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Assessing the Effects of Historical Exposure to Endocrine-Active Compounds on Reproductive Health and Genetic Diversity in Walleye, a Native Apex Predator, in a Large Riverine System

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Abstract In this combined field and laboratory study, we assessed whether populations of native walleye in the Upper Mississippi River experienced altered genetic diversity correlated with exposure to estrogenic endocrine-active compounds (EACs). We collected fin-clips for genetic analysis from almost 600 walleye (13 sites) and subsampled 377 of these fish (6 sites) for blood and reproductive organs. Finally, we caged male fathead minnows at 5 sampling sites to confirm the presence of estrogenic EACs. Our findings indicate that male walleye in four river segments produced measurable concentrations of plasma vitellogenin (an egg-yolk protein and, when expressed in male fish, a biomarker of acute estrogenic exposure), a finding consistent with the presence of estrogenic EACs and consistent with published historical data for at least three of these study sites (Grand Rapids, St. Paul, and Lake City on Lake Pepin). Patterns of vitellogenin induction were consistent for native walleye and caged fathead minnows. No widespread occurrence of histopathological changes, such as intersex was found compared with published reports of intersex at the furthest downstream study site. To assess possible effects of estrogenic exposure on the genetic diversity of walleye populations at the study sites, we DNA-fingerprinted individual fish using 10 microsatellite loci. Genetic differences were observed between populations; however, these differences were consistent with geographic distance between populations, with the largest observed difference in genetic diversity found between fish upstream and downstream of St. Anthony Falls (and/or Lock and Dam 1 of the Mississippi River), traditionally a historical barrier to upstream fish movement. Although the persistent occurrence of endocrine disruption in wild fish populations is troubling, we did not detect degradation of reproductive organs in individual walleye or alteration in genetic diversity of walleye populations.

Contaminants of emerging concern, including endocrine-active compounds (EACs), have received substantial scientific and public attention in recent years as a result of reconnaissance studies indicating widespread occurrence (Kolpin et al. 2002) and reproductive effects (Hinck et al. 2009) of these compounds on native fish populations across North America. However, many of these compounds have likely entered aquatic environments at least since the chemical revolution of the 1940s (for example, bisphenol-A) if not the industrial revolution of the late 18th century or even earlier with the advent of urban settlements (for example, 17-β-estradiol from human and livestock excretions). Yet, despite reports of widespread changes to reproductive organs in male fish (for example, the presence of female ovarian tissues in the testis of male fish; Jobling et al. 1998; Vajda et al. 2008; Hinck et al. 2009) in anthropogenically impacted aquatic environments, long-

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term population level effects have been difficult to establish (Mills and Chichester 2005; Zhou et al. 2010).

The ultimate population question is whether EACs are affecting reproductive fitness and thus population sustainability (Mills and Chichester 2005). Changes in fish population abundance are often difficult to detect, especially in large rivers, but losses of genetic diversity may provide a detectable signal of population bottlenecks as well as reinforce population declines through the detrimental effects of inbreeding and loss of evolutionary potential (Gilpin and Soulé 1986; Theodorakis 2003). Genetic diversity is lost at a rate inversely related to the effective population size (N_e), which is determined by abundance as well as reproductive characteristics of a population (sex ratios and variance in family size) (Hedrick 2005). Thus, EACs may lead to genetic bottlenecks directly if they decrease reproductive output or cryptically if they decrease reproductive fitness of exposed or sensitive male fish and lead to skewed sex ratios or increased variance in reproductive success (Mills and Chichester 2005; Vajda et al. 2008). Decreased genetic diversity associated with pollutants has been shown for fish (Bourret et al. 2008; Silbiger et al. 2001) and other aquatic organisms (Krane et al. 1999; Ma et al. 2000).

Recently, a multiyear controlled exposure of an entire lake to the potent estrogenic EAC ethynylestradiol (Kidd et al. 2007) resulted in the collapse of the fathead minnow population and a decline in pearl dace and lake trout (Palace et al. 2009). Exposure of all three species resulted in greater plasma vitellogenin concentrations, but effects on reproductive organs were only observed in fathead minnows and pearl dace. Impairment of reproductive organs in male trout were observed in fish collected from lakes in Western United States National Parks (Schwindt et al. 2009), although the source of EACs was less clear and hypothesized to be the result of aerial transport. However, hydrological conditions, contaminant residence times, and trophic interactions in lakes are quite different from those observed in river systems, and the applicability of the results from lake studies to river environments is not clear. Despite these differences, observed effects of exposure to EACs on the reproductive anatomy of male fish (intersex condition in male fish) matched histopathological findings in bass collected in nine river basins in North America between 1995 and 2004 (Hinck et al. 2009). Examining the reproductive and genetic effects of historical exposure to EACs in riverine systems is challenging and inherently imperfect due to the ephemeral nature of the system and its longitudinal continuity. The lack of knowledge of contaminant concentrations and fluctuations in the past, as well as the logistical complexity of collecting sufficient numbers of fish across long segments of rivers in a temporally constricted design (to minimize the effects of seasonally changing reproductive conditions), further complicates analyses (Blazer et al. 2010). However, the headwaters of the Mississippi River in Minnesota may offer an opportunity to examine reproductive and genetic consequences of historical contaminant exposure in a large riverine system. The river's first approximately 1,000 km are located in Minnesota, with more than half of this course being located in a sparsely populated, heavily forested part of the state, with few well-defined sources of municipal and industrial effluents. The industrial effluents in this river, which are mostly from pulp-and-paper mill effluents, known to release EACs (Dubé and MacLatchy 2000; Vajda et al. 2011), lack the heavy manufacturing sources and agricultural practices that could contribute contaminants that may confound the effects of EACs. In addition, the few cities on the Upper Mississippi River have been sewered for many years providing a clearly defined pathway for anthropogenic EACs into defined segments of the river. A recent study by Hinck et al. (2009) documented high incident rates of intersex in an apex predator (bass) in portions of this river stretch, suggesting the presence of EACs at sufficient concentrations to cause reproductive impairment. The finding of reproductive impairment consistent with the exposure to EACs is further supported by a plethora of field studies conducted on water and fish in the Mississippi River in Minnesota during the past 15 years that documented the presence (Barber et al. 2000, 2007; Lee et al. 2008) and physiological effects (induction of the egg-yolk precursor protein vitellogenin in male fish) of EACs (Barber et al. 2007; Folmar et al. 1996, 2001; Lee et al. 2008; Schoenfuss et al. 2002). The apex predator walleye (*Sander vitreus*), a fish with a narrow reproductive window each spring, is found throughout the length of the river and is the subject of intense angling pressure during a weekend-long "walleye-opener" when >10% of the state's human population is pursuing this fish with rod and reel. This provides a unique opportunity to gain a large sample size in a small temporal window during the reproductive season of the fish.

In this study, we tested the hypothesis that fish in river segments contaminated by EACs have altered reproductive anatomy and decreased genetic diversity compared with fish populations in river segments less likely to contain these compounds. Walleye blood samples and reproductive tissues were collected in 48-h windows during three successive spring walleye-opener angling events and assessed for physiological and reproductive abnormalities consistent with exposure to EACs. Genetic diversity was measured using these tissue samples and fin-clips from additional catch-and-release collections as a source of DNA. The multiple sites and approaches were used to provide components of the weight-of-evidence approach to establishing causality between contaminants and genetic diversity as

described by Adams (2003) and Theodorakis (2003). This unique collection allowed for the assessment of the effects of historic exposure to EACs in an aquatic apex predator.

