Characterization of vitellogenin concentration in male fathead minnow mucus compared to plasma, and liver mRNA.

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ABSTRACT

The objective of this study was to characterize vitellogenin (VTG) protein in male fathead minnow (*Pimephales promelas*) mucus compared with more conventional measures in plasma and mRNA isolated from liver. To assess the intensity and duration of changes in mucus VTG concentrations, male fathead minnows were exposed to 17α-ethinylestradiol (EE2) for 7 days with a subsequent depuration period of 14 days. The experiment was conducted in a flow-through system to maintain a consistent concentration of EE2 at a nominal EC_{50} concentration of 2.5 ng/L and high concentration of 10 ng/L as a positive control. Mucus, plasma and liver were sampled at regular intervals throughout the study. Relative abundance of *vtg* mRNA increased after 2 days of exposure and returned to control levels after 4 days of depuration. VTG protein concentration displayed similar induction kinetics in both mucus and plasma, however, it was found to be significantly increased after 2 days of exposure using the mucus-based assays and 7 days with the plasma-based assay. Significantly elevated levels of VTG were detected by both assays throughout the 14-day depuration period. The elimination of the laborious plasma collection step in the mucus-based workflow allowed sampling of smaller organisms where blood volume is limiting. It also resulted in significant gains in workflow efficiency, decreasing sampling time without loss of performance.

1. Introduction

For decades, induction of vitellogenin mRNA (*vtg*) and protein (VTG) in male fish have been used as an indicator of exposure to endocrine disrupting compounds (Barber et al., 2012; Costigan et al., 2012; Crago et al., 2011; Garcia-Reyero et al., 2011, 2009; Kirby et al., 2004; Sumpter and Jobling, 1995; Flick et al., 2014; Dammann et al., 2011; EPA, US, 2002). Though increased expression of *vtg* or VTG is generally used to indicate estrogenicity, they have independent strengths and weaknesses both in terms of the nature of the information they provide (i.e. timing of exposure relative to induction) and in practical terms (sensitivity, cost and ease of use).

Differences in the amount of *vtg* mRNA have been detected by various reverse transcriptase (quantitative) polymerase chain reaction (RT-PCR and RT-qPCR) assays (Biales, Bencic, Flick et al., 2007; Dorts et al., 2009; Garcia-Reyero et al., 2011; Kolok et al., 2007; Cavallin et al., 2016; Biales, Bencic, Lazorchak et al., 2007). RT-qPCR can be a fast, sensitive method to measure gene expression changes. Induction of *vtg* can be seen as early as 8 h after exposure to an endocrine disruptor (Gordon et al., 2006), remains substantially elevated throughout the

exposure and returns to baseline levels within days after cessation (Schmid et al., 2002). The high sensitivity and relatively rapid turn-around time of performing RT-qPCR (from RNA to results within days) makes this assay highly appealing for routine monitoring applications.

VTG protein has most frequently been measured by enzyme-linked immunosorbent assay (ELISA) (Korte et al., 2000; Parks et al., 1999; Hemmer et al., 2002; EPA, US, 2002; Ohkubo et al., 2003; Mylchreest et al., 2003; Meucci and Arukwe, 2005; Hoffmann et al., 2008). Measurement of VTG typically has either been performed on blood plasma or homogenized tissue, most often liver. Except for larger fish, from which non-lethal blood collection is possible, obtaining samples for VTG analysis usually requires sacrificing the exposed fish. Because of this, VTG assays have had very limited use for longitudinal studies or in applications specific to threatened and endangered species. VTG has been detected in the surface mucus of fish (Allner et al., 2016; Genovese et al., 2011; Meucci and Arukwe, 2005; Moncaut et al., 2003; Van Veld et al., 2005). This non-invasive sample may sidestep the aforementioned limitations and has the potential to allow researchers to apply these tools in an increasing number of monitoring applications. Moreover, the use of non-invasive samples may satisfy the need for toxicity testing and

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monitoring to move away from sacrificing whole animals. The use of mucus as a sample matrix offers much promise. Before it gains widespread acceptance as an alternative test method, it must be evaluated against currently accepted methods. The objective of this study was to evaluate the performance of the VTG-mucus assay in the fathead minnow (*Pimephales promelas*) relative to gold standard methods, namely blood plasma VTG ELISA and *vtg* expression after exposure to an environmentally relevant dose of the estrogen 17α-ethinyl estradiol.

