part models were calculated according to Nagelkerke [44]. In models in which more than one variable was identified as a predictor of either probability of TO occurrence or TO density, the relative strength of the relationship between TOs and each covariate was compared by rescaling the values of each variable to equal ranges, and comparing the absolute values of the slopes. Datasets with potential outliers (Cook's distance > 0.5) were re-evaluated excluding the group containing the outlier. If the removal of the outlier group did not change the outcome of the models significantly, the group was not considered to be an outlier, and results of the analysis of the complete dataset were reported.

Two-part mixed effects models (package "lme4", R-project [45]) were used to determine whether the gonadal region (anterior, middle, or posterior) was correlated with the presence or absence of TOs (modeled with logistic regression) or the magnitude of TO density among fish with TOs (modeled with linear regression). To account for within-site and within-fish similarities, individual fish ID and collection site ID were added as random effects. Models that examined both logistic and linear effects, logistic only, linear only, and no effects (the null model) were considered statistically equivalent if the AIC differed by less than 3.8 [43].

RESULTS

Scatterplots of age versus weight indicated slight differences among sites; notably, fish from Lake Shagawa (a moderately developed watershed) were relatively large for their ages, and fish from Dumbbell Lake (a minimally developed watershed) were relatively small for their ages (Fig. 2). However, when biometric averages were compared across all spring-collected fish from moderately and minimally developed watersheds, very few differences were seen

(Table 2). Mean age within sites ranged from 3.9 to 7.3 years, with no significant difference in mean age between fish from moderately and minimally developed watersheds. Mean weight was not significantly different between groups ($p = 0.056$), but mean length of fish from moderately developed sites was higher ($p = 0.038$) than that of fish from minimally developed sites (Table 2). Mean condition index (calculated as $[\text{body weight, g}/\text{total length, cm}^3] \times 100$), hepato-somatic index (HSI), and gonado-somatic index (GSI) were not significantly different ($p > 0.05$) between fish from moderately and minimally developed sites. Comparing data between spring and fall samplings (St. Louis River and Lake Shagawa only), there were no significant differences in any biometric characteristic ($p > 0.05$; Table 2).

Mean TO prevalence among spring-collected fish from moderately developed watersheds was 52% (range = 48-56%, $n = 3$ sites). Male fish from minimally developed watersheds had a significantly lower mean TO prevalence (22%; $p < 0.001$), but site prevalence varied widely (range = 7-43%, $n = 5$ sites). Mean TO density among spring-collected fish from minimally developed watersheds ($0.49/\text{cm}^2$) was also significantly lower ($p = 0.045$) compared with moderately developed watersheds ($3.16/\text{cm}^2$). Among fish collected from two sites in moderately developed watersheds in the fall, mean TO prevalence was 71%, and mean TO density was 8.2 per cm^2 . Fish collected from the same two sites in the spring had significantly lower mean TO prevalence (51%, $p = 0.045$) and density (1.9 per cm^2 ; $p = 0.025$). Site-specific TO prevalence and densities and the results of multiple comparisons among all sites are shown in Figure 3.

Several statistically significant two-part multiple regression models identified the strongest simultaneous correlates of both the probability of TO presence and the magnitude of TO density among spring-collected fish with TOs. Among statistically equivalent models, increased human development in the watershed was consistently one of two parameters that predicted the probability of TO presence (Fig. 4A), with the second being either age (Fig. 4B), or length (Fig. 4C). Both age and length were positively correlated with the probability of TO presence, and the absolute values of the scaled slopes were greater than that of human development (2.73 compared with 1.30 in the age + development models, and 2.10 compared with 0.94 in the length + development models). The magnitude of TO density among fish with TOs was correlated with each of the other variables individually (development, population density, and weight are shown in Fig. 4D-F), although the fit of the data was poor ($R^2 = 0.13$ to 0.15) for all models. A summary of all variables that were evaluated for potential correlation with TO presence and density, as well as a list of all statistically significant and equivalent models identified for spring-collected fish from all sites and for both spring-and fall-collected fish from two moderately developed sites is available in the supporting information (Fig. SI 2a-d).

