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CHARACTERIZATION OF VITELLOGENIN GENE EXPRESSION IN ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*) USING A QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY

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Abstract—A growing concern over endocrine disruption in aquatic species has prompted the development of molecular assays to monitor environmental impacts. This study describes the development of quantitative polymerase chain reaction (qPCR) assays to characterize the expression of two vitellogenin (Vtg) genes in the invasive round goby (*Neogobius melanostomus*). Fragments from the 18SrRNA (housekeeping gene), Vtg II, and Vtg III genes were cloned and sequenced. The qPCR assays were developed to detect hepatic Vtg expression in goby. The assays detected induction of both Vtg genes in nonreproductive males following a two-week laboratory exposure to 17β-estradiol (≥1 mg/kg i.p. injection). The assays were applied to goby from Hamilton Harbour, Lake Ontario (Canada), including those from sites where feminization and intersex of goby has been documented. Both Vtg genes had significantly higher expression in females compared to males. Male reproductive goby adopt either parental or sneaker tactics; Vtg II expression was higher in sneaker than in parental males but parental and nonreproductive males did not differ from each other. The Vtg III expression was significantly higher in sneaker males followed by parental males and nonreproductive males, respectively. The Vtg II and III expression in nonreproductive males was elevated in the contaminated site with documented intersex. This assay provides an important tool for the use of an invasive species in monitoring endocrine disruption in the Great Lakes region. Environ. Toxicol. Chem. 2010;29:2751–2760. © 2010 SETAC

Keywords—Gobiidae Hamilton Harbour Biomarker Vitellogenin Endocrine disruption

INTRODUCTION

The concentrations and impacts of endocrine-disrupting chemicals (EDCs) from wastewater and industrial effluents on aquatic species have not been adequately characterized in the Great Lakes region of North America. Increases in human populations, pharmaceutical use, and industrial activity in the region have the potential of increasing harmful loads of EDCs into receiving waters. Endocrine disruption has been observed in aquatic environments and has been linked to both emerging contaminants, such as pharmaceuticals [1] and estrogenic compounds [2], and more traditional contaminant sources, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [3–5]. In vitro and in vivo studies have demonstrated that exposure to EDCs can alter gene transcription [6], induce vitellogenesis in males [7], alter plasma steroid levels [8], and cause developmental impacts [9], intersex [10], reduced or arrested fecundity [11], and multigenerational effects [12] in aquatic species. It is predicted that EDCs could have population-level impacts including population crashes in native fish species [13]. Monitoring biologically relevant exposures is critical to assessing the potential risks of EDCs to populations prior to population level impacts.

One of the most effective and widely used biomarkers to measure estrogenic exposure and effects is the inappropriate expression of the egg yolk precursor lipophosphoprotein vitellogenin (Vtg) in males. Vitellogenin is highly expressed in egg-producing, sexually mature females during the reproductive season, although males may express low levels without significant contaminant exposure [14]. This protein is regulated at the transcriptional level; activation of estrogen receptor alpha (ERα) upregulates Vtg genes in liver of oviparous animals [15]. Aryl hydrocarbon receptor (AhR) ligands have been to shown to upregulate [4] and downregulate [3] estrogen-responsive genes including ERα and Vtg via AhR/ER crosstalk pathways. Expression of high levels of Vtg in males is considered a hallmark of exposure to biologically relevant concentrations of natural or synthetic estrogens [16].

Because Vtg expression is under transcriptional control, it can be measured at the mRNA or protein level; most assays measure circulating Vtg protein since plasma Vtg protein levels tend to persist much longer than transcriptional Vtg levels, particularly after exposure has ceased [17]. An attractive advantage of mRNA-based assays is that they have detected maximal Vtg induction days earlier than protein-based assays [14] and significant induction in exposure studies when protein-based assays failed to do so [17]. Temporal data on intermittent exposure events from sources such as combined sewer overflows may be better characterized with mRNA-based assays, although induction may not be seen unless recent exposure has occurred. Antibodies for Vtg protein typically show poor cross-species reactivity and are only available for a select few model species. Antibody-based assays typically require plasma volumes that would be difficult to obtain from individual small fish, including round goby. Thus, protein-based assays are more limited in the study of many native and/or wild-caught fish because these assays are not available for many desirable species. mRNA based assays, such as quantitative polymerase chain reaction (qPCR), can be readily developed for any fish

All Supplemental Data may be found in the online version of this article.

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species of interest once the Vtg gene sequences for that species are known.

The use of the round goby (*Neogobius melanostomus*) for the study of contaminant research in the Great Lakes has several advantages: these fish are invasive, benthic, highly territorial, and widely distributed throughout the Great Lakes [18]. Catching this species in large numbers is relatively simple and sampling many individuals will not be environmentally detrimental, as it might be for native fish. The round goby have high site fidelity and occupy small territories ($5 \pm 1.2\,m^2$) [19], especially parental males that care for their eggs, making them an ideal species for pinpointing point sources of contamination or local environmental differences between sites. Housing this species for laboratory-based studies requires less room than many other fish species due to their small body size. However, maintaining round goby in a laboratory can be challenging in static tanks and they are better held under flow-through conditions. In addition, round goby have several features that are particularly useful in the study of EDCs. First, these fish have multiple alternative reproductive tactics; adult, reproductively mature males are either parental or sneaker male morphs. Parental males are larger in size, darkly colored, build a nest, and care for the brood. Sneaker males are small, lightly colored, invest in larger gonads, and exploit parental male efforts to attract females and care for eggs [20]. Sneaker and parental males primarily use different androgen hormones, providing the opportunity to examine the impact of contaminants on male physiology dominated by either 11-ketotestosterone (parental male) or testosterone (sneaker male) within a single species [20,21]. Second, this species is sensitive to feminization; round goby have been found with altered secondary sexual characteristics (i.e., urogenital papilla shape) and intersex at selected contaminated sites in Hamilton Harbour [22]. Other available species that have the advantage of large databases of historical data, such as brown bullhead, do not appear to be as sensitive to intersex as the round goby. To our knowledge, there are no documented cases of intersex in brown bullhead, even in Cootes Paradise, an area of Hamilton Harbour with very high intersex in white perch [23].