2. Materials and methods

2.1. Exposure

To assess the intensity and duration of changes in mucus VTG concentrations, reproductively mature adult male fathead minnows were exposed to control (dechlorinated tap water with KCL added to moderate hardness), 2.5, or 10 ng/L 17α-ethinylestradiol (EE2) for 7 days in 10 L tanks with a flow-through diluter system at a rate of 40 mL/min, approximately 6 water changes per day. Nominal test concentrations of 2.5 and 10 ng EE2/L were chosen based on an approximate EC_{50} effect concentration (Flick et al., 2014) as well as consistent effect concentration from previous studies in our laboratory (Biales, Bencic, Flick et al., 2007; Biales, Bencic, Lazorchak et al., 2007). Treatments were blocked in 30 diluter system tanks, 10 tanks per treatment, to account for location-based effects. On day 7, the exposure solutions and tank water were exchanged for control water for a depuration period of 14 days. Fish were placed at an initial density of 6 per 38 L tank and maintained at a 16 hr light/8 hr dark photoperiod. They were fed frozen adult brine shrimp twice daily to satiation. All animal handling protocols were approved by the Institutional Animal Care and Use Committee of U.S. EPA Cincinnati.

2.2. Exposure water chemistry

A composite water sample, approximately 100 mL per tank = 1 L total, was collected from each treatment on study days 0, 1, 2, 4, 7, 11, 14 and 21. Samples were stored at 4 °C and concentrated within 48 h by solid phase extraction on C-18 columns. Samples were then analyzed by ultrahigh pressure liquid chromatography tandem quadrupole mass spectrometry (UPLC/MS/MS). The method detection limit for EE2 was 0.1 ng/L with a lowest-concentration minimum reporting level of 0.9 ng/L.

2.3. Sample collection

A random sample of 10 fish from the stock culture used for this study was sacrificed on day 0. One fish was sampled from each exposure tank on days 2, 4, 7, 11, 14 and 21, resulting in 10 fish per treatment per day. Fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma, USA) and samples of epidermal mucus, blood and liver obtained. Mucus was collected by gently rolling about half a swab (TECO® Mucus Collection Set, Diapharma, USA) down the length of the fish from just posterior to the gills to just anterior of the caudal fin, turning the fish over and rolling the other side of the swab on the other side of the fish. The swab was broken off into a 1.5 mL microcentrifuge tube and kept on wet ice until all swabs were collected. Samples were stored at − 20 °C until analyzed. For plasma, the caudal artery was severed, and blood was collected in heparinized hematocrit tubes, centrifuged for three minutes, transferred to a 1.5 mL microfuge tube and stored at − 20 °C. Liver tissue was removed, placed in centrifuge tubes and immediately flash frozen in liquid nitrogen. Frozen liver samples were subsequently stored at − 80 °C until analyzed.