Materials and Methods

We collected walleye from eight river segments that were classified as contaminated with EACs and five segments lacking an obvious source of such contamination ("reference sites"). Sites were classified as contaminated based either on a priori information from previous studies (Lee et al. 2000, 2008, 2010) or results from the current analysis (i.e., vitellogenin induction in male fish). We assessed plasma vitellogenin concentrations, gonad histology, and genetic diversity of walleye. In addition, we measured plasma vitellogenin concentrations in caged fathead minnows at three sites.

Collection Sites

We collected fish tissue samples (fin-clips) from 13 sites on the Upper Mississippi River in Minnesota (Fig. 1, Table 1). All sites are separated from each other by dams (hydroelectric or for river traffic) that likely restrict movement of fish between study sites (complete upstream barriers above St. Anthony Falls, but locks are present here and downstream) (Fig. 1). At 6 of these sites, we were able to collect fish for analysis of plasma vitellogenin concentrations and reproductive organ histopathology (walleye at the other sites had to be released after a fin-clip was taken). Based on the presence of human populations upstream and of nearby pulp-and-paper mill effluents with considerable contribution to river flow (>5%), seven sites were considered a priori likely to have experienced chronic exposure to EACs. These sites include Bemidji (treated wastewater effluent since 1985 = 4.5 million L/day); Grand Rapids

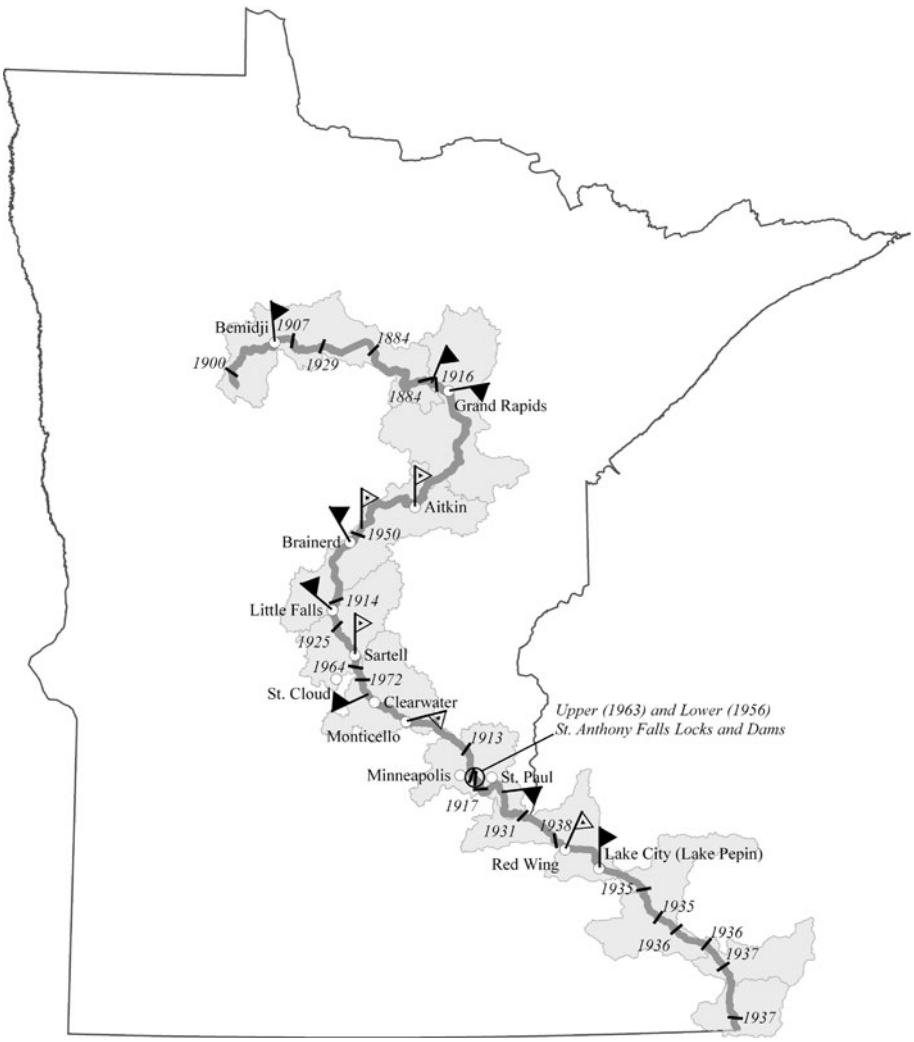

Fig. 1 Map of the Upper Mississippi River in Minnesota with sites from which walleye were collected. Dams are indicated by *black bars* accompanied by the year of dam construction. *Circles* indicate treated wastewater effluent discharge >4 million L/day. *Black flags* indicate sites a priori or after confirmation of vitellogenin induction proposed to be exposed to EACs. *White flags* indicate reference sites

(treated wastewater effluent since 1975 = 5.6 million L/day and 22 million L/day pulp-and-paper mill effluent); Brainerd ([below dam] treated wastewater effluent since 1981 = 12 million L/day); Little Falls (treated wastewater effluent 1959 = 9 million L/day [soy-processing facility]); St. Cloud/Clearwater (treated wastewater effluent since 1956 = 49 million L/day); St. Paul (treated wastewater effluent since 1938 = 1 billion L/day); and Lake City on Lake Pepin (treated wastewater effluent since 1934 = 5.7 million L/day). In addition to the long-term presence of point-source effluent producers at these sites, they have also been identified in previous studies to exhibit chemical and biological indications of the presence of EACs (Lee et al. 2008). An eighth site (Upstream Grand Rapids) was a potential reference site later classified as an EAC site based on our data for vitellogenin induction in male fish. Five other sites (Fig. 1, Table 1) were considered reference sites based on previous studies (Lee et al. 2008) or because they lacked obvious sources of EACs. However because all study sites are segments of the Mississippi River, we recognize that even at these reference sites, fish will likely receive some exposure to EACs, albeit at much lower concentrations. Furthermore, classification of sites as likely to be exposed to EACs did not affected analysis parameters and was only used to determine whether patterns of genetic diversity correlated with the occurrence of endocrine disruption in fish.

Fish Collection and On-Site Processing

All walleye tissues for analysis of plasma vitellogenin concentrations and histology were collected on the walleye-opener weekend (second weekend in May) in three successive years (2007–2009) from six of these field sites (Table 1) using fish caught by anglers with rod-and-reel. Some collection sites did not yield fish in some years due to inclement weather or river conditions (in 2008, ice covered part of the river during the walleye-opener, and flooding made some river segments inaccessible in 2009). Walleye tissues for genetic analysis only were collected from additional river segments throughout these years by various means, including electroshocking and trap nets by the Minnesota Department of Natural Resources. These collections were designated catch-and-release operations and yielded only scales or a small fin-clip for genetic analysis before fish were released back into the river. One site (St. Cloud/Clearwater) had few individuals and several produced poor genotypes, so this sample was removed from genetic analysis.