The present study was conducted in Hamilton Harbour, a 2,150-ha body of water, on the westernmost end of Lake Ontario. The Harbour is surrounded on all sides by heavily populated and industrialized land; 46% of the 42 km shoreline is dedicated to heavy industry [24]. The harbor is nearly cut off from Lake Ontario, except for a narrow canal on its eastern shore (Fig. 1) that strongly limits water exchange with the lake. Contaminants released into Hamilton Harbour are likely to remain there since water flow is restricted. Cootes Paradise, a protected wildlife sanctuary at the western tip of Hamilton Harbour, is an important fish nursery for Lake Ontario and has been greatly impacted by dredging, invasive carp, wastewater effluent discharge, and untreated sewage from a combined sewer system. Water quality in Cootes Paradise has been significantly improved by a physical barrier to invasive fish species, improvements to wastewater treatment, and decreased untreated sewage discharge, yet fish therein continue to display elevated levels of PCBs [24] and intersex [23]. Currently, there are four wastewater treatment plants (WWTP) and 23 combined sewer overflows that discharge into Hamilton Harbour and Cootes Paradise. Potential EDCs from WWTP effluents have been found in sediments in Cootes Paradise and appear to persist in sediment for decades [25]. Potential EDCs from industrial sources such as PAHs [26] and PCBs [27] are known to persist in sediment deposits in the harbor. Hamilton Harbour has been identified as an area of concern (AOC) and is presently home to the second-most contaminated PAH deposit in Canada [24].

The present study describes the establishment of a qPCR assay for Vtg gene expression in round goby. The assay was optimized using hepatic mRNA from reproductively active females. The capacity for induction of Vtg in males was determined by laboratory exposures of nonreproductive males to 17β-estradiol. The assay was then used to assess Vtg expression in male and female round goby collected from several sites in Hamilton Harbour. Two Vtg genes were cloned from round goby and gene-specific primers were developed to determine if both genes were equally responsive to estrogens. This assay may be widely applicable for determining the presence and impacts of EDC contamination in the Great Lakes region.

MATERIALS AND METHODS

17β-estradiol exposure design

Sixty nonreproductive male round goby were collected from LaSalle Marina (Burlington, ON, Canada) between July 15 and August 4, 2009 (LS site, Fig. 1). Fish were acclimated to a flow-through system consisting of dechlorinated tap water in 40-L tanks for a period of at least 6 d prior to intraperitoneal (i.p.) injection with 17β-estradiol (E2; Sigma-Aldrich) suspended in

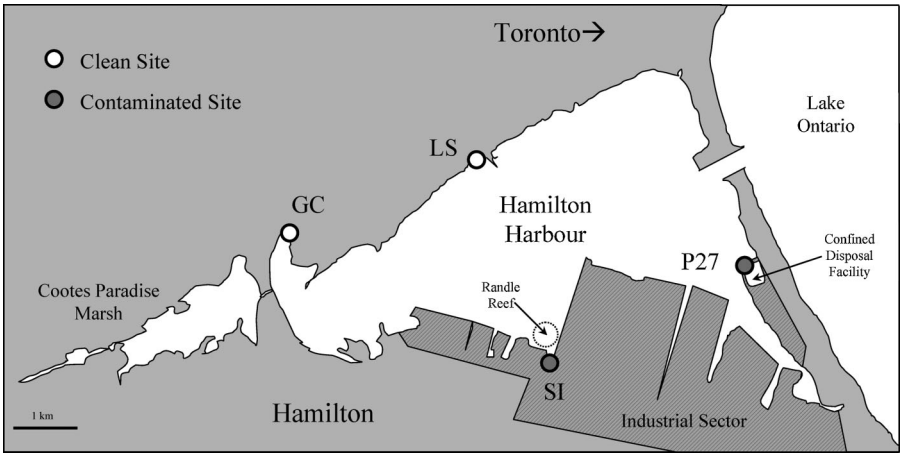

Fig. 1. Field sites for round goby collections in Hamilton Harbour, Canada (adapted from [22]). Cleaner sites are indicated by open circles and contaminated sites are indicated by closed circles. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet.

a coconut oil carrier (Alpha Health Products). Coconut oil is a solid below 25°C, providing a slow E2 release after bolus injection because the water temperature was kept at approximately 17°C. Each of the six tanks held 10 fish; two fish per treatment group were included in each tank to remove possible tank effects ($n = 12$ fish per treatment). Fin clips were used to identify treatment groups. Fish were randomly assigned to treatment groups; the control group received an injection of coconut oil only, the remaining treatment groups were dosed with 1, 5, 10, or 20 mg/kg E2 suspended in coconut oil. Fish were fed once daily with Nutrafin tropical flake food (Hagen). Artificial light was used to maintain a 14:10 h light:dark photoperiod. Two fish died in the 5 mg/kg E2 treatment group, leaving 10 fish in this group; there was no mortality in any other treatment group. After two weeks of exposure, fish were sacrificed and tissues collected (see *Tissue and plasma sampling,* below). The average (±standard deviation [SD]) fish mass and total length was 15.3 (±8.18) g and 10.3 (±1.59) cm, respectively.

Field sampling

Nonreproductive and reproductive (parental and sneaker) male and gravid and nongravid female round goby were collected between April 25 and September 12, 2007. Field sites were located in Hamilton Harbour, on the northwest tip of Lake Ontario. The field sampling sites included two cleaner sites at Grindstone Creek ([GC]; 43° 17′ 21″ N, 79° 53′ 13″ W) and LaSalle Park Marina ([LS]; 43° 18′ 1″ N, 79° 50′ 47″ W), a site presumed to be contaminated at Pier 27 ([P27]; 43° 17′ 3″ N, 79° 47′ 33″ W) and a highly contaminated site at Sherman Inlet ([SI]; 43° 17′ 3″ N, 79° 47′ 33″ W; Fig. 1). The cleaner sites were not near any known sources of contamination and the sediments contain low levels of contaminants [28]. The Sherman Inlet site was adjacent to Randle Reef, a site highly contaminated with PAHs from historical coal tar deposits and a site of intermittent combined sewer overflow discharge [24]. The Pier 27 site was adjacent to a closed disposal facility (CDF) that received contaminated sediments from dredging activity [24]. High levels of PCBs have been documented near Pier 27 [28]; its close proximity to the CDF and two WWTP effluent sources and the presence of feminization in round goby collected at Pier 27 [22] strongly suggested that this was a contaminated site.