2.4. ELISA

Mucus proteins were extracted from swabs by adding 0.5 mL

Extraction Buffer (TECO® Mucus Collection Set, Diapharma, USA) vortexing and incubating 30 min per the manufacturer's instructions. Samples were assayed using the TECO® Cyprinid Vitellogenin ELISA (Diapharma), a sandwich ELISA utilizing pre-coated plates, an anti-VTG antibody with broad cyprinid fish reactivity and horseradish peroxidase detection, according to the manufacturer's instructions. Absorbance was measured on a Synergy HTX multimode plate reader (Biotek, USA). Experimental control and low dose mucus samples were analyzed neat (undiluted). High dose mucus samples were analyzed neat on day 2, 1:10 or 1:20 for days 4 and 7, 1:50 or 1:100 for days 11, 14 and 21. Analyses were repeated for samples that were above the standard curve at the stated higher dilution. Plasma experimental control and day 2 low dose samples were assayed at 1:100. Low dose plasma samples from the remaining days were assayed at 1:5000 or 1:10,000. High dose plasma samples were analyzed at 1:1000,000. The sample size for mucus ranged from n = 8–10 and plasma was n = 4 – 5 per treatment per time point. The variability in mucus sample size is due to random mortality across treatments. The smaller number of plasma samples was due to resource limitations and selected based on power analysis for EC_{10} of 0.9 – 1.5 ng/L EE2 from (Flick et al., 2014). Total protein concentration was measured but not used for normalization because of sample size limitations (see Supplementary Information Section 1). Each ELISA plate included a standard curve with a range of 0.4 – 70 ng/mL and manufacturer provided externally validated positive controls. Target values (acceptable range) for the manufacturer's controls were control 1 = 2.0 (1.3 – 2.7), control 2 = 6.0 (3.9 – 8.1) and control 3 = 29 (19.0 – 39.4) ng/mL. Additionally, mucus (1:100) or plasma (1: 1000,000) collected from breeding fathead minnow (FHM) females was used as an in-lab positive control. Samples were blocked so a single dilution of a sample, control 1, control 3 or female FHM from each experimental condition was included on a plate. That same group of samples was repeated on a second plate as technical replicates. Two technical replicates of control 2 were included on each plate. This control was used to calculate intra- and inter-plate variability, 16% and 14% respectively. The lowest concentration control, control 1, was used to calculate the method detection limit (MDL). This was 0.544 ng/mL which is 3 times the standard deviation of all measurements of control 1. Values below the MDL were recorded as 0.272 ng/mL, half the MDL. Values above the standard curve after repeat analysis were recorded as 73.5 ng/mL. The cutoff for including measurements from biological samples ≥ 1.3 ng/mL (second lowest standard) was 14% coefficient of variation (CV), the empirically determined inter-plate CV. Eleven samples needed to be excluded. Samples ≤ 1.3 ng/mL were all included as reported though CVs ranged from 0% to 80%.

2.5. RT-qPCR

RNA was isolated from liver tissue (n = 8 – 10 except Day 21; n = 4 due to sample loss from freezer failure) with TRI Reagent (Ambion, USA) and Phasemaker tubes (Invitrogen, USA) according to the manufacturers' instructions. Samples were treated with TURBO DNA*free* (Ambion, USA) to remove any remaining genomic DNA. Total RNA was quantified by UV-Vis absorbance with a Take3 microspot plate on a Synergy HTX plate reader (BioTek, USA). All samples had 260/280 ratio of 2.0 ± 0.2 indicating they were of high quality, and free of contaminants.

RT-qPCR was performed with RNA-to-Ct 1-step kit, which contains proprietary pre-mixes of RT enzyme and qPCR reagents (Applied Biosystems, USA) according to the manufacturer's instructions using gene specific primers and TaqMan probes for *vtg* (see Supplementary Information Section 2), TATA box binding protein (*tbp*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) or SYBR Green for ribosomal protein l8 (*rpl8*, Table 1). The RT-qPCR assay met the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). RNA standards, generated from a pool of total RNA, were included on each plate (see Supplementary Information

Table 1

Primer and probe sequences for *vtg, tbp, hprt1* and *rpl8* recorded 5′ to 3′. References are GenBank accession numbers for sequence used to design primers and probes or publication they were copied from. See Supplementary Information Section 2 for details on *vtg*. Primers with MGB probes were designed using PrimerExpress v3.0.1 (Applied Biosystems, USA).

Primer Name	Sequence 5' – 3'	Final RT-qPCR concentration (nM)	Amplicon (bp)/qPCR Efficiency	Reference
vtg Q F3738	CACCACATACGCCAAAAAGCT	250	67 / 94%	AF130354.1
vtg Q R3805	CAAGTCTAAAGCCCGTCTGGTT	250		
vtg Q PB3766	6FAM-CACATTCCTATGGCGGC-MGBNFQ	300		
hprt1 Q F422	ATTCCGATGACAGTGGACTTCA	300	65 / 92%	DT085800.1
hprt1 Q R487	GATGTCACCTGTAGATTGGTCATTTT	300		
hprt1 Q PB445	6FAM-CCGACTCAAGAGTTAC-MGBNFQ	250		
tbp Q F657	AGGAGCCAAAAGTGAGGAACAG	300	60 / 93%	DT344258.1
tbp Q R717	CTGCACCACTCTGGCATATTTC	300		
tbp Q PB680	6FAM-CCCGATTGGCAGCCA-MGBNFQ	250		
rpl8 Q F84	TCAAGGGGATTGTGAAGGAC	100	72 / 92%	Flick et. al. 2014
rpl8 Q R156	TCACGGAAAACCACCTTAGC	100		

