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Vitellogenin Induction in Mucus from Brook Trout (*Salvelinus fontinalis*)

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Abstract

Induction of vitellogenin (VTG) is widely used as a biomarker of exposure of male or immature fish to chemicals that are agonists of the estrogen receptor (i.e., xenoestrogens). Analysis of VTG in samples of epidermal mucosa collected from fish is a non-invasive method for evaluating whether wild fish are exposed to xenoestrogens. In this study, the mean levels of VTG in the mucus of immature brook trout (*Salvelinus fontinalis*) collected from the Credit River in Ontario, Canada downstream of aging residential septic systems and in an agricultural watershed were 0.67 ng per mg protein, which was significantly elevated relative to the mean VTG levels of 0.22 ng per mg protein in the mucus of immature brook trout collected from a less impacted site. To validate the mucus assay, immature brook trout were exposed in the laboratory to 17α-ethinylestradiol (EE2) at nominal concentrations of 10, 50 and 100 ng/L and VTG levels in mucus from these fish showed a concentration-dependent increase relative to fish from the control treatment. This study illustrates the utility of this non-lethal method for assessing whether wild fish have been exposed in situ to xenoestrogens. Exposures to xenoestrogens from non-point sources may be impacting brook trout populations in urban watersheds in southern Ontario.

Keywords Vitellogenin · Mucus · Brook trout · Xenoestrogens · Wastewater · Credit River

Vitellogenin (VTG) is an egg-yolk protein that is present during the reproductive cycle in oviparous vertebrates, including fish. Increased expression of the VTG gene occurs naturally during the spawning cycle in mature female fish, although low quantities of VTG are present in juvenile fish and in adult males (Folmar et al. 1996). However, expression of VTG can be induced in immature or male fish that have been exposed to chemicals that are agonists of the estrogen receptor, or "xenoestrogens". Previous studies have identified elevated VTG in immature fish in areas impacted by discharges of domestic wastewater (McGovarin et al. 2018) and runoff from agricultural lands (Sellin et al. 2009).

In wild fish, VTG is usually analyzed either in samples of liver or from blood plasma. Analyzing liver tissue for VTG requires the fish to be sacrificed, and while it is possible to collect blood from fish without causing mortalities, this requires some skill and is a difficult procedure in smaller fish. Recently, assays have been developed for the analysis of VTG in the epidermal mucosa of fish (Maltais et al. 2010; Allner et al. 2016).

For this study, brook trout (*Salvelinus fontinalis*) were sampled within the Burnt and Credit River watersheds in Ontario, Canada. Leakage from aging septic systems and inputs of agricultural runoff have been identified as sources of coliform bacteria and nitrates within the Credit River watershed (Credit Valley Conservation 2015; Ainley Group 2017). Brook trout populations are under stress in southern Ontario because of habitat loss and pollution (Haxton et al. 2020), so a non-invasive method is preferred for monitoring exposure to endocrine disrupting chemicals.

In the present study, an ELISA assay for VTG in mucus was used to evaluate exposure to xenoestrogens from non-points sources in wild immature brook trout collected at locations in the Credit River in southern Ontario, Canada. VTG concentrations normalized to the protein content in the mucus were compared to VTG levels in the mucus of brook trout collected from tributaries of the Burnt River, a less impacted watershed in the Haliburton region of central Ontario. Thermal, fluvial and physical restrictions during the summer months may limit the ability of brook trout to avoid chronic exposure to contaminants, making brook trout a

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valuable bioindicator of environmental stressors (McLaughlin and Noakes 1998). The objective of this study was to conduct a preliminary analysis of VTG levels in the mucus of wild brook trout to evaluate whether these fish have been exposed to xenoestrogens from non-point sources. To validate the mucus assay, immature brook trout were exposed in the laboratory to environmentally relevant concentrations of the potent estrogen agonist, 17α-ethinylestradiol (EE2), and the VTG responses were assessed in mucus samples.

Materials and Methods

Samples of water collected from the EE2 exposures were prepared for analysis without preconcentration. The samples were thawed, and the 10 mL volumes were filtered through 1.0 µm glass fiber filters. To a 2.5 mL aliquot of the filtered sample, 2.4 mL of methanol and 10 µL of 1% formic acid were added. Also added were a 40 µL volume of an internal standard, estrone-$^{13}C_2$, which was purchased from Cambridge Isotopes (Tewskbury, MA, USA). All samples were prepared in triplicate.