All TOs observed in this study were primary (pre-vitellogenic) oocytes that were usually embedded in the germinal epithelium of spermatid tubules (Fig. 5A), but occasionally appeared as free-floating cells within the tubule lumen. TOs were typically located near the central region of the testis cross-section, close to a major blood vessel. Less frequently, TOs were found around the perimeter. Most fish with TOs only had a few scattered oocytes in each testis cross section, although in rare cases, several dozen oocytes appeared across more

than one field of view (1.0 mm with 20× objective lens) (Fig. 5B). TOs were almost always in an early stage (chromatin nucleolar), measuring approximately 15-20 µm in diameter, with densely stained cytoplasm and a clearly defined nucleus. However, in two individuals from Lake Vermilion (a moderately developed site), at least one TO was in a late stage of primary growth (perinucleolar stage) with larger diameters (35-100 µm), nuclei containing multiple nucleoli around the periphery, and small vacuoles in the cytoplasm (Fig. 5C).

Histologically, all testes with oocytes appeared to be undergoing normal spermatogenesis with no indication of impaired reproductive function or feminized structure. Germ cell degeneration, fibrosis, or granulomatous inflammation were occasionally observed, but did not appear to coincide with presence of TOs. In tissues that were most extensively affected by TOs, the affected region still had a predominantly male phenotype, rather than appearing as a discrete section of ovarian tissue within the testis (Fig. 5B).

The mean TO density among sections collected from the anterior, middle, and posterior regions of the gonads were 1.7, 1.1, and 3.1 TOs per cm², respectively, with wide ranges (Supporting Information). The mixed effect model results suggested that there were significant effects of section location on TO presence and density. Specifically, sections collected from the middle region of the gonad had 72% lower odds of containing TOs than the anterior or posterior regions ($p = 0.002$), and sections collected from the posterior region of the gonad had 1.8 times higher TO density than the anterior portion ($p < 0.05$). There was no significant difference in TO density between the middle and anterior regions.

DISCUSSION

These data, showing smallmouth bass TO prevalence ranging from 7-59% in Northeast MN, are largely consistent with reports of TO prevalence in bass populations in other parts of North America [10]. Within the range of development density represented in this study, the presence and extent of TOs in mature male smallmouth bass were only weakly related to the intensity of human activities, and by proxy, the inferred likelihood of fish exposure to EDCs. While mean TO prevalence was significantly higher in bass populations from moderately developed watersheds (55%) compared to those from minimally developed watersheds (22%), it was not consistently low in populations from minimally developed watersheds, as might be expected if EDCs or other human impacts were a unique or sole cause. Mean TO density showed a similar pattern - significantly higher in populations from moderately developed watersheds (3.16 per cm², range 2.22 – 4.21 per cm²) compared to those from minimally developed watersheds overall (0.49 per cm²), but not consistently low in this latter set (range 0.07 – 1.22 per cm²). This lack of consistency is illustrated by Figures 3A and 3C, in which the sites are ordered from left to right by decreasing human population density, but the TO prevalence and mean TO density do not decrease monotonically along with the population.

Of the 10 independent variables evaluated for their relationships to TOs, five were highly correlated with each other (e.g. $r = 0.95$ between weight and length; Fig. SI 2b). This may partly explain the large number of parameters identified as being related to TOs in statistically significant models. Despite the relatively poor fit of those models ($R^2 =$