Fish caught by anglers were usually killed and processed quickly (<60 min). Three to 5 mL of blood was drawn from the caudal vasculature and transferred into a heparinized hematocrit tube, which was stored on wet ice. The fish was then killed, and measurements were recorded for the fish's weight, total length, and standard length. Body condition factors were calculated as [(total weight/standard length^3) × 1000000]. Body condition factor is a useful measure in examining whether fish are of similar nutritional condition across field sites.

In male walleye, both testes were removed, and a tissue sample was collected and placed into a histological cassette. If gravid ovaries were present in the abdominal cavity, the sex was noted on the data sheets as female, but no attempt was made to weigh or collect these tissues for later histological analysis. The rationale for the exclusion of female reproductive tissue was that a gravid female ovary was too fragile to be removed intact under field collection conditions. All histological cassettes were then placed into a site-specific container with 10% formalin. Fin-clips were collected from the dorsal or caudal fin of each fish, placed into 95% ethanol, and refrigerated until laboratory processing. During collection, an effort was made to return collected fish samples (blood and testis) to the laboratory within 15 h, but not <36 h, from collection time. All specimens were maintained on ice in the field until they could be processed according to analysis needs in the laboratory. It is noteworthy that the catch restrictions and/or angler preference during the walleye-opener yielded fish of comparable size (440 ± 94 mm [mean ± SD]) and during a short window of time (<36 h), thus eliminating some of the natural variability in reproductive activity for a species that spawns only during a short period of time each spring. Most male fish captured were releasing milt at the time of capture, and many female walleye were passively releasing eggs during processing.

Walleye Plasma Vitellogenin Analysis

Vitellogenin concentrations were determined in fish plasma using enzyme-linked immunosorbent assay (ELISA) techniques (Denslow et al. 1999). Whole-blood samples were centrifuged in heparinized hematocrit tubes (Phoenix Research Products, Hayward, CA) for 5 min at 5,800×g at 4°C. Triplicate aliquots from each sample were stored in two separate −80°C freezers before analyses. An ELISA antibody for striped bass was used to analyze vitellogenin concentrations in walleye plasma (Biosense Laboratories, Bergen, Norway). Standard curves were calculated based on five to seven dilution points (after removing the highest and lowest dilution points). Each ELISA plate included two blanks and a series of purified vitellogenin standards at 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μg/mL. The r^2 values for the standard curves generated by four-parameter logistic regression were >0.95.

Table 1 Summary of samples collected in this study for assessment of endocrine disruption and genetic diversity from 2007 to 2009

Sampling location	River km (mile[a])	2007		2008			2009		Sum	
		Morphology and physiology	Genetics[b]	Morphology and physiology	*Caged Minnows*	Genetics[b]	Morphology and physiology	Genetics[b]	Morphology and physiology	Genetics[b]
Lake Bemidji	2063 (1282)		30[c]							**30**
Upstream of Grand Rapids	1934 (1202)	79	81	25		20	30	24	**134**	**125**
Grand Rapids	1903 (1183)	40	39	26		59	37	33	**144**	**131**
Aitkin	1706 (1060)		25							**25**
Brainerd (above dam)	1622 (1,008)		28							**28**
Brainerd (below dam)	1617 (1005)		34							**34**
Little Falls	1553 (965)		19							**19**
Sartell	1495 (929)	27	30		*7*				**27**	**30**
St. Cloud/ Clearwater	1485 (923)	9			*20*				**9**	**0**
Monticello	1445 (898)					18				**18**
St. Paul	1345 (836)	22	47		*19*				**22**	**47**
Red Wing	1275 (792)							47		**47**
Lake City	1241 (771)	15	15	26		32			**41**	**47**

Site names in bold print indicate their designation as sites with likely fish exposure to EAC as suggested by previous studies (i.e., Lee et al. 2008, 2010) or increased vitellogenin concentrations in male fish according to the current study. Sites names in italics were considered reference sites based on previous studies or lack of obvious sources of EACs

[a] River km/mile as measured above Cairo, IL. Although not an SI unit, river miles are included in this table because they are readily found on most maps of the Mississippi River (compared with km readings)

[b] Genetic sample size differs from morphology/physiology sample due to extra fin clips added to the study from other sources and occasional DNA amplification failure

[c] Sampled in 2006 by MN DNR

Walleye Histological Analysis

After at least a 1-week fixation period, testis tissue samples were processed for histological analysis. Histological cassettes were processed in a Jung TP1050 automated tissue processor (Leica, Wetzlar, Germany) according to an established histological protocol of dehydration and embedding in paraffin wax (Gabe 1976; Carson 1996). Once embedded, histological sections (two sections per histological cassette, sectioned at 4-μm thickness) were produced and stained with haematoxylin-and-eosin stains (two sections). The slides were examined for reproductive condition (immature, gravid, spawned out) and occurrence of intersex or other histopathological findings (i.e., parasitic cysts) (Vajda et al. 2008). Fish were designated as intersex if microscopic evaluation determined the simultaneous occurrence of oocytes in testicular tissue.

Genotyping

Genotypes were determined for 10 walleye microsatellite DNA loci (*Svi4, Svi18, and Svi33,* Borer et al. 1999; *SviL2 and SviL6,* Wirth et al. 1999; *Svi2, Svi6, Svi16, Svi20, and Svi26*, Eldridge et al. 2002). DNA was extracted from tissue samples using a chelating resin (Chelex, Sigma Chemical, St. Louis, MO), and subsequent polymerase chain reaction (PCR) amplifications were performed using standard procedures as described in Logsdon et al. (2009). Each run included a water blank as a negative control to detect possible contamination of PCR solutions. Products of PCR amplifications were submitted to a genetics core facility (Biomedical Genomic Center, University of Minnesota, St. Paul, MN) for electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Allele scores were determined relative to an internal size standard in each lane using Genemapper v.4.1 (Applied Biosystems).

Statistical Analysis of Genetic Diversity Data

We first estimated the measure of genetic differentiation F_{st} (the analog theta from Weir and Cockerham 1984) between temporal replicates at sites using FSTAT (Goudet 1995) and 1000 permutations to test significance. Replicates within sites were combined because little or no differentiation was detected (F_{st} values were not significantly different than zero, except for one low value between the 2007 and 2009 upstream Grand Rapids samples [$F_{st} = 0.008$, $p = 0.02$]). Observed and expected heterozygosities were then calculated, and conformance with Hardy-Weinberg expectations was confirmed for each locus in each sample using exact test procedures of Guo and Thompson (1992), as implemented by GENEPOP v4 (Raymond and Rousset 1995). Allelic richness was estimated for each sample by the software HP-Rare (Kalinowski 2005), using rarefaction techniques to standardize to an equal sample size of 30 genes.