Samples were analyzed for EE2 and the internal standard by injection of a 50 µL volume of the sample without pre-concentration into an Agilent 1100 Series HPLC and autosampler, coupled to a Sciex 5500 QTrap tandem mass spectrometer operated with electrospray ionization (i.e., LC–ESI–MS/MS). Analytes were separated chromatographically on a Thermo Acclaim RS LC120 C18 column (2.2 µm, 2.1 × 50 mm) equipped with a C18 guard cartridge. The binary mobile phases were 20 mM ammonium acetate in high purity water (A) and 20 mM ammonium acetate in methanol (B), and the flow rate was 400 µL/min. The mass spectrometer was operated in negative polarity mode and ions were monitored using multiple reaction monitoring (MRM). The MRM transitions monitored for EE2 and estrone-$^{13}C_2$ (internal standard) were 295 > 145 and 271 > 147, respectively. EE2 was quantified using a 5-point calibration curve with an external standard, with the concentrations adjusted according to the recoveries of the internal standard.

The Credit River watershed flows into the western part of Lake Ontario at a location to the west of the Greater Toronto Area, Ontario, Canada (Fig. 1 inset). The Burnt River is a relatively less impacted watershed in central Ontario (Fig. 1 inset). These watersheds are historical habitat for brook trout. In the Credit River watershed, the population of approximately 11,000 inhabiting the Municipality of Erin does not have a centralized wastewater treatment plant and so domestic wastewater is treated using residential septic systems. There is potential for leakage of poorly treated sewage containing xenoestrogens, as the average household septic system in this municipality is 30 years old (Ainley Group 2017). Land use in the municipality also includes extensive agricultural lands.

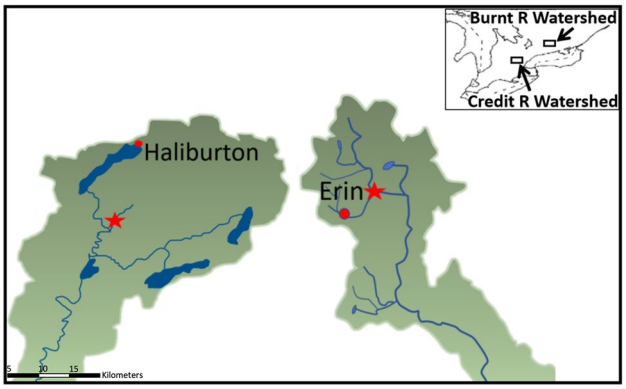

Fig. 1 Map showing the locations in Ontario, Canada of the watersheds of the Credit River and Burnt River (inset) and the two watersheds, with locations of the Municipality of Erin and the village of Haliburton. The red stars indicate the sites for sampling of immature brook trout

Immature brook trout were collected in the fall of 2018 from the Credit River in cooperation with the Ontario Ministry of Natural Resources and Forestry (OMNRF) as part of an annual population survey conducted at specific collection sites. The fish were collected at a location approximately 5 km downstream from the Municipality of Erin (Fig. 1). Immature brook trout were collected at a location in the Burnt River watershed in cooperation with a graduate research project (Fig. 1). Sampling occurred on September 28th in the Credit River and on October 1st in the Burnt River, for a two-day difference in sampling times. The movements of brook trout in the tributary downstream of Erin are restricted by a dam. Using backpack electrofishing gear, 15 brook trout were sampled from the Burnt River and 20 brook trout were collected from the Credit River. The average total lengths of sampled brook trout were 93.2 mm from the Burnt River and 95.7 mm from the Credit River, which corresponds with the immature stage of development (Wydoski and Cooper 1966). Fish collection was approved under the Animal Care Committee of Trent University.

Upon collection of fish from these rivers, mucus samples were collected from the flank of individual fish using swabs from the kits for the TECO® Salmonid Vitellogenin ELISA assay (protocol number TE1034) supplied by TECO Medical Group (Geneva, Switzerland). Each swab was gently run from the head to tail, slightly above the lateral line of each fish. The swab was rotated as the action was performed. Each swab was then placed in the collection vial provided in the kits, transported on ice to the laboratory, and subsequently stored in a – 80°C freezer.