Section 3). All samples were analyzed in triplicate RT-qPCR reactions. A cutoff of > 0.3 standard deviations was set but no samples were removed from analysis due to this cutoff. Samples from every experimental condition were included on each plate. A subset of 20% of the samples were analyzed on each plate as technical replicates. Intra- and inter-plate variability were < 3% CV for all genes tested. Reaction efficiency was 92–95%. Samples reported as undetermined, > 40 cycles, were replaced with a cycle threshold (Ct) value of 40. RT-qPCR reference genes, *tbp, hprt1* and *rpl8,* were analyzed with the NormFinder algorithm using genorm in R v3.5.1 to determine the most stably expressed genes and create a normalization factor for *vtg* expression (Andersen, Jensen, and Orntoft, 2004). Relative *vtg* expression was calculated using the ΔΔCt method (Schmittgen and Livak, 2008) where:

$$relative\ vtg\ expression = (1 + 0.95_{vtg\ efficiency})^{-[(vtg\ Ct_{treatment} - normalization\ factor_{treatment}) - (vtg\ Ct_{Day\ 0} - normalization\ factor_{Day\ 0})]}$$

2.6. Data analysis

Statistical analyses were done in R version 4.0.0 (Team, 2020). Outliers were identified and removed from further analysis by Cook's distance using a cutoff of 8 standard deviations. The 5 outliers removed had no relation to treatment or time (Supplementary Figs. S4 and S5, shapes with green fill). The high cutoff was chosen due to the high variability in VTG typically seen in male FHM. Treatment groups for each sample type were tested for heteroscedasticity with Levene's test (Fox and Weisberg, 2019). All sample sets had unequal variation and unequal sample sizes between treatment groups. Log transformations did not alter the results of Levene's test. Differences between means within a day were tested using Welch's ANOVA with a Games-Howell post-hoc test. Differences were considered significant with a p-value ≤ 0.05.

3. Results and discussion

3.1. Exposure water chemistry and FHM health

Measured concentrations of EE2 remained relatively constant with mean ± SD values of 0.01 ± 0.01, 1.33 ± 0.20 and 5.48 ± 1.13 ng/L (n = 1 composite sample per treatment) in the control, low and high treatments, respectively. The exposure concentrations were approximately half of the nominal concentration. The maximum measured concentrations for the low and high treatments were 1.66 and 7.41 ng/

L, respectively, on day 0. This result suggests EE2 was lost to the system, possibly through adsorption to mixing pots or tubing. The minimum concentration in the low treatment was 1.16 ng/L on day 1. The minimum concentration in the high treatment was 4.4 ng/L on day 2. No detectable EE2 was observed in the depuration phase of the study. The FHM mortality rate was 4%. There was no association between death and treatment or time.

3.2. VTG concentration in mucus compared to plasma

Changes in VTG frequently have been studied in fathead minnows exposed to the well-characterized endocrine disrupting compound, 17α-ethinylestradiol (EE2). First, we evaluated VTG concentration between controls over time as it is known that altered social structure as a consequence of crowding can affect this endpoint (Ivanova et al., 2017). There were no significant differences found between controls over time in this study. In mucus samples, protein concentration was significantly increased in the high concentration compared to the control beginning on day 2 (Fig. 1 A). VTG concentration continued to increase during the depuration phase, peaked on day 14, and dropped off sharply by day 21. Plasma VTG followed a similar trend, however, a significant elevation was not observed until day 7, and then increased more rapidly, peaking at day 11 (Fig. 2 A). The delay in reaching a statistically significant level relative to control in the plasma likely results from reduced statistical power due to the lower sample size used in the plasma analysis. Power analysis conducted by Flick et. al. 2014 estimated n = 5 for plasma from FHM males exposed to an EC_{10} of 0.9 – 1.5 EE2 ng/L. While this is within our actual concentration of 1.33 ± 0.2 ng/L, Flick et. al. also calculated a sample size of n = 17 – 18 for an EC_5 of 0.64 – 1.2 ng/L, which overlaps our actual concentration and may account for our lack of statistical power. Differences in VTG concentration, μg/mL vs. ng/mL, and kinetics between the plasma and mucus are consistent with the life cycle of VTG and the physiological function of mucus. VTG is produced in the liver and transported via blood circulation to the ovaries where it is deposited in developing oocytes (Wallace and Selman, 1990; Selman and Wallace, 1983). In male fish, where VTG is generally not expressed and serves no known biological function, VTG must be eliminated through other routes such as urine, feces, gill or epidermis. The lower VTG concentration and subsequent later peak in mucus could be due to branchial or epidermal excretion of the excess protein.