Goby were caught in minnow traps baited with kernel corn and left overnight. Goby were processed on-site or brought back to the lab and processed within 2 d, as described below (see *Tissue and plasma sampling*, below). Male and female goby were identified based on external secondary sexual characteristics and visual inspection of the gonads upon dissection. Female and male goby have a broad, U-shaped or triangular urogenital papilla, respectively. Gravid and nongravid females have traditionally been defined as those fish with either a gonadosomatic index (GSI) above or below 8%, respectively [29]. Reproductive males were defined as having a GSI over 1% and a developed papilla [20]. Parental male morphs were clearly identified in the field based on physical characteristics including a developed papilla, black body coloration, a large body, and swollen cheeks. Sneaker males were identified by a small body, developed papilla, and large gonads. Nonreproductive males ranged widely in size but lacked a developed papilla and gonads [20,22]. Eggs and seminal fluid were typically visible upon dissection in animals above the GSI cutoffs.

Tissue and plasma sampling

Goby were placed in an ice bath prior to caudal puncture with a 26.5G needle and a 1-ml syringe to collect blood samples. When this method failed to produce large enough volumes of blood, caudal severance followed by plasma collection with 10 μl coagulation capillary tubes was used. Immediately after blood collection the fish were sacrificed by spinal severance using a scalpel blade or scissors. Blood was stored on ice until it was centrifuged at 14,500 rpm for 10 min at 4°C. Plasma was removed and stored at −80°C until extraction for determining plasma hormone concentrations. Liver samples were removed, flash-frozen in 2-ml cryogenic vials in liquid nitrogen, and stored at −80°C.

Steroid hormone quantification

Plasma samples were extracted by vortexing three times for 30 s in diethyl ether. The ether layer was removed between each vortex, transferred to a new vial, and allowed to evaporate prior to reconstitution in reaction buffer to the desired concentration. The extraction recovery, based on the addition of a known amount of hormone into the samples prior to extraction, was $94.7 \pm 3.3\%$ ($n = 4$), $88.0 \pm 3.5\%$ ($n = 4$), and $90.5 \pm 10.8\%$ ($n = 4$) for E2, T, and 11KT, respectively. Samples were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical). All standards and samples were run in duplicate. Standard curves of 6.6 to 4,000 pg/ml, 3.9 to 500 pg/ml, and 0.78 to 100 pg/ml with an average lower detection level (80% binding limit) of 26.79 ± 8.7 pg/ml ($n = 5$), 7.36 ± 1.9 pg/ml ($n = 4$) and 2.26 ± 0.7 pg/ml ($n = 3$) were used for E2, T, and 11KT, respectively. The intraassay coefficients of variation (calculated from sample duplicates) was $8.10 \pm 5.46\%$ ($n = 52$), $8.11 \pm 5.85\%$ ($n = 73$), and $8.83 \pm 5.54\%$ ($n = 68$) for E2, T, and 11KT, respectively. The interassay coefficient of variation at 50% binding was 18.32% ($n = 5$), 17.80% ($n = 4$), and 15.30% ($n = 3$) for E2, T, and 11KT, respectively. In some cases, low plasma sample volume precluded determination of steroid hormone concentrations.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from liver samples using the TRIzol® Reagent protocol (Invitrogen) using ≤100 mg of liver tissue in 1 ml of reagent. Tissues were homogenized in TRIzol for 10 to 30 s using an OMNI GLH homogenizer (OMNI International) and the extraction followed the manufacturer's protocol. The total RNA was reconstituted in molecular biology-grade water, quantified spectrophotometrically, and absorbance at 260 and 280 nm was used as a measure of relative purity and quantity. Samples showed 260:280 nm ratios of 1.86 or greater. The total RNA samples were analyzed for structural integrity using agarose gel electrophoresis. Samples were diluted to 400 ng/μl with molecular biology-grade water. The cDNA was made using random hexamer primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase according to the manufacturer's protocol (Invitrogen).

Cloning and sequencing of round goby genes

Using pools of liver from reproductively mature gravid females as a total RNA source, Vtg II, Vtg III, and 18SrRNA genes were cloned and sequenced for the development of a qPCR assay. Based on known Vtg sequences in the closely related Japanese common goby and pufferfish, the only other percomorpha species with a complete genome or exhaustive

Table 1. Degenerate and quantitative polymerase chain reaction (qPCR) primer sequences for vitellogenin (Vtg) II, vitellogenin III, and 18SrRNA genes in round goby

Primer		Primer sequence	Tm[a]	Size[b]	Amplicon length[c]
qPCR primers					
Vtg II	F[d]	ACATGCTTGAGCCATCTAGTGATA	50	24	90
	R[e]	GCAAGACTGGTCCATAGTTTTCTT		24	
Vtg III	F	GCAGCTGTGCAGGCCATGAGA	60	21	96
	R	AGCCTCCAGCTCCCGGTTCA		20	
18SrRNA	F	CCTGAATACCGCAGCTAGGA	50	20	124
	R	ACCTCTAGCGGCACAATACG		20	
Degenerate primers					
Vtg II	F	**HHB** GAG TAC A**R**T G**RH** **RTB** TGG CC	50	23	1,886
	R	A**ND** GT**N** GCA GC**R** TC**R** TTG AT		20	
Vtg III	F	T**BW** CCT **WY**G GCT C**Y**C TGG TG	50	20	1,658
	R	**GW**A TCC **V**AG GAA **RTR** GTA CAG		21	
18SrRNA	F	GCG GCG ACG **WY**T C**W**T TCG AAT GTC	54	24	719
	R	CAT **B**GT TTA **R**GG TCG GAA CT		20	

IUPAC degenerate bases are in bold for degenerate primers used in cloning genes from round goby. All sequences are listed 5' to 3'.
[a] Tm = melting temperature.
[b] Size = primer length, in basepairs.
[c] Amplicon length = PCR product length, in basepairs.
[d] F = forward.
[e] R = reverse.

approaches for Vtg gene identification, we expected to find multiple Vtg genes in round goby. Both pufferfish and Japanese common goby contain two Vtgs, type II and III [30], and these genes, along with 18SrRNA, were targeted with gene-specific degenerate primers (Table 1). For each gene a multiple sequence alignment of available fish sequences (Supplemental Data, Table S1) was made using Clustal X ([31]; http://media.wiley.com/CurrentProtocols/0471250937/0471250937-sampleUnit.pdf) and used to add degeneracy to primers (Table 1) designed in Primer3 [32] based on Japanese common goby (Vtg II and III) or previously described for fathead minnow (18SrRNA) [33]. Polymerase chain reaction amplification of target genes was completed with the Platinum taq polymerase kit (Invitrogen) according to the manufacturer's protocol. Amplified products were gel purified using the Wizard® SV gel and PCR clean-up kit, ligated into the pGEM-T easy vector, and transformed into JM109 bacterial cells (Promega). Four colonies per amplicon were grown in LB broth and those colonies with a confirmed insert were plasmid purified using the QIAprep® Spin Miniprep kit (Qiagen) and bidirectionally sequenced using T7 and SP6 primers in the MOBIX lab (McMaster University, Hamilton, ON, Canada). Sequences were assessed for read quality and consensus sequences were used to create primers for qPCR.