0.13-0.15), the results suggest that parameters that are both likely related and unrelated to EDC exposure may be associated with probability of TO presence and TO density. For instance, while watershed development was associated with the probability of TO presence, the correlations between TO presence and fish age and length were stronger. Similarly, TO density was associated with watershed development and human population density, but also with each of the biometric characteristics evaluated. HSI has been associated with vitellogenin induction by EDC exposure in rainbow trout [46], although there is likely not a causal relationship between vitellogenin and TOs [10, 26]. Weight, length, and age have no obvious relationship with EDC exposure. Overall, our results provide little support for a relationship between TOs and human activities in our study area, and suggest that in smallmouth bass, TOs may develop (at least in part) in response to unknown factors, or develop spontaneously. In contrast, an exposure-response pattern has been demonstrated between WWTP discharges and intersex in roach in the UK and darters in Ontario, suggesting stronger evidence for a causal relationship [5, 8, 47, 48]. However, degree of human influence in those systems is likely greater than in our NE MN study area.

The likelihood of EDC exposure at our study sites could be influenced by unknown EDCs or unaccounted EDC sources, such as atmospheric deposition or leaks from private septic systems, or by biotic or abiotic factors not considered in our analysis. It can also be difficult to compare the relative likelihood of EDC presence among rivers (such as the St. Louis River), large lakes (Lake Vermilion), and small lakes (all other study sites), with widely varying retention rates and watershed sizes. In addition, differences in primary productivity and/or phytoestrogens, population genetics, behavior, and the extent of an individual fish's home range relative to potential chemical inputs may also influence the relationships we identified between TOs and human development and fish biometrics at our sites. For instance, our size and weight data is consistent with the hypothesis that sites in moderately developed watersheds were more productive than those in minimally developed watersheds, which could explain the association of weight with TO occurrence. Consistent with this theory, a study of largemouth bass (*M. salmoides*) from Georgia impoundments with very little influence from agriculture or municipal wastewater found that TO prevalence (as determined by evaluations of longitudinal testis sections) was negatively correlated to water surface area, which corresponded with smaller impoundments being more eutrophic than larger ones [28].

Our observation that TOs were most frequently found in the chromatin nuclear stage, and only rarely in the perinucleolar stage, are consistent with other data from *Micropterus sp.* [9, 11]. Histologically, the predominance of normal testicular tissue in gonads from individuals with even the most extensive TO development does not suggest that TOs were hindering spermatogenesis, but no functional measures of sperm quality were made. Laboratory studies have demonstrated that sperm production or quality is impaired in some species (e.g., roach, zebrafish) with feminized testicular tissue [47, 49], but there are few data on the relationship between intersex and fertility in bass. Blazer et al. [11] demonstrated a significant inverse relationship between sperm motility and TOs in wild smallmouth bass. In that study, fish collected from a site with low likelihood of EDC exposure had a higher percentage of motile sperm (86%) than fish from sites with higher potential exposure

(17-29% motility) as well as lower TO prevalence (11% compared with up to 50-100%). However, there was no difference in sperm viability among sites in that study.

We detected an increased likelihood of TO development in the posterior region of the gonad compared with the middle and anterior regions. Although this observation has unknown biological significance, it indicates that attention should be paid to sampling techniques, and may suggest a need for developing a common set of sampling practices among researchers in this field. Similarly, the slightly increased TO prevalence and density in fall-collected fish is noteworthy, but not consistent with other studies, which have reported either no difference between spring and fall TO prevalence or decreased TO prevalence in fall compared with spring [10, 11]. The method of determining TO density used in the present study was based on the total tissue area, including the germinal epithelia where germ cells develop and the tubule lumens where mature spermatozoa are stored. Seasonal differences in the proportional areas of these two regions may influence TO density; the size of testes in actively spawning fish collected in the spring may be greater, due to the presence of large volumes of sperm, compared with fish collected in the fall. However, in the present dataset, the total cross-sectional area of tissue evaluated from spring-collected fish was not significantly larger than that of fall-collected fish from the lower St. Louis River and Lake Shagawa ($p = 0.11$), nor was the GSI significantly different between seasons ($p = 0.11$). Seasonal differences in TO prevalence and/or density could be important to future investigations into the mechanism by which TOs develop, given that the functional state of the gonad is quite different at the end of the reproductive season compared with the beginning [50]. Many of the testicular tissue samples collected during the fall season appeared to be undergoing recrudescence, in which the germinal epithelium is rebuilt via proliferation and differentiation of germ cell precursors. We speculate that this period of increased differentiation may lead to an enhanced likelihood of TO development, whether they are caused by some exogenous stimulus or develop spontaneously.