To determine if mucus and plasma VTG assays were sensitive enough to identify estrogenic exposures at environmentally relevant
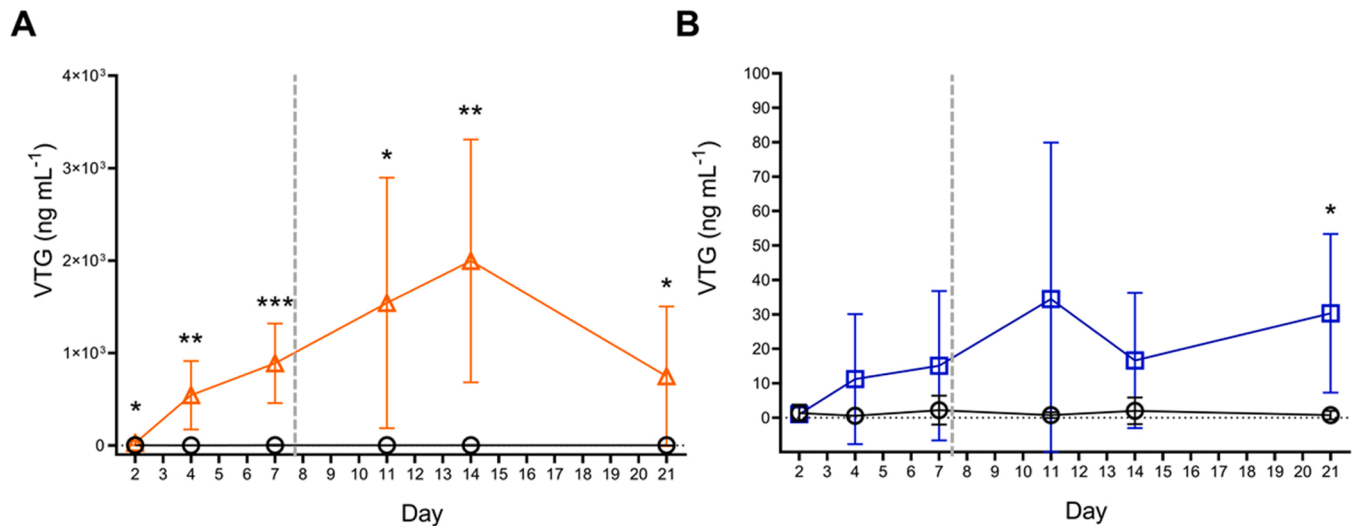
Fig. 1. Concentration of vitellogenin (VTG) over time in mucus of male fathead minnows exposed to **A,B** Control (0.00 ng/L, black circle), **A** High (5.48 ng/L, orange triangle), or **B** Low (1.33 ng/L, blue square) EE2 for 7 days followed by a 14-day depuration in clean water. Each point represents the mean with vertical lines displaying standard deviation (n = 8–10). Vertical dashed gray line indicates the beginning of the depuration period. Horizontal dotted line marks VTG (ng mL^{-1}) = 0. Asterisks indicate significant difference between the means of Control and High or Low within that day, determined by Welch's ANOVA with Games-Howell post-hoc test (*p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). Individual sample values may be found in Fig. S4.
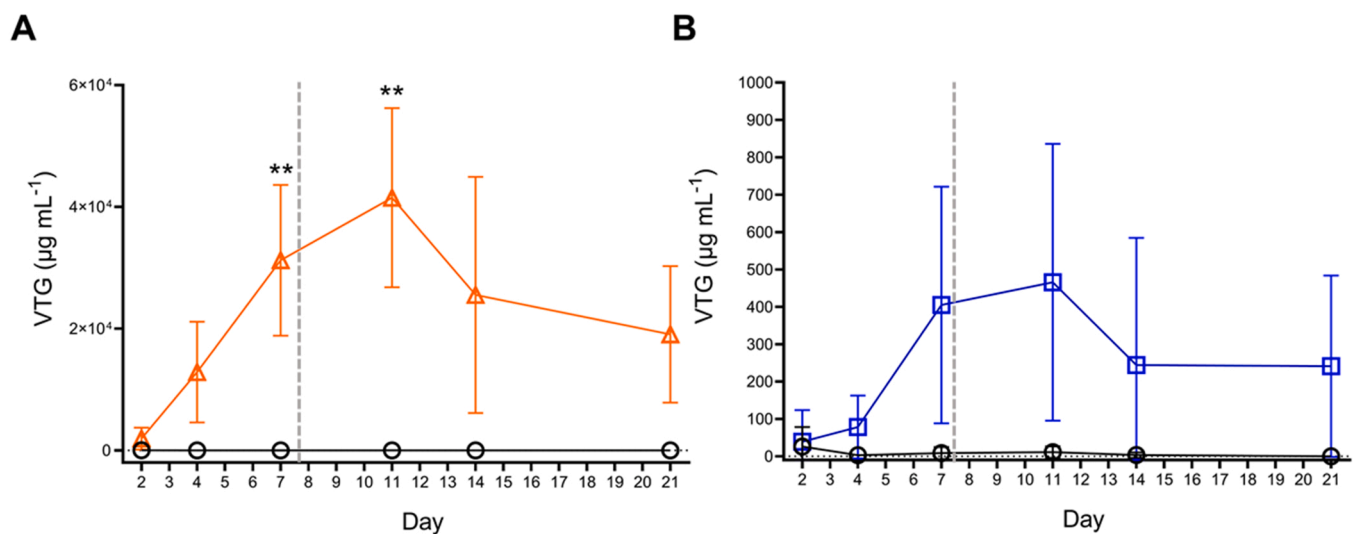