Quantitative PCR

All qPCR primers were designed in Primer3 [32], straddled an intron–exon boundary, and had an amplicon size of approximately 100 base pairs (bp) (Table 1). Standard curves for two Vtg genes (Vtg II and Vtg III) and one housekeeping gene (18SrRNA) were made using cDNA from a pool of five reproductively active females using 10-fold serial dilutions (1 μg to 100 pg). Reaction parameters included assay efficiencies between 90 to 105%, r^2 values of 0.98 or better, similar cycle threshold (C_t) values for replicate wells (less than 0.5 C_t difference), and the production of a single peak in the melt-curve analyses to ensure single product synthesis. All qPCR reactions were done on a Stratagene Mx3000™ thermocycler using the Platinum® SYBR® Green qPCR kit with ROX (Invitrogen). The cDNA template, made from 1 μg total RNA, was used in each reaction. The reactions were carried out as follows: 2 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 50°C (60°C for Vtg III), and 30 s at 72°C. The melt curve analyses were done by terminating the reaction at 95°C then stepping up one degree every 30 s from 50°C (60°C for Vtg III) to 95°C. Expression levels of Vtg II and III for individual fish were normalized using 18SrRNA expression levels and fold changes were calculated based on the expression levels in the laboratory exposure control group.

Gonad histology

Gonad samples were collected from 30 reproductively mature common carp (*Cyprinus carpio*), pumpkinseed (*Lepomis gibbosus*), and brown bullhead (*Ameiurus nebulosus*) between August 27 and November 5, 2009 from Cootes Paradise (43° 16′ 46″ N, 79° 53′ 36″ W; Fig. 1). Fish were caught by electrofishing or live trap. The average (±SD) fish mass and total length was 3.20 (±0.69) kg and 56.0 (±4.28) cm for carp, 42.43 (±15.24) g and 11.6 (±1.12) cm for pumpkinseed, and 517.68 (± 92.45) g, and 33.9 (±2.46) cm for bullhead. Entire gonads were taken for pumpkinseed and bullhead. A small piece, approximately 8 cm^2, was removed from the caudal tip of each carp gonad. Gonads were stored in formaldehyde for one week, rinsed in 50% ethanol for 20 min, and stored in 70% ethanol. Tissues were embedded in paraplast (McCormick) and sectioned at 5 μm. Ten serial sections were taken from each tissue and mounted on superfrost slides (Fisher Scientific). Slides were stained with hematoxylin and eosin (Richard-Allen Scientific). Sections were scanned using a Nikon Eclipse TE2000-S light microscope at 100× magnification and images were captured using a Nikon DXM1200F digital camera.

Statistics

Statistical analyses were performed using SigmaPlot® 11.0 (Systat Software). Data were log-transformed and statistical differences were determined using one-way analysis of variances (ANOVAs), except Vtg II and III expression in field-caught goby, which were assessed using a two-way ANOVA on field sites and reproductive classes. Data from sneaker males were omitted from the two-way ANOVA because samples were unavailable for this reproductive class from one field site (GC). To determine whether sneaker male Vtg levels were different

across field sites a one-way ANOVA was used. Following one- or two-way ANOVAs, significant differences were determined using a Holm-Sidak post-hoc test. Data which were non-normal after log-transformation (Vtg III expression from exposure, GSI for males and females) were tested using Kruskal–Wallis analysis of variance on ranks followed by a Dunn's post-hoc test. Sneaker male data were omitted from the statistical test for 11KT due to low sample size. A Spearmen rank order correlation test was performed to evaluate the relationship between plasma E2 and Vtg mRNA levels. The significance level for all statistical tests was $p \leq 0.05$.

RESULTS

Development of a qPCR assay for Vtg expression

Two Vtg genes were specifically targeted based on data from a complete genome (pufferfish) and DNA library screening (Japanese common goby) in the most closely related species with available Vtg sequences. The Japanese common goby [30], a closely related gobiidae, and the pufferfish [7], a fellow percomorpha with a complete genome, were known to have two Vtg genes, one type II and one type III Vtg gene per species. Our cloning strategy targeted Vtg II and III genes using liver from reproductively mature, gravid female goby. Approximately 1,900, 1,650, and 720 basepairs (bp) were amplified from Vtg II (GenBank Accession HM238184), Vtg III (GenBank Accession HM238185), and 18SrRNA (GenBank Accession HM238183) genes from round goby. The 18SrRNA sequence was 89 to 97% identical to other fish sequences; Vtg II and III sequences were 19 to 70% and 57 to 78% similar to other fish Vtg genes, respectively.

The qPCR assays were optimized for all genes; products were tested by melt-curve analysis to confirm the amplification of no more than a single product in each reaction well. Single peaks were generated for every sample with a melting point of 77.3, 83.8, and 82.3°C for Vtg II, Vtg III, and 18SrRNA, respectively. Amplification of all genes was seen in all laboratory control animals and most field samples. Negative controls (no cDNA template) did not produce amplification products, indicating that nontarget amplification or primer dimerization was not an issue for quantification.