An additional outcome of this work is the development of an alternative method for estimating the extent to which fish are affected by TOs. The large size of mature *Micropterus* gonads makes it infeasible to serially section the entire organ; therefore, the presence of TOs must be estimated by some sampling procedure. Most other studies of *Micropterus* TOs have employed a severity grading system, which is a well-established approach to histopathological evaluation [9, 34, 51]. The TO density estimation method developed for the present study is similar to severity grading in that it hypothesizes the existence of a dose-response relationship, where increased exposure to some stimulus results in more extensive TO development. However, there are several key differences in the method used for the present study compared with the most commonly used severity index for *Micropterus* TOs developed by Blazer et al. [9]. The Blazer method assigns a grade from 0 to 4 based on the most extensively affected field of view (approximately 4 mm²), focusing on the central region of the cross-section; this is rapid and simple, but introduces a sampling bias, in that the grade is based on a larger proportion of tissue in small gonads compared with large gonads. In contrast, the TO density method avoids gonad-size bias by normalizing the total number of TOs by the area of the entire section. Additionally, whereas the Blazer method assigns a higher severity grade to sections with clustered TOs than to sections with diffusely distributed TOs, the TO density estimate used here does not presume that clusters

of TOs represent a more severe condition. Severity indices developed for other species (i.e. European flounder, *Platichthys flesus*, and roach [51, 52]), also consider TO distribution, but the relationship between TO distribution and likelihood of adverse reproductive outcome in bass is unclear. One study in which the highest TO severity indexes were assigned to bass testis sections with clustered TOs found a weak, but significant correlation ($R^2 = 0.32$, $p < 0.001$) between TO severity and percent of motile sperm [11], but we are unaware of similar evaluations for TO density. Understanding the biological significance of TO spatial distribution could inform development of the most appropriate metrics for TO presence and severity.

A separate, but related issue is that the severity levels in existing grading systems are ordinal categories without continuous quantitative basis, which means that averaging the grades of many sections from a single individual or the grades of many individuals from a single collection site is not statistically defensible [38]. Although appropriate statistical methods for ordinal categories have been established (e.g. RSCABS [38]), as a continuous variable, TO density data can be more easily analyzed with statistical methods that are inappropriate for categorical indexes.

For comparative purposes, we re-evaluated our field samples using the severity index method used by Blazer et al. [9]. Figure 6A indicates that, while TO severity was higher among fish from moderately developed watersheds overall, as for TO density data (Fig. 3C), there were two minimally developed sites (CRT and TWI) with ranges of TO severity scores that intersected with those of the moderately developed sites. Comparing the two methods directly by plotting severity scores and TO density for individual fish aggregated across sites (Fig. 6B), indicates a broad correlation between the two metrics; in general, fish with higher mean TO density estimates also scored higher in mean TO severity ($R^2 = 0.63$; $p < 0.001$). However, Figure 6B also reveals differences in the sample qualities driving each measure; namely, that the TO severity index method is sensitive to TO position within the central region, and especially in sections with clustered TOs, while the TO density method is insensitive to clustering, but is sensitive to diffusely scattered TOs. For example, fish with TO severity scores of 0.5 had TO density estimates ranging from 1-10 per cm^2 , while fish with TO severity scores of 1.6 had TO density estimates that coincided and extended that range, from 2-20 per cm^2 . This likely occurred because tissue sections with clustered TOs received high TO severity scores (3-4), even if there were very few TOs present in the overall section (low TO density). Conversely, fish with mean TO densities of 7-8 per cm^2 had mean TO severity indexes ranging from 0.5-2.2, which may be partly accounted for by sections with TOs scattered throughout the section (high TO density) that had a low TO severity score because the score was based on a single field of view and TOs were not clustered.