Fig. 2. Concentration of vitellogenin (VTG) over time in plasma of male fathead minnows exposed to **A,B** Control (0.00 ng/L, black circle), **A** High (5.48 ng/L, orange triangle), or **B** Low (1.33 ng/L, blue square) EE2 for 7 days followed by a 14-day depuration in clean water. Each point represents the mean with vertical lines displaying standard deviation (n = 3–5). Vertical dashed gray line indicates the beginning of the depuration period. Horizontal dotted line marks VTG (ng mL^{-1}) = 0. Asterisks indicate significant difference between the means of Control and Low or High within that day, determined by Welch's ANOVA with Games-Howell post-hoc test (*p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). Individual sample values may be found in Supplementary Fig. S5.

concentrations, VTG levels were assessed in the low EE2 treatment. The measured concentration of EE2, 1.33 ng/L, is approximately half of the EC$_{50}$ (Flick et al., 2014) and well within the range of environmentally relevant (Kolpin et al., 2002; Kostich, Flick, and Martinson, 2013). Mucus VTG was significantly increased over control only during the depuration period on day 21 (Fig. 1B). The VTG response observed in plasma was similar to mucus, but was not significantly different from control, again likely due to reduced statistical power (Fig. 2B). We demonstrated that VTG can be detected in the mucus of fish exposed to environmentally relevant levels of an estrogenic compound. Though a trend in higher concentrations of VTG were observed in earlier time-points, the magnitude was not sufficient to overcome the high variability, suggesting increased sample size would increase sensitivity.

3.3. Vtg compared to VTG expression

Gene expression measured by RT-qPCR is often used as a fast, sensitive assay for exposure to environmental contaminants. Expression of *vtg* in liver was increased above control in fish exposed to the high concentration by the first time point, day 2 (Fig. 3A). The expression level peaked on day 7 where it was also significantly higher than control. *Vtg* expression dropped sharply during the depuration period where it was indistinguishable from control except for 4 days into depuration (day 11) in fish exposed to the high concentration. A similar trend was observed in the low EE2 dose however *vtg* was only significantly higher than control on day 7 of exposure (Fig. 3B).