We assessed genetic structure, or patterns in the distribution of genetic variation among sites, using several approaches. First, we estimated F_{st} between all population pairs. A neighbor-joining tree depicting genetic relationships among populations based on the pairwise F_{st} distances was constructed in TreeFit (Kalinowski 2009) and visualized using TreeView. Bayesian clustering algorithms in the program STRUCTURE (version 2.2.3; Pritchard et al. 2000, 2007; also refer to http://pritch.bsd.uchicago.edu) were used to estimate the number of genetically distinct populations contributing to our samples. Three replicates were run to test the likelihood of there being 1 to 5 distinct genetic clusters in the Mississippi River data. The burn-in period was 50,000 Markov chain Monte Carlo (MCMC) replications, which was followed by an additional 150,000 replications run under a model that assumed possible admixture and correlated allele frequencies. The FSTAT and STRUCTURE approaches to assessing genetic structure among populations do not work well when sampling occurs along a gradient of genetic differentiation rather than among distinct isolated populations. To test for this phenomenon, called isolation-by-distance, we conducted Mantel tests to determine if genetic distances between pairs of populations ($F_{st}/[1 - F_{st}]$) were correlated with geographic distances (river kilometers).

Statistical Analysis of Genetic Diversity and EACs

We assessed the relationships of genetic diversity and structure with EACs in several ways. We first used analysis of molecular variance (AMOVA) in the software Arlequin (Excoffier et al. 2005) to partition genetic variation into differences among individuals within population, differences among populations, and differences among groups of populations. Populations were grouped as reference or sites of EAC exposure to test for a relationship with EACs and as above or below St. Anthony Falls to test for a relationship with geography. Statistical significance was tested with 16,000 permutations of the data. A second test directly compared measures of genetic diversity between sites with EAC presence and references sites. Differences in the genetic diversity measures expected, heterozygosity and allelic richness, were compared between pairs of populations using Wilcoxon signed-rank tests and one-sided *p*-values to test the hypothesis that fish from sites with exposure to EACs had lower diversity those from reference sites. All samples were also tested for evidence of bottlenecks using Wilcoxon sign-rank test and two-phased model of mutation in the software Bottlenecks (Cornuet and Luikart 1996). According to our hypothesis, the sites with

EAC exposure might be expected to show evidence for bottlenecks, whereas the reference sites should not.

Fathead Minnow Caging and Analysis

In addition to the collection of walleye tissues, we also caged male fathead minnows at five sites in July 2008 (above Grand Rapids, Grand Rapids, Sartell, St. Cloud/Clearwater, St. Paul) for 21 days to assess whether fish exposed to water at these sites will exhibit physiological alterations consistent with exposure to estrogenic EACs. These sites were selected because they were logistically feasible (access, river conditions) and because they presented a mix of sites a priori considered as likely historically exposed to EACs or to be reference sites (see Table 1). Six-month-old mature male fathead minnows were obtained from a laboratory fish supplier (Environmental Consulting and Testing, Superior, WI), and acclimated to river water temperatures in the laboratory (48–72 h). After acclimation, the fish were transferred in aerated containers to their respective field sites for 21-day deployments. At each site, a wire-mesh cage (10 cm × 10 cm × 24 cm) containing 20 male fathead minnows was secured at a depth of approximately 50 cm against the streambed to allow fish to forage from the substrate below the cage. At the end of the deployment, fish were retrieved and returned to the laboratory in aerated coolers. Fish were killed and sampled for blood and reproductive tissues within 15 h of retrieval from the field sites as described for walleye. Briefly, plasma vitellogenin levels were measured by way of a competitive antibody-capture ELISA (Hyndman et al. 2010) using a polyclonal anti–fathead minnow vitellogenin antibody (provided by Gerald LeBlanc, North Carolina State University). The standard curve was prepared as a 7-step twofold serial dilution with a range of 4.8–0.075 µL/mL. Each ELISA plate included two blanks and a series of purified vitellogenin standards at 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/mL. The r^2 values for the standard curves generated by 4-parameter logistic regression were >0.95. The lowest standard was periodically removed from the curve to maintain linearity. The samples were diluted 1:75, 1:825, and 1:7700 in 0.075 M phosphate-buffered saline assay buffer, giving an assay quantitation range of 5.6 µg/mL to 37 mg/mL. All experimental procedures were approved by the St. Cloud State University Institutional Animal Care and Use Committee.

Results and Discussion

In this study we tested the hypothesis that native walleye populations confined to Mississippi river segments by dams would exhibit differing degrees of genetic diversity as result of differing exposure history to EACs. As expected, plasma vitellogenin concentrations from walleye collected from these sites were four to six time greater than in walleye collected from sites classified a priori as reference sites, suggesting acute exposure of walleye to estrogenic EACs at sites assumed to contain historical presence of EACs, although our experimental design did not allow for the identification of the specific EAC(s) responsible. Furthermore, caging of laboratory-reared male fathead minnows provided further evidence for the presence of estrogenic EACs because fathead minnows from the one site considered a priori to contain historical EACs pollution also exhibited vitellogenin concentration 4 to 5 times greater than caged minnows from reference sites.

Physiological and Reproductive Analysis

During three field seasons (2007–2009), we collected 377 adult walleye from 6 field sites in the Upper Mississippi River, including four previously identified sites that likely contained EACs (Grand Rapids, St. Cloud/Clearwater, St. Paul, and Lake Pepin near Lake City) and two putative reference sites (Upstream of Grand Rapids, Sartell) (Table 1). Fish were collected by various means with a sizeable portion being donated by anglers on the Mississippi River on walleye-openers in 2007 through 2009. Although our collections targeted male fish, we were able to assess parameters related to endocrine disruption in 66 female fish (18% of the total catch). Analysis of fathead minnows caged in 2008 was restricted to 3 sites because inclement river conditions near Grand Rapids during the caging period prevented analysis of caged fathead minnows from the two sites near Grand Rapids (upstream/downstream of the Grand Rapids dam; Fig. 1).

Our analysis of the collected walleye showed that fish in eight sections of the Upper Mississippi River in Minnesota are exposed to estrogenic EACs that are persistent enough to result in physiological responses (vitellogenin induction; Fig. 2A, B) but not severe enough to cause histopathological changes (data not shown). The body condition index, which is frequently used as an overall measure of fish health, was similar among sites (Fig. 2C, D). We documented plasma vitellogenin concentrations in male walleye approaching 50% of the value measured in female fish in four of the six river segments from which we collected fish for tissue-level analysis: Upstream of Grand Rapids, Grand Rapids, Pool 2 in St. Paul, MN, and Lake Pepin near Lake City. No induction was seen in Sartell, where plasma vitellogenin concentrations were near the detection limit for wild-caught walleye (Fig. 2) as well as caged male fathead minnows (Fig. 3). Caged male fathead minnows exhibited slightly increased plasma vitellogenin