Exposure to 17β-estradiol

Nonreproductive males were collected from a cleaner site (LaSalle Marina) and maintained in the laboratory for a two-week, controlled E2 exposure study. Average plasma levels of E2 increased across treatment groups. Control fish had a mean (±SD) of 0.38 (±0.24) ng/ml E2 while E2-exposed fish ranged from 55 to 1,290 (±67 to 1,531) ng/ml in the 1 and 20 mg/kg groups, respectively (data not shown). Mean E2 levels were significantly higher in fish from all treatment groups compared to control fish. Fish E2 levels in the 5, 10, and 20 mg/kg groups were significantly higher than the 1 mg/kg group ($p \leq 0.05$) but did not vary from each other. The average concentrations of plasma E2 in the males (from all treatment groups) were much higher than levels observed in reproductively active female goby; the average plasma E2 concentration in pooled and nonpooled gravid female goby plasma was 7.92 ± 5.62 ng/ml ($n = 9$; data not shown).

Expression of Vtg II and Vtg III (normalized to 18SrRNA) in all treatment groups were significantly higher than controls, but treatment groups were not statistically different from each other ($p \leq 0.05$, data not shown). A linear trend in dose-response was seen when Vtg II and III expression levels were compared to measured plasma E2 levels (Figs. 2, 3). Plasma E2 levels were significantly correlated with Vtg II ($R = 0.574$; Fig. 2) and Vtg III ($R = 0.416$; Fig. 3) mRNA levels. Vitellogenin II and Vtg III mRNA levels were significantly correlated with each other ($R = 0.761$; data not shown). The levels of Vtg II and Vtg III expression in E2-exposed male fish were similar to levels seen in field collected females (Figs. 4, 5). Based on normalized cycle threshold values, Vtg III expression levels were approximately 18 times higher than Vtg II levels overall (data not shown). Vitellogenin II expression appears to be more sensitive to induction by E2; Vtg II expression increased approximately 10 times more than that seen with Vtg III induction (1238% increase vs. 100% increase at the highest dose of E2; Figs. 2, 3).

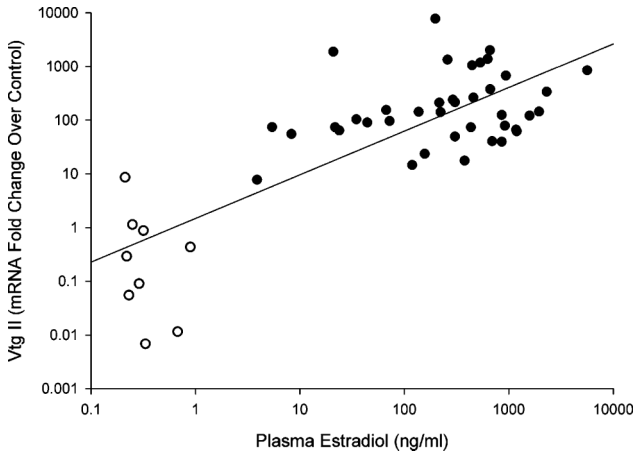

Fig. 2. Plasma 17β-estradiol and hepatic vitellogenin II (Vgt II) mRNA levels in nonreproductive male round goby after two weeks of injection with 17β-estradiol in a coconut oil carrier. Gene expression was normalized for the expression of 18SrRNA housekeeping genes. Data are shown as the fold change relative to control (coconut oil carrier injection only) animals. Open and closed circles represent control and treated fish, respectively. $R = 0.574$; Spearman rank order correlation test.

Vtg expression in field-caught fish

Females were identified as gravid or nongravid based on clear differences in GSI ($p \leq 0.05$), yet gravid and nongravid females did not differ significantly in body mass or total length

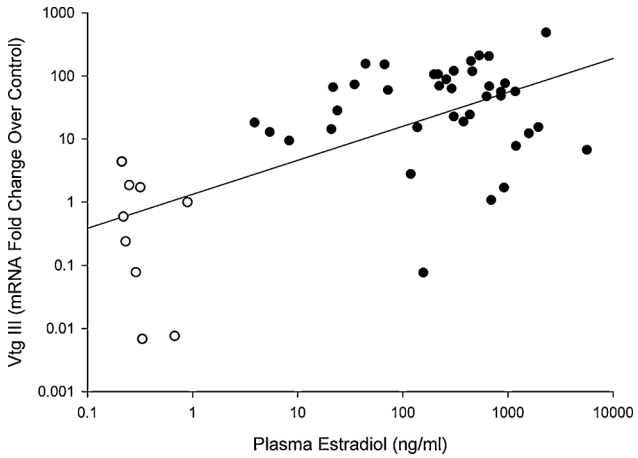

Fig. 3. Plasma 17β-estradiol and hepatic vitellogenin III (Vtg III) mRNA levels in nonreproductive male round goby after two weeks injection with 17β-estradiol in a coconut oil carrier. Gene expression was normalized for the expression of 18SrRNA housekeeping genes. Data are shown as the fold change relative to control (coconut oil carrier injection only) animals. Open and filled circles represent control and treated fish, respectively. $R = 0.416$; Spearman rank order correlation test.

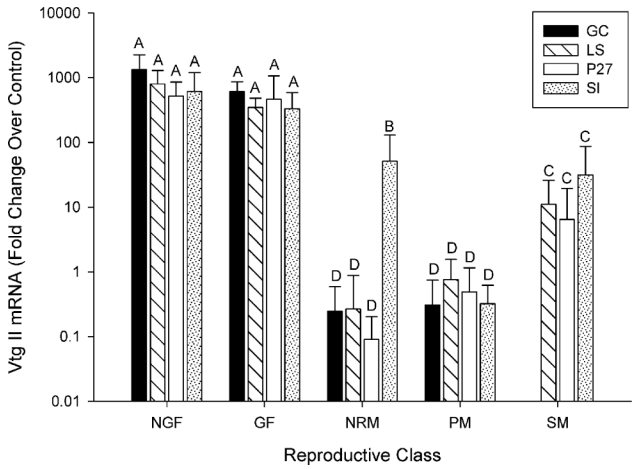

Fig. 4. Vitellogenin (Vtg) II gene expression levels in round goby liver collected from Hamilton Harbour, Canada. Gene expression was normalized by 18SrRNA expression and is shown as fold change from expression in the laboratory control group (i.e., nonreproductive males collected from LS and maintained under laboratory conditions). Error bars are standard deviations from group means. Uppercase letters indicate statistical differences ($p \leq 0.05$) in a two-way analysis of variance; Holm-Sidak post-hoc test. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet, NGF = nongravid female, GF = gravid female, NRM = nonreproductive male, PM = parental male, SM = sneaker male.