A total of 40 immature brook trout, ranging in total lengths from 80 to 120 mm were provided by the OMNRF

Harwood Fish Culture Station. Fish were acclimated for 14 days at Trent University to sand filtered and ozone disinfected water from the Otonabee River, Ontario. The water temperature was maintained at 10–12°C and the photoperiod was a 16 h:8 h light/dark cycle. During acclimation, the fish were fed daily ad libitum with commercial trout chow pellets. Following acclimation, fish (n = 10 per treatment) were exposed to EE2 in static assays at nominal concentrations of 10, 50 and 100 ng/L. The EE2 (98% purity) was supplied by Sigma-Aldrich (Oakville, ON, Canada) and stock solutions of EE2 dissolved in acetone were spiked into river water (20 L) in volumes of < 1 mL. Fish in the control treatment (n = 10) were placed in 20 L of river water spiked with the carrier solvent only. The water was contained in polyethylene bags placed in plastic buckets and the water was aerated with aquarium air stones. Exposures were conducted for 96 h in an environmental chamber maintained at 10°C and with a 16 h:8 h light/dark cycle. Fish were not fed during exposures. At the end of the exposure period, mucus was collected from the fish as described previously for wild brook trout. The experimental protocol was approved by the Animal Care Committee of Trent University. At the beginning and end of the exposures, water samples (10 mL) were collected from each treatment and immediately frozen for later analysis of the concentrations of EE2.

VTG concentrations in mucus swabs were analyzed using the TECO® Salmonid Vitellogenin ELISA assay kit (protocol number TE1049) using methods previously described by McGovarin et al. (2018). All solutions and reagents that were required to complete the assay were provided in the kit. The analysis was conducted in a sterile 96-well microplate. A series of aspiration, washing, and incubation steps at room temperature was followed by adding a reaction mixture to the wells, which were read by the absorbance at λ 405 nm, using a SpectraMAX Plus 384 UV–Vis plate reader (Molecular Devices, Sunnyvale, CA, USA). VTG concentrations were determined by establishing a standard curve with a known VTG dilution buffer provided in the ELISA kit. Samples that fell below the limit of detection (0.06 ng/mL) were excluded from further analysis. The VTG concentrations were normalized to the amounts of protein in the swabs.

Kits for a protein assay based on the Bradford method were purchased from Bio-Rad Canada (Mississauga, ON, Canada). A volume of 0.5 mL of extraction buffer was added to the mucus swabs, and a volume of 10 µL of each of the samples was transferred to the wells in a sterile 96-well plate. Protein assay dye reagent concentrate (Bio-Rad) was diluted 1:4 with distilled water, and 200 µL of this solution was added to each well. Standards were created using a serial dilution of bovine serum albumin (Bio-Rad) diluted with extraction buffer. Absorbance was measured in the wells at 405 nm using a SpetraMAX Plus 384 UV–Vis plate reader.

Statistical analysis was conducted using R Studio version 4.0.2. Normality and homogeneity of variance were tested using Shapiro-Wilks and Bartlett's test, respectively. The data overall conformed to a normal distribution, so that Student's two sample *t* tests and one-way ANOVA and post hoc Dunnett test were applied for statistical analysis. A non-parametric test for Kendall Correlations was used to evaluate relationships between fish length and VTG responses.

Results

The mean concentrations of VTG (ng/mg protein) in the mucus of immature brook trout exposed in the laboratory to EE2 and in the control treatment are illustrated in Fig. 2. Mean VTG concentrations in the mucus of fish from all EE2 treatments were significantly elevated relative to the fish from the control treatment. There was a concentration related increase in the levels of VTG in the mucus of the exposed fish (Fig. 2). The reported EE2 values in water samples collected at the beginning of the exposures showed that they were consistent with the nominal concentrations for treatments with 50 and 100 ng/L (Table 1). The concentrations in these treatments declined to approximately 50% of the nominal concentration by the end of the 96-h exposure period. The concentrations in the 10 ng/L treatment were below the limits of detection of 25 ng/L