Given the current lack of understanding of the relationship between TO position or distribution and adverse reproductive outcomes in bass, the TO density method provides an alternative for objectively estimating the extent to which fish are affected by TOs without presuming more than necessary, and results in field survey conclusions that appear to be comparable to those reached using the TO severity index method. A major logistical drawback of the TO density estimation method is that it is more time-consuming than

severity indexing. It is also somewhat dependent on the requirement that the TOs are of similar size across all samples, an assumption that was borne out in the current study for all but two fish, in which large, late stage primary oocytes were observed (Fig 5C).

In summary, we have compared TO occurrence in smallmouth bass populations from watersheds that are minimally influenced by WWTPs, agriculture, and urbanization, with populations from moderately developed areas. Based on two hypotheses, that human activities increase the likelihood of EDC presence, and that EDCs increase TO prevalence and/or density, we predicted that fish from moderately developed watersheds would have more TOs. While this was true to a degree, our findings suggest that within our study area, TO prevalence and density in smallmouth bass are not strongly related to WWTP presence or human population density, and notably, that factors unrelated to EDC exposure are also correlated with the presence of TOs. Questions regarding exposure to undocumented or unknown EDCs, as well as the possibility that TOs might be a normal or transient developmental feature in black basses, remain open. Resolving these uncertainties is a critical research issue, given the continuing public interest in the risks of EDCs, the high cost of wastewater treatment facility upgrades, the cultural significance of these species, and their emerging status as a model species for monitoring endocrine disruption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Map of NE Minnesota region where mature male smallmouth bass were collected and evaluated for presence of testicular oocytes

Orange circles indicate collection sites within watersheds that were considered either moderately developed (Lower St. Louis River, Lake Vermilion, and Lake Shagawa) or minimally developed (Dumbbell Lake, Crescent Lake, Pike, Lake, Two Island Lake, and Devilfish Lake). Immediate watersheds (the portion draining directly into the sample sites) are outlined in black; extended watersheds (i.e. subbasins; area that drains into the sample site through tributaries) are outlined in red. The direction of water flow through the watersheds is indicated with blue arrows. A continental divide is indicated by the green line. Locations of major towns are indicated for reference.

Figure 2. Scatterplot of ages and weights of male smallmouth bass collected from eight sites in NE Minnesota

Solid triangles represent fish collected in the spring; open triangles represent fish collected in the fall. The dashed line represents the overall trend among all fish collected; the green line represents fish collected from Lake Shagawa (SHG), which were larger than average for their age; the orange line represents fish from Dumbbell Lake (DMB), which were smaller than average for their age.

Figure 3. Testicular oocyte (TO) prevalence and average TO density among male smallmouth bass collected from eight sites in NE Minnesota located in either moderately or minimally developed watersheds in the spring and in two moderately developed sites in both spring and fall. Percent of mature males with at least one TO observed among 6 transverse sections is shown for fish collected from all sites in the spring (**A**), and fish collected from two sites in both spring and fall (**B**). TO prevalence was significantly higher in spring-collected fish from moderately developed sites (grey bars) compared with minimally developed sites (white bars; $p = 0.002$), and in fall-collected fish from two sites compared with spring-collected fish from the same sites ($p = 0.045$).

Mean TO density per cm^2 of tissue evaluated is shown for fish collected from all sites in the spring (**C**), and fish collected from two sites in both spring and fall (**D**). The boxes represent the 25-75th percentiles; the heavy lines within the boxes are the medians; means are represented by solid dots; dashed lines represent the highest values still within $1.5 \times$ interquartile range; outliers are indicated by open dots. TO density was significantly higher ($p = 0.045$) in spring-collected fish from moderately developed sites (grey boxes) compared with minimally developed sites (white boxes), and in fall-collected fish from two sites compared with spring-collected fish from the same sites ($p = 0.025$).