(Table 2). As expected, sneaker males were significantly smaller than both parental and nonreproductive males in both length and weight, had a developed papilla, and a significantly higher GSI (Table 2; $p \leq 0.05$). Nonreproductive males were not significantly different from parental males in length or weight and had a significantly lower GSI values than both parental and sneaker males (Table 2; $p \leq 0.05$). The number of fish collected varied across sites for each reproductive class and no sneaker males were available from GC (Table 2).

The Vtg II and Vtg III expression in gravid and nongravid females were not significantly different, but were significantly higher than all male expression levels (Figs. 4, 5). Vitellogenin

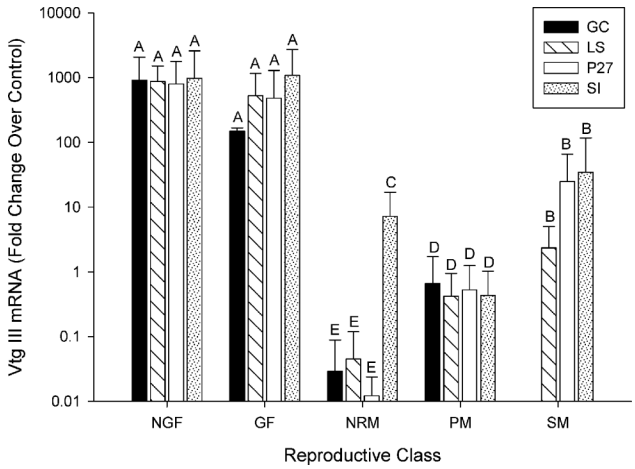

Fig. 5. Vitellogenin (Vtg) III gene expression levels in round goby liver collected from Hamilton Harbour, Canada. Gene expression was normalized by 18SrRNA expression and is shown as fold change from expression in the laboratory control group (i.e., nonreproductive males collected from LaSalle Marina [LS] and maintained under laboratory conditions). Error bars are standard deviations from group means. Uppercase letters indicate statistical differences ($p \leq 0.05$) in a two-way analysis of variance; Holm-Sidak post-hoc test. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet, NGF = nongravid female, GF = gravid female, NRM = nonreproductive male, PM = parental male, SM = sneaker male.

Table 2. Morphometrics of field-caught round goby

Class[b]	Length (cm)	Weight (g)	GSI[c]	Sample number per site[a] GC	LS	P27	SI	Total
Females[d]								
NGF	7.44 A ±1.09	5.44 A ±2.23	4.62 A ±2.37	5	15	11	19	50
GF	7.41 A ±1.21	6.01 A ±3.05	13.87 B ±4.70	6	7	9	7	29
Males[e]								
NRM	9.16 C ±1.71	10.99 C ±6.16	0.29 C ±0.79	9	10	11	7	37
PM	10.06 C ±2.24	14.97 C ±11.34	2.32 D ±0.76	3	12	14	12	41
SM	6.79 D ±1.09	4.18 D ±2.24	6.59 E ±11.43	0	9	12	18	39

Mean values (± standard deviation) of all sites combined are given for round goby of different reproductive classes caught in Hamilton Harbour, Canada in 2007.

[a] GC = Grindstone Creek; LS = LaSalle Marina; P27 = Pier 27; SI = Sher-Sherman Inlet.

[b] NGF = nongravid female; GF = gravid female; NRM = nonreproductive male; PM = parental male; SM = sneaker male.

[c] Gonadosomatic index.

[d] One-way analysis of variance (ANOVA) comparing NGF and GF for differences in length, weight, or GSI. The same letter indicates means are not statistically different at $p \leq 0.05$.

[e] One-way ANOVAs comparing NRM, PM, and SM for differences in length, weight, or GSI. The same letter indicates means are not statistically different at $p \leq 0.05$.

II expression was not different between nonreproductive males and parental males, but sneaker males had higher Vtg II expression levels than the other two male reproductive classes (Fig. 4). Nonreproductive males had the lowest Vtg III expression, parental males intermediate expression levels, and sneaker males the highest expression (Fig. 5). Both Vtg II and Vtg III levels were significantly higher in nonreproductive males from Sherman Inlet compared to nonreproductive males from the other sites ($p \leq 0.05$). No site differences were found among other reproductive classes tested. The Vtg III had approximately 18 times higher overall expression than Vtg II in both our field and laboratory exposed fish (data not shown).

Plasma steroid hormone concentration

Average plasma concentrations of testosterone (T), 11-ketotestosterone (11KT), and 17β-estradiol (E2) were compared between the three male reproductive classes, averaged over all field sites. Sneaker males had a significantly higher concentration of T compared to the other two male reproductive classes ($p \leq 0.05$; Fig. 6). Although there were only three samples from sneaker males, a significant difference was detected due to the large difference in plasma T levels. The lowest measured value for sneaker male plasma was 29.79 ng/ml while the highest value for parental males was 4.15 ng/ml. Parental males had a significantly higher concentration of 11KT compared to nonreproductive males (sneaker males omitted from analysis due to small sample size; Fig. 6). No significant differences were found in E2 concentrations between the three male reproductive classes (Fig. 6).

Gonad histology

Intersex has been characterized for the round goby used in the present study by Marentette et al. [22]. No incidence of intersex was found in any pumpkinseed, brown bullhead, or carp screened in the present study.

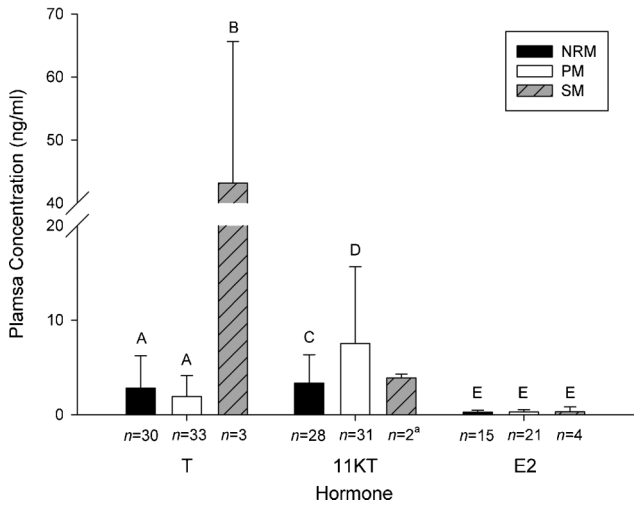

Fig. 6. Hormone concentrations in round goby plasma collected from Hamilton Harbour, Canada. Error bars are standard deviations from group means. Uppercase letters indicate statistical differences ($p \leq 0.05$) in a one-way analysis of variance; Holm-Sidak post-hoc test. T = testosterone, 11KT = 11-ketotestosterone, E2 = 17β-estradiol, NRM = nonreproductive male, PM = parental male, SM = sneaker male. [a]SM data were omitted from the statistical test for 11KT due to low sample size.