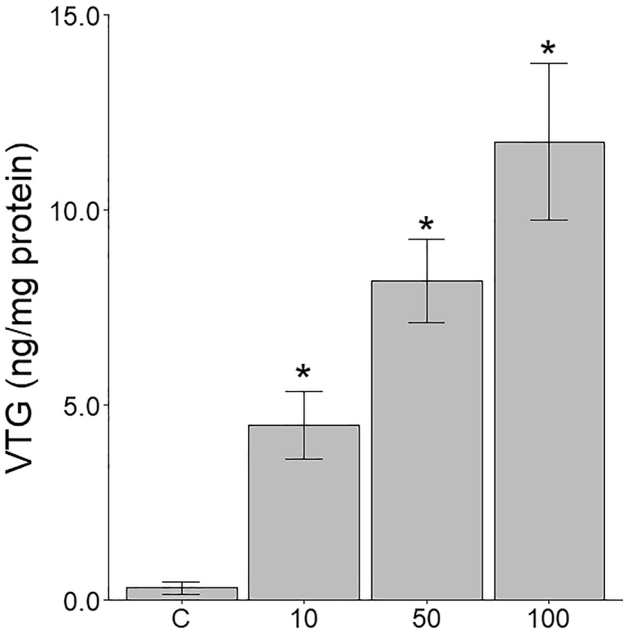

Fig. 2 Mean (±S.E.) concentrations of VTG (ng per mg protein) in the mucus of immature brook trout (n = 10 per treatment) exposed for 96 h to EE2 at nominal concentrations of 10, 50 and 100 ng/L and in the control (C) treatment. Significant differences between groups are identified with asterisks (*)

Table 1 Mean (±S.D.) measured concentrations in samples of water analyzed in triplicate that were collected at the beginning and end of the period for 96-h exposures of immature brook trout to EE2 and in the control treatment

Sample	Treatment			
	Control	10 ng/L	50 ng/L	100 ng/L
Beginning (0 h)	ND	ND	55.7 (±8.5)	111 (±12.4)
End (96 h)	ND	ND	27.8 (±5.3)	57.2 (±7.7)

ND Not detected at concentrations above the limit of detection of 25 ng/L

using the analytical method with no pre-concentration of the samples (Table 1). It was assumed that EE2 was present in the water in this treatment, even though it could not be detected using the analytical methods. No EE2 was detected in water samples collected from the control treatment.

Figure 3 shows the distribution of VTG concentrations (ng per mg protein) in the mucus of individual brook trout collected from the Credit River and the Burnt River. Even though there was considerable variation in the levels of VTG in the mucus of brook trout collected from the Credit River, the mean concentration of VTG of 0.67 ng per mg protein in fish from the Credit River was significantly elevated relative to the mean VTG concentration of 0.29 ng per mg protein in trout from the Burnt River (Student's t test; $df = 21$, $p \leq 0.05$). In 12 of the 20 fish collected from the Credit River, the VTG concentrations in mucus exceeded 0.5 ng per mg protein, while VTG levels in the mucus of all fish collected from the Burnt River were < 0.5 ng/mg protein (Fig. 3). Fish length was not significantly correlated with VTG concentrations.

It should be noted that the mean concentration of VTG in the mucus of brook trout collected from the Credit River was also significantly greater than the VTG concentrations in brook trout from the control treatment in the laboratory experiment (Student's t test, $df = 16$, $p = 0.04$). However, the levels of VTG in all wild brook trout were less than the VTG levels in trout exposed in the laboratory to EE2 at the lowest nominal concentration of 10 ng/L (Student's t test, $p \geq 0.05$).