In the present study, the relative kinetics of plasma VTG and hepatic *vtg* induction were similar to previously reported observations (Flick
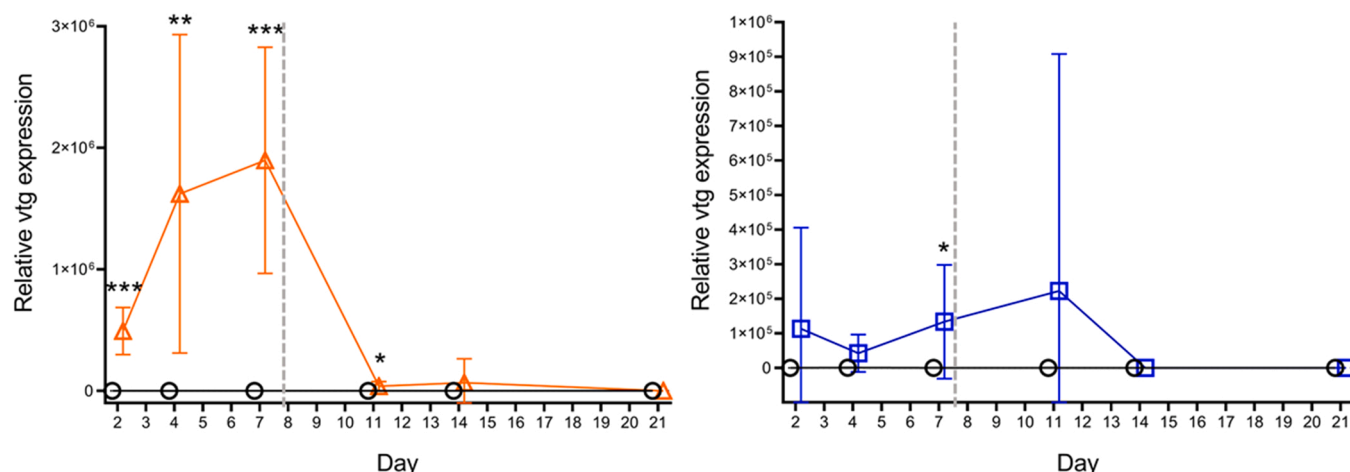
Fig. 3. Expression of vitellogenin (*vtg*) over time in liver of male fathead minnows exposed to **A,B** Control (0.00 ng/L, black circle), **A** High (5.48 ng/L, orange triangle), or **B** Low (1.33 ng/L, blue square) EE2 for 7 days followed by a 14-day depuration in clean water. Each point represents the mean with vertical lines displaying standard deviation (n = 8 – 10, Day 21 n = 4). Vertical dashed gray line indicates the beginning of the depuration period. Asterisks indicate significant difference between the means of Control and Low or High within that day, determined by Welch's ANOVA with Games-Howell post-hoc test (*p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). Individual sample values may be found in Supplementary Fig. S6.

et al., 2014; Hiramatsu et al., 2006; Korte et al., 2000; Moncaut et al., 2003; Schmid et al., 2002). Interestingly, the mucus VTG concentration was significantly increased at the earliest time point and remained elevated throughout the duration of the study, including the 14 day depuration period. Though not significant until day 7, this pattern was reflected in the plasma VTG, suggesting detection at day two was not a false positive. Our results suggest that no additional information is gained from monitoring both mucus and plasma VTG and that mucus VTG may be the more sensitive of the two. Additionally, the data here suggest a monitoring approach that relies on the increased concentration of both mucus (protein) and hepatic (mRNA) vitellogenin may provide finer scale temporal resolution to the characterization of estrogenic exposures. For example, the simultaneous detection of both mucus VTG and hepatic *vtg* mRNA would suggest a very recent or ongoing estrogenic exposure, whereas detection of only mucus VTG would indicate that an estrogenic exposure lasting at least 2 days had occurred sometime within the last several weeks. Clearly, this is a simplistic example and confidence in the accurate interpretation of the spectrum of potential responses of the combined approach would require additional finer scale studies over varying time scales, however, our preliminary results suggest the feasibility of the approach.