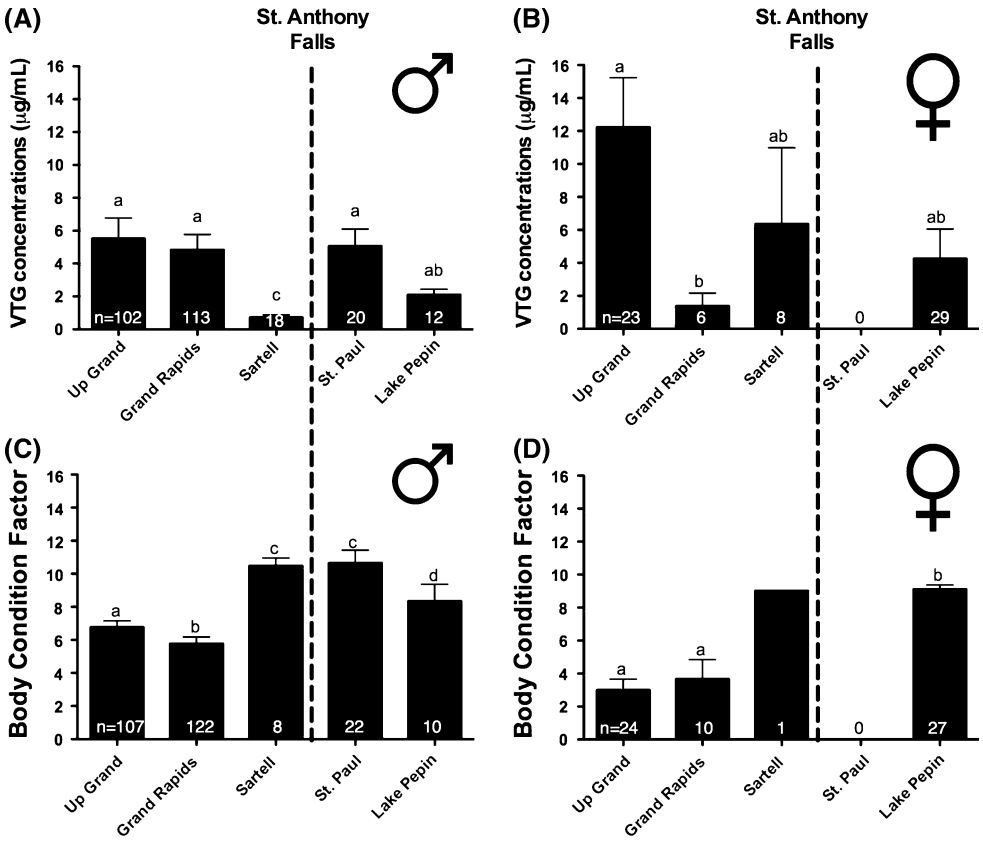

Fig. 2 **A**, **B** Physiological (vitellogenin induction [µg/mL]) and **C**, **D** morphological (body condition factor) characteristics of walleye collected in the Mississippi River during 2007 to 2009. The St. Cloud/Clearwater site is excluded from this figure due to the low sample size. *Letters* above each column indicate significant differences ($p < 0.05$) between treatments

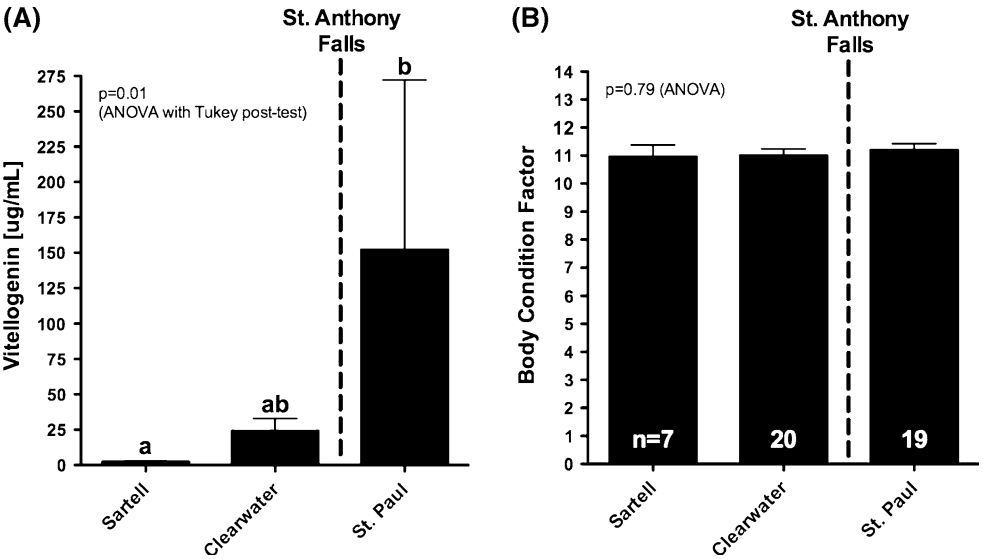

Fig. 3 **A** Physiological (vitellogenin induction [µg/mL]) and **B** morphological (body condition factor) characteristics of male fathead minnows caged for 14 days in the Mississippi River in 2008 near walleye-collection sites. *Letters* above each column indicate significant differences ($p < 0.05$) between treatments

concentrations at St. Cloud/Clearwater compared with fish from Sartell, but the difference was not significant. Vitellogenin induction in male fish was consistent for walleye and caged fathead minnows in St. Paul (Figs. 2, 3). Interestingly, three of the four sites that were found to contain wild male walleye with plasma vitellogenin concentrations approaching 50% of female values have been reported to be estrogenic in past studies (Grand Rapids, St. Paul, and on Lake Pepin near Lake City) (Barber et al. 2007; Folmar et al. 2001; Hinck et al. 2009; Lee et al. 2000). At none of these sites was induction of vitellogenin correlated with histopathological changes to reproductive organs. In fact, only sporadic histopathological changes were observed across all walleye collected in this study (data not shown). The lack of histopathological alterations in field-collected walleye is noteworthy because a recent study found high incident rates (73%) of intersex in male smallmouth bass collected in Lake Pepin (Hinck et al. 2009). The absence of

intersex in most walleye analyzed in our study (intersex frequency <1%) suggests interspecies differences in testis sensitivity to pathological changes because the life span of the fish collected in both studies overlapped (thus suggesting that exposure history would be comparable between walleye and smallmouth bass in Lake Pepin near Lake City, MN). However, Hinck et al. (2009) also sectioned the entire testis of each fish, greatly enhancing the likelihood of finding oocytes embedded in testis tissue. Alternatively, the natural history of smallmouth bass and walleye may differ enough to result in differing lifetime exposure to estrogenic EACs, even within the same river segment, as has been reported for fish species exposed concurrently in a whole lake–exposure study (Palace et al. 2009).

The fourth site to exhibit estrogenic activity as assessed by vitellogenin induction in male walleye was just upstream of Grand Rapids. No clear source of estrogenicity is apparent upstream of this collection site, which was assumed not to be impacted by point sources of EACs. However, other studies have suggested that nonpoint sources, such as septic systems (contributing natural estrogens, pharmaceuticals, and personal care products; Conn et al. 2006), agricultural runoff (contributing natural estrogens, growth hormones, and pharmaceuticals; Nichols et al. 1997; Burnison et al. 2003), and phytoestrogens released from decomposing leave litter (Hermelink et al. 2010) may contribute to estrogenicity. Lee et al. (2000) took sediment samples near the Upstream Grand Rapids site and reported the presence of the plant sterol beta-sitosterol as well as the presence of coprostanol (often used as a biomarker for human fecal presence) and bisphenol-A, suggesting nonpoint source influx of anthropogenic effluent in this segment of the Mississippi River.