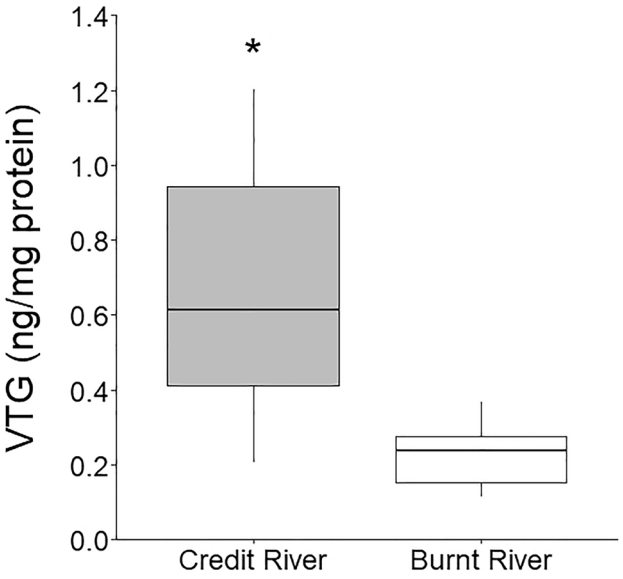

Fig. 3 Whisker boxplots of the median, upper and lower quartiles and upper and lower range of concentrations of VTG in mucus (ng per mg protein) in individual immature brook trout collected from the Credit River and the Burnt River in Ontario, Canada. Significant differences between groups are identified with asterisks (*)

Discussion

This study demonstrated the potential for using a non-invasive technique for monitoring VTG in fish that minimizes confinement and stress to the organism. The analysis of VTG in the epidermal mucosa of fish is useful for evaluating biomarker responses in small or immature fish and is an ideal approach when monitoring endangered species (Maltais and Roy 2014). Previous studies show that there is a good correlation between the induction of VTG in epidermal mucosa samples and VTG in blood plasma of fish exposed in the laboratory to xenoestrogens, although the concentrations of VTG induced in epidermal mucosa are lower than the levels reported in blood plasma (Maltais et al. 2010; Allner et al. 2016). Although plasma sampling can have little impact on fish when conducted properly (Cooke et al. 2006), mucus sampling offers an alternative approach that is non-invasive, takes only seconds to complete, requires little training of the sampler and results in low mortalities (Barkowski and Haukenes 2014).

Mucus VTG concentrations of 0.98 ± 0.61 ng/mg measured by Müller et al. (2021) in a control treatment with immature rainbow trout (*Oncorhynchus mykiss*) were greater than the mean value of 0.42 ± 0.27 ng/mg for brook trout from the control treatment in the present study. These baseline VTG concentrations reported for the mucus of rainbow trout were also within the range of the VTG concentrations measured in wild brook trout collected from the Credit River. However, significant differences in mucus VTG levels found in wild brook trout from both river systems provide some assurance that the VTG levels in fish in the Credit River were elevated. The higher levels in mucus of unexposed immature rainbow trout reported by Müller et al. (2021) relative levels in the mucus of immature brook trout in the present study may be due to inter-species differences in expression of VTG or could reflect variations in the sensitivity of the ELISA kits.

The data from the present study indicates that wild immature brook trout from the Credit River are exposed to

xenoestrogens, but identification of the specific compound(s) causing this response were beyond the scope of this preliminary study. Previous assessments of the Credit River watershed have identified elevated levels of coliform bacteria and nitrates, likely originating from agricultural runoff and/or leakage from aging septic systems (Credit Valley Conservation 2015; Ainley Group 2017). Old and leaking septic systems have been linked to contamination by xenoestrogens in surface waters (Peed et al. 2011). Endocrine disruption in fish has been associated with exposure to contaminants originating from domestic wastewater (Griffin and Harrahy 2014; Lee Pow et al. 2017) and in agricultural runoff (Sellin et al. 2009). The present study indicates that further investigations are warranted to identify the xenoestrogens present in the Credit River, possibly using non-targeted screening for unknown compounds (Tian et al. 2020).

In southern Ontario, Canada, brook trout within urbanized watersheds are increasingly under threat from habitat loss and exposure to pollutants (Haxton et al. 2020). The present study indicates that exposure to xenoestrogens may be an additional stressor contributing to threats to brook trout populations in this mixed-use watershed. Brook trout is a cold-water fish, and the movements of this species are restricted to habitats with colder temperatures and high flows (McLaughlin and Noakes 1998). Thermal, fluvial, and physical barriers during the summer months may limit the ability of brook trout to avoid chronic exposure to contaminants that are carried into the watershed.

This study demonstrates the potential for using a non-invasive biomarker of exposure of fish to xenoestrogens.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest related to this study.

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