DISCUSSION

We recommend the use of the round goby as a very suitable model species for studying endocrine disruption in the Great Lakes region. In Hamilton Harbour this species has demonstrated intersex [22], while other species including goldfish (*Carassius auratus*), gizzard shad (*Dorosoma cepedianum*), bluegill (*Lepomis macrochirus*) [23], common carp, pumpkinseed, and brown bullhead (the present study and Kavanagh et al. [23]) have not. This lack of intersex in several species is in stark contrast to the high intersex (50–80%) documented in white perch from the same location [23]. Thus, many alternative species do not appear as sensitive to feminization and intersex as round goby. With such a small home range and high site fidelity, round goby offer a significant advantage over more mobile species for determining sources of EDCs in the aquatic environment. These fish are very easy to capture in large numbers with minimal effort. The round goby offers an invasive, benthic, widely distributed species with the added advantages of having multiple reproductive tactics driven by unique androgens and a prominent secondary sexual characteristic (urogenital papilla) that has been shown to be sensitive to endocrine disruption [22].

Fish populations in the Great Lakes are declining [34] and at some sites, show signs of endocrine disruption [22,23]. Although the compounds responsible for endocrine disruption in the lakes may be as diverse as the species that inhabit them, little work has been done to identify which anthropogenic factors are responsible and which areas are at the greatest risk. If more information were available on the nature of biological effects and EDC sources, remediation and prevention efforts could be better guided. One major category of pollutants is natural and synthetic estrogens; these compounds have been well documented as endocrine disruptors [2,9–13]. The induction of Vtg transcription and translation after ER activation has been well characterized, making Vtg expression in male fish a useful biomarker of EDCs [7,8,14–16,35–37].

The ability to monitor endocrine disruption is currently limited to a small number of biomarker assays that are typically only available for common model species. Quantifying Vtg expression has proven to be a reliable and sensitive approach to demonstrate endocrine disruption mediated through the ER [36]. Such assays are generally less expensive and require less time to develop than protein-based immunoassays, providing an opportunity to expand the number of species available for use in biological monitoring. This approach seems particularly relevant in a comparative context because species appear to vary greatly in their sensitivity to estrogen insults. A 50-fold increase in sensitivity to a phytoestrogen (the isoflavone genistein) between rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baeri*) [38] suggests high interspecies variability in sensitivity to estrogenic compounds. Transcriptional assays have measured Vtg responses after 24-h exposures to environmentally relevant concentrations of E2 [14], indicating that qPCR assays may provide better data on temporal aspects of EDC contamination associated with transient and intermittent events such as combined sewer overflow.

Fish species have a variable number of Vtg genes; seven Vtg genes are present in the zebrafish (*Danio rerio*) genome [7] and up to 20 Vtg genes have been identified in rainbow trout (*O. mykiss*) [39]. Vitellogenin genes can be classified based on the motifs and subdomains present; Vtg genes may contain a signal peptide, heavy chain lipovitellin (LvH), phosvitin (Pv), light chain lipovitellin (LvL), beta component, and C-terminal coding region motif and five homologous subdomains (HSDs). The first three HSDs are found in LvH, a conserved motif found in all fish Vtg genes; when present, the last two HSDs are found in LvL [7]. Three distinct Vtg gene types exist. Type I Vtg genes contain all three major motifs (LvH, Pv, and LvL) with LvL lacking HSD IV and V [7]. Type II Vtg genes have all three motifs and all five HSDs [7]. Type III Vtg genes lack the Pv motif and HSD IV and V from the LvL motif [7].

The diversity of Vtg genes among species and a lack of knowledge of how sensitive each gene is to estrogens made it prudent to target multiple Vtg genes for the present study. Round goby were assumed to have at least type II and III Vtg genes, based on the genes found during DNA library screening for Vtg genes in the Japanese common goby, a close relative [30]. The possibility of other Vtg genes in round goby cannot be ruled out as our approach was not exhaustive, nor are there genome data for this species. The cloned round goby Vtg II and III sequences were part of the LvH motif and shared low homology with each other; only four regions of high identity (>93%) were present, totaling <3% of the total sequence coverage (≈1,650 bp; data not shown). Primers for each gene were required since amplifying large fragments on both genes would be difficult, if not impossible, with the same primer set. Full-length gene sequences were not obtained for either gene as this was not necessary for the development of a qPCR assay.

Initially, a preliminary waterborne exposure was conducted in an attempt to mimic likely field exposure to estrogens. The round goby does not fare well in static holding conditions at high densities. As a result, significant induction of Vtg expression was difficult to confirm due to small sample sizes. Based on our preliminary data, we exposed larger numbers of round goby to E2 in a flow-through tank system and using a coconut oil i.p. injection for slow release estrogen delivery that has been previously used in Vtg induction studies [40–43]. The coconut oil carrier provided significant E2 exposure over a two-week period and the increase in plasma E2 concentrations was approximately proportionate to the doses given (data not shown). Higher concentrations of E2 were observed in laboratory-exposed males than those found in reproductive females.

Laboratory exposure to E2 showed that Vtg II and III mRNA expression levels were inducible. Both Vtg II and III mRNA expression levels correlated with plasma E2 levels (Figs. 2, 3) and with each other, demonstrating that these assays are able to detect increased activation of the ER pathway. After 48 h of exposure, Davis et al. [40] observed 100- to 10,000-fold induction of three Vtg genes in male tilapia (*Oreochromis mossambicus*) liver after injecting 5 mg/kg E2 via a coconut oil carrier. The lowest dose (1 mg/kg) in the present study was below that used in tilapia [40] yet produced a similar fold induction (data not shown). Lomax et al. [42] injected English sole (*Pleuronectes vetulus*) with 1 and 5 mg/kg E2 and observed approximately 450- and 600-fold increases in plasma Vtg protein levels, respectively. In the present study the same treatment doses produced a 250- and 1,200-fold increase in mRNA Vtg II expression, respectively. Waterborne studies using rainbow trout (*O. mykiss*) found that exposed fish with average measured plasma E2 levels of approximately 10 ng/ml had 100-fold induction of plasma Vtg protein levels [44]. Similar fold induction of Vtg II mRNA was observed in the present study for fish with plasma E2 levels near 10 ng/ml (Fig. 2).