Differences in vitellogenin induction and in body condition indices in female walleye across the study sites are likely related to differences in reproductive state of these fish. Because most fish were collected on the same day during each of the three field seasons (walleye-opener), fish in the southern most sites (St. Paul, Lake City) were further advanced in their reproductive process than fish at the northern most sites (Upstream of and in Grand Rapids).

The totality of collected fish data suggest that fish in substantial segments of the Upper Mississippi River are exposed to estrogenic EACs at concentrations high enough to induce biomarkers of acute exposure (increased plasma vitellogenin concentrations) but not high enough to structurally alter testis tissues in this species (histopathology). However, the pattern of exposure was not as linear as hypothesized. Along with several sites with known historical exposure to estrogenic effluents (treated wastewater effluent, pulp-and-paper mill effluent [e.g., Grand Rapids, Lake City]), another site lacking obvious point sources also was found to harbor male walleye with increased plasma vitellogenin concentrations (Upstream Grand Rapids).

Genetic Analysis

The 10 loci showed moderate variation and conformed to Hardy–Weinberg equilibrium expectations, making them suitable for subsequent analyses of genetic diversity. Mean heterozygosity for loci across sample sites ranged from 0.55 for *SviL6* to 0.84 for *Svi26*, and mean allelic richness ranged from 3.5 for *Svi18* to 9.4 for *Svi26* (Table 2). Eleven of 120 locus by sample Hardy–Weinberg tests resulted in $p < 0.05$, but none were significant after sequential Bonferroni correction for multiple testing. Because Bonferroni adjustment can be conservative, we note that individual tests with $p < 0.05$ were spread among 6 samples and 7 different loci (Table 2), providing no indication of possible disequilibrium for any sample or locus.

Several approaches to analyzing genetic population structure indicated that there are genetic differences among walleye along the length of the river but that most of the differentiation is attributed to a split between samples collected above and below St. Anthony Falls in Minneapolis (historically a barrier to upstream fish movement) (currently a dam at Mississippi River mile 846 acts as a barrier). The measure F_{st} was small and sometimes not statistically significant in comparisons among populations above (F_{st} range 0 to 0.021) or below (F_{st} range 0 to 0.008) St. Anthony Falls (Table 3). In contrast, F_{st} was greater and significant for all comparisons but one between above and below St. Anthony Falls populations (F_{st} range 0.012–0.046). The exception was for Monticello and St. Paul, i.e., the samples immediately above and below the barrier, and Monticello had the smallest sample size. It is not surprising that some populations above St. Anthony Falls had slight genetic differences because they came from >500 river kilometers with numerous dams between some of them. The tree diagrams depicting genetic relationships among populations showed a similar picture: There was large branching between populations above and below St. Anthony Falls and slightly separated clusters of upper Mississippi (Grand Rapids and Bemidji) samples and middle Mississippi (Aitkin to Monticello) samples (Fig. 4).

The approach to identifying genetic clusters with the program STRUCTURE, which relied on the genetic data without information on the location of samples, identified only two main groupings: samples collected above and below St. Anthony Falls. The two clusters were not completely distinguished (as indicated by the presence of both colors in each sample in Fig. 5), but populations below St. Anthony Falls averaged 74% to 90% assignment to one cluster, whereas those above averaged 58% to 79% assignment to the second cluster.

Table 2 Measures of genetic diversity, H_O, H_E, and allelic richness (R [standardized to 30 alleles]) for 10 microsatellite DNA loci at 12 sample sites of walleye in the Mississippi River, MN

Locus and statistic	Bemidji	Upstream Grand Rapids	Grand Rapids	Aitkin	Brainerd above dam	Brainerd below dam	Little falls	Sartell	Monticello	St. Paul	Red Wing	Lake Pepin
Svi16												
H_O	0.71	0.76	0.71	0.5	0.83	0.72	0.71	0.79	0.67	0.86	0.93	0.79
H_E	0.72	0.72	0.74	**0.74**	0.77	0.63	0.6	0.8	0.8	0.87	0.86	0.85
R	6.9	7	7	6.7	6.9	5.3	6.6	8.2	6	10.4	10.8	10.4
Svi18												
H_O	0.62	0.66	0.73	0.58	0.57	0.74	0.74	0.63	0.44	0.55	0.68	0.7
H_E	0.63	0.66	0.66	0.65	0.64	0.66	0.69	0.71	0.64	0.65	0.7	0.66
R	3.5	3.5	3.4	3	3	3.8	4	3.9	3	3.8	4.2	3.3
Svi2												
H_O	0.73	0.71	0.67	0.74	0.86	0.56	0.85	0.74	0.89	0.79	0.77	0.8
H_E	0.73	0.7	0.7	0.65	0.74	0.63	0.74	0.67	0.73	**0.81**	0.74	0.74
R	6.4	5.6	6.3	5.6	5.9	5.6	7	6.4	5.8	6.7	6.7	6.4
Svi20												
H_O	0.74	0.77	0.69	0.7	0.79	0.76	0.74	0.76	0.5	0.63	0.77	0.7
H_E	0.72	0.74	0.68	0.8	0.74	0.77	0.76	0.79	**0.82**	0.79	0.87	**0.85**
R	8.3	8.1	7.3	7.6	6.1	7	7.9	7.3	8	9.2	9.9	9.9
Svi26												
H_O	0.82	0.79	0.83	0.78	0.82	0.81	0.84	0.8	0.81	0.87	0.91	0.91
H_E	0.81	0.81	0.82	**0.9**	0.82	0.79	0.85	0.86	0.82	0.86	0.86	0.9
R	8.4	7.8	9.4	11.9	9.7	7.3	9.4	9.3	6	10.5	10.6	11.9
Svi33												
H_O	0.73	0.72	0.82	0.71	0.68	0.68	0.79	0.77	0.89	0.83	0.83	0.81
H_E	0.77	0.74	**0.77**	0.72	0.68	0.72	0.71	0.73	**0.76**	0.81	0.81	0.79
R	5.3	4.7	5.5	6.4	4.8	4.7	4.8	4.9	5	7.3	7.6	7.6
Svi4												
H_O	0.63	0.74	0.71	0.78	0.71	0.62	0.64	0.58	0.81	0.77	0.82	0.68
H_E	0.73	0.73	0.7	0.68	0.71	0.76	0.74	0.72	0.74	0.73	0.72	0.73
R	4.7	4.7	4.7	4.8	4.8	4.8	6	5.7	4	5.2	5.3	5.5
Svi6												
H_O	0.87	0.88	0.82	0.73	0.56	0.82	0.86	0.71	0.6	0.78	0.7	0.55
H_E	0.81	0.78	0.83	0.78	**0.79**	0.82	0.81	0.81	**0.82**	0.78	0.77	**0.67**
R	9	8.5	9.1	8.4	6.2	7	8	6.5	11	9.7	8.9	10.1
SviL2												
H_O	0.54	0.56	0.67	0.71	0.7	0.67	0.74	0.68	0.71	0.7	0.82	0.76
H_E	0.53	0.52	0.64	0.72	0.73	0.71	0.68	0.78	0.65	0.74	0.76	**0.81**
R	5.8	5.4	6	6.2	7.1	6.3	5.7	7	5.8	5.7	5.3	6.4
SviL6												
H_O	0.64	0.59	0.53	0.38	0.39	0.62	0.74	0.53	0.59	0.57	0.59	0.63
H_E	0.58	0.58	0.5	0.49	0.46	0.52	0.56	0.57	0.58	0.63	0.58	0.57
R	4	3.7	3	3.5	2.5	3.4	3.6	3.4	3.9	4.8	5.6	5.3