Individual sites that share a lowercase letter were not significantly different ($p < 0.05$).
Sites are ordered from left to right by decreasing watershed human population density.

Figure 4. Relationships between watershed and biometric characteristics and testicular oocytes (TOs) in smallmouth bass collected in spring

Two-part multiple regression models were used to identify predictors of both the probability of TO presence using logistic regression, and the magnitude of TO density per cm² of testicular tissue in specimens with TOs using linear regression. Among statistically significant models, the probability of TO presence was positively correlated with (A) watershed development and age (covariate not shown), (B) fish length and watershed development (covariate not shown); and (C) age and watershed development (covariate not shown). TO density in fish with TOs was positively correlated with (D) watershed development, and (E) population density in the extended watershed, and negatively

correlated with (**F**) fish weight. A complete list of predictors identified in all statistically significant models appears in the Supporting Information.

Superscripts indicate covariates that influence the y-axes shown in these graphs as follows: ^a age, ^b watershed development

Numbers on bars represent the total number of fish in each category.

In D, boxes represent the 25-75th percentiles; the heavy lines within the boxes are the medians; dashed lines represent the highest values within a 1.5× interquartile range; outliers are indicated by solid dots.

Figure 5. Photomicrographs of testicular tissue of smallmouth bass (*M. dolomieu*) collected from NE Minnesota

Testicular oocytes (TOs; arrows) appeared most commonly as scattered chromatin nucleolar stage oocytes (A) in the germinal epithelium of spermatid tubules with developing sperm (DS) and mature sperm (MS). Rarely, numerous TOs appeared in a more extended region of testicular tissue (B), or as perinucleolar stage oocytes (C). H&E stain.

Figure 6. TO severity index evaluations

(A) Mean TO severity indexes of spring-collected fish at each site. From left to right, sites are ordered from highest to lowest human population density in the watershed (also see Table 1). Boxes represent the 25-75th percentiles; the heavy lines within the boxes are the medians; means are indicated by solid dots; dashed lines represent the highest values within the 1.5× interquartile range; outliers are indicated by open dots. Sites are ordered from left to right by decreasing watershed human population density.

(B) Scatterplot of mean TO severity index vs. mean TO density per cm² in individual fish collected in both spring and fall.

Table 1

Characteristics of the extended and immediate watersheds around sites where smallmouth bass were collected in NE Minnesota.

Study Site (ID)	Surface area, km ²	Watershed Area km ²		WWTP ^a		Population density/km ²		% Agricultural land		Feedlots	
		Full ^b	Immed. ^c	Full	Immed.	Full	Immed.	Full	Immed.	Full	Immed.
St. Louis River (STL)		9250.24	41.71	25	0	10.9	32.5	3.21	6.80	44	0
Lk. Vermilion (VRM)	158.93	1264.75	387.14	1	1	4.0	3.9	0.71	0.11	0	0
Lk. Shagawa (SHG)	9.49	257.74	34.57	1	1	12.3	80.7	0.18	0.41	0	0
Crescent Lk. (CRT)	3.06	32.09	11.20	0	0	0.1	0.0	0.00	0.00	0	0
Two Island Lk. (TWI)	3.05	24.55	24.55	0	0	0.1	0.1	0.00	0.00	0	0
Dumbbell Lk. (DMB)	1.64	6.11	6.11	0	0	0.5	0.5	0.00	0.00	0	0
Pike Lk. (PIK)	3.30	16.01	16.01	0	0	1.0	1.0	0.00	0.00	0	0
Devilfish Lk. (DVL)	1.64	10.78	10.78	0	0	0.1	0.1	0.00	0.00	0	0

^aWastewater treatment plants

^bExtended watershed, i.e. subbasins; area that drains into the sample site through tributaries

^cImmediate watershed; area that drains directly into the sample site

Table 2

Summary of biometrics and testicular oocytes in smallmouth bass collected from NE Minnesota waters considered either moderately or minimally developed. Presented as mean (standard error).