Vitellogenin II and III were not expressed at the same level, nor were they induced to the same degree in treated versus control fish. The Vtg III expression was higher than Vtg II expression overall in round goby. In contrast, hepatic expression of type III Vtg genes was lower than expression of type II Vtg genes in zebrafish [7]. Zebrafish, which have seven Vtg genes, likely have many more Vtg genes than round goby; the difference in gene copy number may account for these differences in relative expression. After exposure of male goby to E2, overall induction of Vtg II expression (above control fish Vtg II levels) was approximately 10 times higher than induction of Vtg III expression (above control fish Vtg III levels; Figs. 2, 3). Similar to the round goby, zebrafish Vtg II genes were more inducible than Vtg III genes with E2 exposure [7]. Clearly, the differences in expression and inducibility of Vtg genes, and the biological relevance of these differences, will require further study.

Both gravid and nongravid female round goby showed high expression of Vtg II and III genes (Figs. 4, 5). Round goby spawn multiple times during an extended reproductive season (May to October) [45]. Considering that gravid and nongravid females were not significantly different in either length or weight (Table 2), both groups were likely reproductively active. The nongravid females may have recently spawned, resulting in a lower GSI, rather than being immature. The fact that Vtg levels were relatively constant between gravid and nongravid females and that round goby spawn continually during their long reproductive season [45] suggest that Vtg mRNA expression levels may not fluctuate during the reproductive season of this species. Further studies validating these points would provide a reasonable basis for using these assays to monitor antiestrogenic effects in female fish.

Male round goby had significantly lower expression of both Vtg genes compared to females, yet the assays were sensitive enough to detect differences between male reproductive classes. Sneaker males had significantly higher Vtg II levels than the other two male reproductive classes and there were differences between all three male classes in Vtg III expression, although it is currently unclear why this might occur. The difference in Vtg levels between male reproductive classes may be due to the type and amount of circulating steroid hormones; higher levels of 11KT have been reported in parental male goby [20]. We also found that parental males had higher levels of 11KT and that sneaker males use higher levels of T. No differences were found in circulating E2 levels between male reproductive classes (Fig. 6). Sample sizes for steroid analyses were small, particularly in sneaker males, and the samples sizes were not equal between the field sites for either nonreproductive males or parental males. As such, an examination of the potential differences in steroid hormone concentrations between sites was difficult. With these samples, we did not detect a statistical difference in mean steroid hormone concentrations across the field sites in nonreproductive or parental males. However, the possibility of site differences in steroid hormone concentrations could not be robustly tested and must be revisited with a more complete dataset, particularly by increasing sample sizes for sneaker males and ensuring equal sample numbers across the field sites. The present study demonstrates for the first time that there are differences in Vtg expression between male reproductive classes in round goby. Our findings suggest that these assays are sufficiently sensitive to determine differences in expression between reproductive classes and that the use of different male morphs may provide unique information regarding EDC exposure and effects.

Both Vtg II and III assays detected a site difference in nonreproductive males, showing higher levels of Vtg expression in fish caught at Sherman Inlet. Sherman Inlet is near Randle Reef, a large sediment deposit of PAHs, that receives intermittent discharge from combined sewer overflow. Limited data exist characterizing the mechanism of estrogenic effects of PAHs and PCBs in aquatic species, and most of these compounds have not been shown to be estrogenic. Yet several studies have reported an increase in vitellogenesis after PAH and PCB exposures [3–5]. Because PAHs are such a dominant contaminant at Sherman Inlet and intersex has been reported at this site in round goby [22], it cannot be discounted that PAHs are a possible culprit for endocrine disruption. Interestingly, elevated Vtg expression was not seen at Pier 27, a site in Hamilton Harbour where PCB exposure is expected and where feminization of secondary sexual characteristics (urogenital papilla), but no gonadal intersex, has been observed [22]. The lack of significant differences in Vtg II and III expression across some field sites may be attributed to lower sample numbers for some sites. For example, there were no sneaker males collected from one clean site (GC) and GC had lower numbers of fish for most reproductive classes (Table 2).

One potential issue with using Vtg as an endpoint is the variability of expression in response to EDCs. It has been noted that even genetically identical fishes can display large variability in response to the same dose of an ER agonist under identical holding conditions [35]. Prior exposure may be a critical factor in the responsiveness of an individual animal to estrogens. Fish appear to be more sensitive to estrogens and produce much higher levels of Vtg during subsequent exposures compared to animals that have never been exposed [15,17]. When animals have been previously exposed, long-term exposures to relatively low estrogen concentrations can elicit much larger responses in Vtg gene transcription [15]. In addition, variability between fish may be accounted for by the kinetics of transcription and clearance of mRNA. Plasma E2 levels showed high variability, suggesting that uptake and metabolism of E2 could have also been a contributing factor to the variability of Vtg expression. Fish exposed in the laboratory to E2 were collected in 2009, two years after the field samples for the present study were collected. Hence, the variability observed between laboratory control group fish and field-caught fish from cleaner sites may have been a result of year-to-year differences

in field sites. Increasing sample sizes and expanding datasets with multiyear studies would help to clarify this issue.

Measurement of hepatic Vtg gene expression by qPCR appears to be a sensitive assay, capable of detecting differences between laboratory-exposed and control fish, major reproductive classes, and across field sites with and without intersex. The overall expression of Vtg II was lower than Vtg III, and Vtg II was more inducible than Vtg III, but both assays were able to detect significant differences in Vtg expression in laboratory and field samples. We suggest that these are suitable assays for measurement of Vtg gene expression in the field. The round goby appears to be a useful sentinel species for determining the presence and impacts of EDC pollution in the Great Lakes. These fish are widely distributed, easily caught, and have been shown to be sensitive to several important endpoints including intersex, abnormal secondary sex characteristics [22] and, now with the present study, Vtg induction by an ER agonist.

SUPPLEMENTAL DATA

Supplemental Table (13 KB).

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