H_Es in bold text indicate Hardy–Weinberg equilibrium tests with $p < 0.05$, but none were significant after Bonferroni correction for multiple testing

H_O observed heterozygosity

Mantel tests provided strong evidence for patterns of isolation-by-distance in Mississippi River walleye (Fig. 6). For all sample pairs, river distance between them was positively correlated with genetic distance ($p = 0.0012$, $R^2 = 0.42$), but this was driven in part by the greater genetic differentiation between samples from above and

Table 3 Pairwise F_{st} between 12 walleye samples in the Upper Mississippi River

Sample	Bemidji	Grand Rapids up	Grand Rapids	Aitkin	Brainerd above	Brainerd below	Little Falls	Sartell	Monticello	St. Paul	Red Wing
Grand Rapids up	–0.002	–									
Grand Rapids	0.000	**0.005**	–								
Aitkin	**0.012**	**0.014**	**0.010**	–							
Brainerd a	0.007	**0.010**	0.002	0.001	–						
Brainerd b	**0.017**	**0.014**	**0.012**	0.004	0.004	–					
Little Falls	0.005	0.003	0.007	0.001	0.003	–0.002	–				
Sartell	**0.019**	**0.021**	**0.012**	0.001	0.006	0.008	0.002	–			
Monticello	0.004	0.004	0.004	0.010	0.003	0.004	0.009	–0.001	–		
St. Paul	**0.027**	**0.032**	**0.033**	**0.015**	**0.022**	**0.034**	**0.016**	**0.021**	0.005	–	
Red Wing	**0.041**	**0.046**	**0.040**	**0.020**	**0.035**	**0.042**	**0.030**	**0.025**	**0.022**	**0.006**	–
Lake City	**0.035**	**0.038**	**0.035**	**0.013**	**0.030**	**0.037**	**0.025**	**0.025**	**0.012**	**0.008**	0.002

Significant values according to permutation tests in FSTAT (Goudet 1995) are in bold text

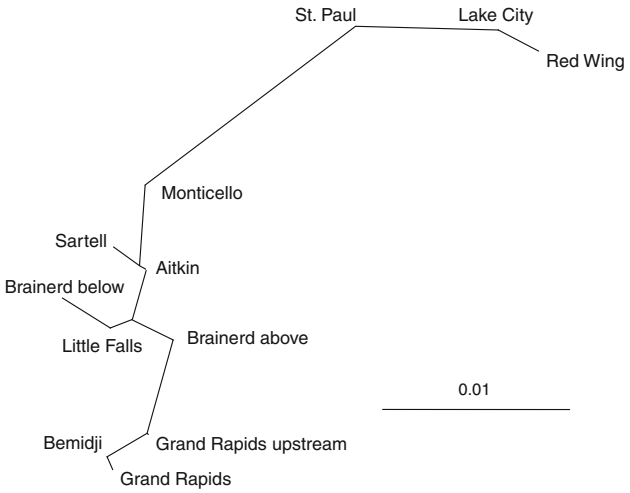

Fig. 4 Tree diagram of genetic relationships among Mississippi River walleye populations based on the genetic distance F_{st}

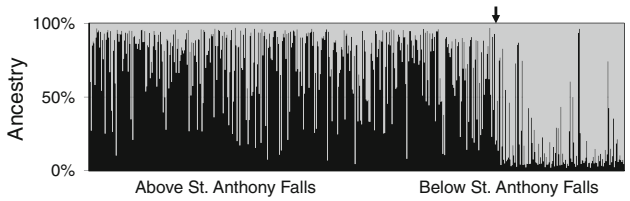

Fig. 5 Assignment of ancestry based on STRUCTURE analysis for 12 samples of Mississippi River walleye. Each *vertical bar* represents a single fish with the proportion of ancestry assigned to each of two genetically distinct clusters indicated by *color*. The *arrow* separates individuals from the three sample sites below St. Anthony Falls, a historical barrier to fish movement, from individuals from nine sites above the barrier

below St. Anthony Falls. When the tests were repeated with only the samples from above St. Anthony Falls, the relationship was still significant but not as strong

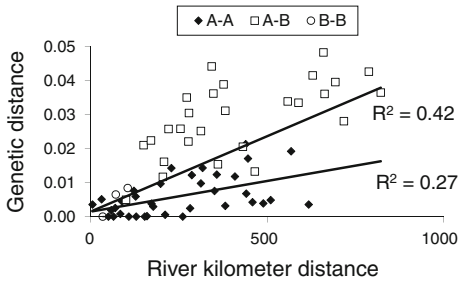

Fig. 6 Genetic distance ($F_{st}/[1 - F_{st}]$) *versus* river kilometer distance between sample sites. The *symbols* indicate comparison within and between sites above (*A*) and below (*B*) St. Anthony Falls, a historical barrier to fish movement. The *top line* is the linear regression for all pairs of sites, whereas the *bottom line* is for comparisons of above St. Anthony Falls sites only (A–A)

($p = 0.012$, $R^2 = 0.27$). Isolation-by-distance patterns may arise in rivers if individuals can disperse but tend to mate with nearby populations. Populations in distant locations can develop genetic differentiation but in fact may have genetic exchange over generations as descendents move up or down the river. Although numerous dams now block upstream movements of fish, walleye still show isolation-by-distance patterns consistent with historical population connectivity throughout the upper Mississippi River above St. Anthony Falls.

We found no detectable relationship between genetic diversity as measured by allele frequencies and estrogenicity as implied by plasma vitellogenin concentration in Mississippi River walleye. AMOVA analyses run with samples grouped by the likely presence of EACs (*vs*. reference sites) showed that this factor explained little of the genetic structure of populations (0.15% of variation; $p > 0.05$), but geography (above or below St. Anthony

Table 4 AMOVA results (percentage of genetic variation explained by the grouping factor) for samples of Mississippi River walleye grouped according to the likelihood of historical exposure to EACs or geography (i.e., above or below St. Anthony Falls in the Twin Cities of Minneapolis, St. Paul, MN)

Samples included	Grouping factor	Variation (%)	Statistically significant ($p < 0.05$)
All sites	Historical EAC site or reference	0.20	No
Above St. Anthony Falls only	Historical EAC site or reference	0.46	No
All sites	Above or below St. Anthony Falls	2.78	Yes