Study Site (ID)	Season	N	Age ²	Weight ^b	Length ^c	Condition Index ^d	GSF ^e	HSI ^f	TO Prev. ^g	Mean TO Density ^h
St. Louis River (STL)	Spring	44	5.9 (0.3)	502 (40)	324 (8)	1.4 (0.0)	0.7 (0.0)	1.0 (0.0)	48%	4.21 (1.26)
	Fall	14	5.1 (0.5)	349 (40)	289 (14)	1.3 (0.1)	0.7 (0.1)	1.0 (0.1)	71%	11.85 (4.57)
Lk. Vermilion (VRM)	Spring	20	7.3 (0.8)	831 (103)	371 (17)	1.4 (0.0)	1.0 (0.1)	1.3 (0.1)	55%	2.28 (1.52)
Lk. Shagawa (SHG)	Spring	36	6.3 (0.3)	892 (69)	366 (11)	1.5 (0.0)	0.9 (0.0)	1.4 (0.1)	56%	2.22 (0.67)
	Fall	10	4.2 (0.6)	677 (155)	332 (25)	1.5 (0.1)	0.8 (0.1)	1.5 (0.2)	70%	3.08 (1.78)
Average, moderately developed:										
All mod. developed	Spring	100	6.3 (0.3)	708 (41)	353 (7)	1.4 (0.0)	0.9 (0.0)	1.2 (0.0)	52%	3.16 (0.69)
STL & SHG only	Spring	80	6.1 (0.2)	677 (43)	348 (7.6)	1.4 (0.0)	0.8 (0.0)	1.2 (0.0)	51%	1.87 (0.40)
STL & SHG only	Fall	24	4.8 (0.4)	486 (76)	307 (13)	1.4 (0.1)	0.7 (0.1)	1.2 (0.1)	71%	8.20 (2.86)
Crescent Lk. (CRT)	Spring	21	6.3 (0.2)	291 (37)	274 (9)	1.3 (0.0)	0.7 (0.0)	1.2 (0.1)	43%	1.03 (0.34)
Two Island Lk. (TWI)	Spring	21	6.9 (0.5)	573 (63)	327 (19)	1.5 (0.0)	0.6 (0.0)	1.2 (0.1)	38%	1.22 (0.60)
Dumbbell Lk. (DMB)	Spring	21	6.5 (0.5)	240 (29)	258 (12)	1.2 (0.0)	1.0 (0.1)	1.4 (0.1)	19%	0.25 (0.18)
Pike Lk. (PIK)	Spring	24	5.6 (0.3)	359 (49)	282 (13)	1.4 (0.0)	1.0 (0.1)	1.2 (0.1)	8%	0.11 (0.09)
Devilfish Lk. (DVL)	Spring	29	3.9 (0.2)	231 (40)	246 (12)	1.2 (0.0)	0.7 (0.0)	1.6 (0.1)	7%	0.07 (0.05)
Average, minimally developed	Spring	116	5.7 (0.2)	332 (23)	276 (6)	1.3 (0.0)	0.8 (0.0)	1.3 (0.0)	22%	0.49 (0.14)

^aTotal number of male fish collected and evaluated

^bDetermined by sagittal otolith annuli

^cWet weight

^dTotal length

^eCondition index (body weight, g/total length, cm³)*100

^fGonado-somatic index (gonad weight, g/body weight, g)*100

^gHepato-somatic index (liver weight, g/body weight, g)*100

^hPercent of males with testicular oocytes

ⁱNumber of TOs per cm² of tissue evaluated