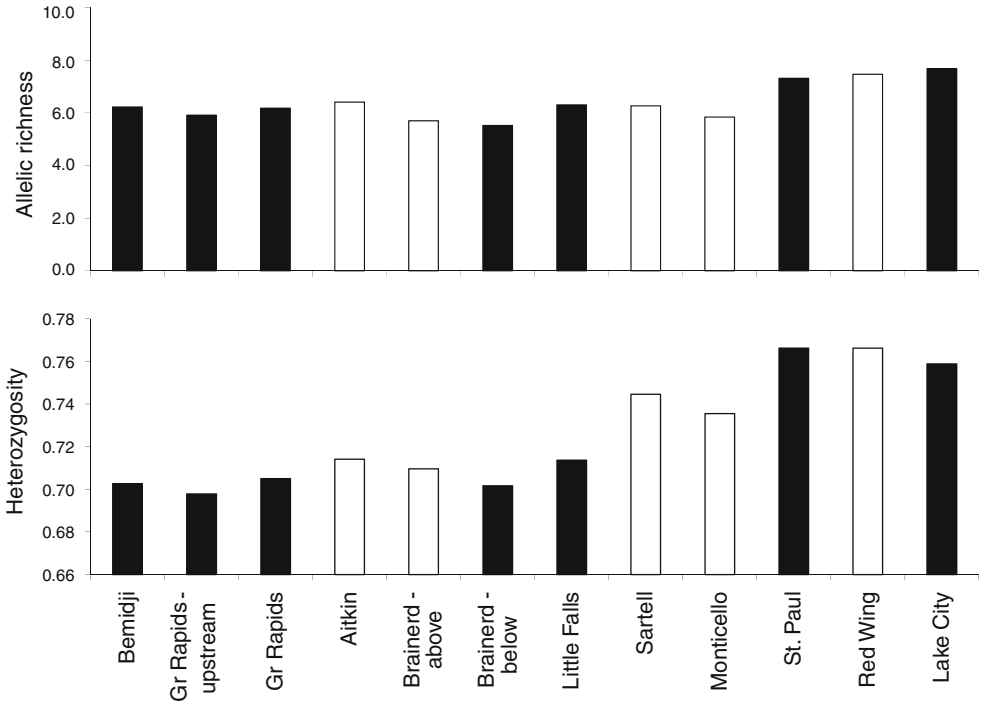

Fig. 7 Measures of genetic diversity, average allelic richness (*upper figure*), and average H_E (*lower figure*) for 10 microsatellite DNA markers in walleye samples from 12 sites in the Upper Mississippi River. Sites are in order going downstream from Bemidji to Lake City. *Black bars* indicate EAC sites, and *white bars* indicate reference sites

Falls) had significant effects (2.78% of variation; $p = 0.004$) (Table 4). Because geography had a strong effect, we repeated the analysis with only populations above St. Anthony Falls, but we still found no patterns of genetic variation that differentiated sites of likely exposure to EACs from references sites (0.65% of variation; $p > 0.05$).

Neither heterozygosity nor allelic richness, a more sensitive indicator of population bottlenecks (Allendorf 1986), showed decreases at EACs sites. Instead, there was a trend of increased diversity from upstream to downstream, especially for heterozygosity, with substantial increases in samples below St. Anthony Falls (Fig. 7). Allelic richness was significantly greater for all samples below compared with all samples above St. Anthony Falls (Wilcoxon signed-rank tests; $p < 0.05$), but it did not differ significantly for any comparisons within groups of samples above or below St. Anthony Falls. For heterozygosity, fish from EACs and reference sites from Aitkin upstream did not differ significantly among themselves but were significantly lower than Sartell and all samples downstream. The increasing diversity going downstream may result from one-way gene flow since the dams were built. The notable increase in diversity in samples below St. Anthony falls may reflect historical separation. Populations have been isolated above St. Anthony for thousands of years, whereas the downstream populations potentially had connectivity to others in the entire lower Mississippi basin. Below St. Anthony Falls, the two sites assumed to contain high estrogenic endocrine activity (St. Paul and Lake Pepin near Lake City) did not differ from the reference site. Tests for bottlenecks were not significant for any EAC or reference site.

The detection of vitellogenin induction at one site chosen as a reference may indicate broader riverwide EAC effects than expected, but the samples from the Mississippi River did not show decreased genetic variation compared with regional populations. We have data from eight loci used in this study for four other populations in the Upper Mississippi drainage above St. Anthony Falls. Expected heterozygosity (H_E) for these populations averaged 0.74 and ranged from 0.71 to 0.76, whereas the Mississippi

River populations also averaged 0.74 and ranged from 0.72 to 0.77 (L. Miller, unpublished data).

Our genetic measures, decreased diversity and bottlenecking, are likely difficult to detect except in situations of severe bottlenecks (Cornuet and Luikart 1996). Bourret et al. (2008) detected decreased diversity in populations of a related percid (*Perca flavescens*) associated with metal contamination as did Maes et al. (2005) for eels (*Anguilla anguilla*), but others failed to detect decreases related to polychlorinated biphenyl loads (Roark et al. 2005; McMillan et al. 2006). We hypothesized that detectable decreases in genetic diversity might result from exposure to EACs because of their possible effects on genetic effective population size directly through abundance and indirectly through altered sex ratios or reproductive success. Our study also reinforces the need to assess biogeographical structure in studies on genetic effects of pollutants as stressed by Whitehead et al. (2003). Similar to their findings, we observed patterns of increasing diversity downstream and isolation-by-distance that would be expected from biogeographical hypotheses. Furthermore, difference in genetic diversity between sites above and below St. Anthony Falls may be attributable to this natural barrier to fish movement rather than anthropogenic factors.

We studied walleye because of their importance to the Minnesota sport fishery and our ability to use anglers to assist in a wide-scale sampling effort, but they are not representative of all fish species in the river. River walleye can be mobile (Gangl et al. 2000), so a portion of a population could spend time distant from major point sources of pollutants. In addition, stocking has occurred in lakes adjoining the Mississippi River (but not in the river itself), particularly in the Grand Rapids area, to meet angler demands. The within-basin source populations are similar to the Mississippi River samples (F_{st} 0.00 to 0.03 between source populations and any Mississippi River sample above St. Anthony Falls; L. Miller, unpublished data, 8 loci), so any influence of stocked fish should not enhance diversity through mixing distinct populations. Stocked fish could be helping to maintain genetic diversity at some study sites if they are contributing to the populations we sampled in the river. Cena et al. (2006) provided evidence for genetic introgression by stocked walleye into native populations in Ontario lakes, but both Stepien et al. (2004) and Wilson et al. (2007) identified native walleye populations little influenced by years of stocking. Other more sedentary and nonstocked species should be extensively sampled by fish biologists to determine if EACs are affecting those populations.

Conclusion

Despite historical exposure to estrogenic EACs, walleye in the Upper Mississippi River did not exhibit detectable organismal or population level changes. However, fish were affected by acute exposure to these compounds at many sites, thus confirming the widespread presence of estrogenic EACs in the Upper Mississippi as suggested by previous studies. Our findings highlight the importance of not assuming reproductive impairment solely on indicators of acute exposure to EACs, although the challenge may be our ability to detect impairment in wild fish populations (Mills and Chichester 2005). Additional species should be studied because some of our populations could have been influenced by stocking, and the mobility of walleye may decrease their exposure to point sources of pollutants. The distribution of sites with estrogenic activity yielded both predictable sites with known point sources as well as surprising locations void of obvious sources of estrogenic EACs. The latter finding deserves further study because identification of nonpoint sources of estrogenic EACs is a crucial step in mediating the effects of these aquatic contaminants.

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