Precision was calculated for ELISA using Control 2 with 2 replicates analyzed over 8 runs. Percent coefficient of variation (CV) in the table correspond to 6.2 ± 1.0 ng/mL intraplate and 6.2 ± 0.8 ng/mL interplate variability. The higher intraplate variation is likely due to having only two replicates per plate. Precision was calculated for RT-qPCR using in-house control female fathead minnow liver total RNA along with 14 experimental samples. Three replicates were analyzed on two plates for each gene, per sample. *Vtg* precision was the most variable;

intraplate average standard deviation = $Ct_{mean} \pm 0.17$, and interplate $Ct_{mean} \pm 0.36$.

3.4. Assay comparison

Cost, reliability, and ease of use are important factors to consider when employing these assays for environmental monitoring. Table 2 displays these factors using variability as a measure of reliability and time as a measure of ease of use. The per sample cost of ELISA was double that of RT-qPCR. The cost of the particular kit used was in the same range as other commercially available VTG ELISA kits. Assay reliability was evaluated using measures of intra- and inter-plate variability. RT-qPCR is considered a very precise assay and this case was no different with CV < 3%. The CV for inter-plate variability in ELISA was 14% for samples ≥ 1.3 ng VTG/mL. Both assays require the same skill level and training in good laboratory practices, precise pipetting and basic molecular biology to perform correctly. Therefore, time to perform the assay from sample collection to data acquisition was used to evaluate ease-of-use. In this experiment, samples were collected at each time point and stored until they could all be processed together. The time estimates are based on processing a batch of fish from the beginning to end of the protocol, which is a best-case scenario where samples are collected and processed at the same site. Surface mucus sample collection was the easiest requiring about a minute to swab both sides of the fish and break off the swab in the sample tube. Preparing the sample to dilute requires a 30-minute incubation with extraction buffer (TECO® Mucus Collection Set, Diapharma, USA). Plasma was collected by removing the tail and holding a heparinized hematocrit tube to the caudal vein until filled or blood stopped flowing. Then the tubes were centrifuged for 3 min, scored, split and the plasma was transferred to a microcentrifuge tube. The whole process took up to 10 min and many samples were not usable due to low blood volume, clotting during collection or hemolysis. Mucus or plasma samples have to be diluted and added to the appropriate well of the ELISA plate then the assay takes about 4 h to perform. All steps can be performed within one day.

For RNA time estimates include all steps from tissue collection through data collection. Tissue necropsy, RNA isolation, quantification and quality assessment are typically completed in one day. RNA is then diluted to a standard concentration and added to the RT-qPCR plate (384 well plate = 96 samples due to technical replication). The RT-qPCR protocol takes approximately 2.5 – 3 h depending on the specifics of the assay and the instrumentation.

Table 2

Costs are rounded to the nearest dollar based on current list price.Sample prep = TECO Mucus collection set vs. TRIzol + Phasemaker tubes + TURBO DNA-free Per sample assay cost = 2 replicates of one dilution in TECO Cyprinid Vitellogenin ELISA vs. 3 replicates of 4 target genes in RNA-to-Ct™ 1-Step Kit.

	ELISA	RT-qPCR
Total cost per sample	$31	$14
Sample collection/prep	$5	$5
Per sample assay cost	$26	$9
Intraplate variability	16%	≤ 3%
Interplate variability	14%	≤ 3%
Time (sample collection to data acquisition)	1 day	2 days

Data processing and analysis for either the protein or mRNA can take a few hours depending on the number of samples and streamlining of the workflow. Overall, the mucus ELISA was by far the easiest method used in this study because no dissection was required and the protocol was simpler requiring minimal sample manipulation and pipetting.

4. Conclusion

Increased expression of vitellogenin in male fish has long been used an indicator of exposure to estrogenic compounds in an aquatic environment. The employed methods required researchers to sacrifice fish in order to measure vitellogenin protein or RNA (VTG and *vtg* respectively) limiting their application to longitudinal studies and protected species. Recently, mucus has been recognized as a viable non-invasive sampling material for a number of biomarkers including VTG (Allner et al., 2016; Church et al., 2008; Dzul-Caamal et al., 2016; Genovese et al., 2011; Guardiola et al., 2015; Meucci and Arukwe, 2005; Moncaut et al., 2003; Van Veld et al., 2005; Ekman et al., 2015; Mosley et al., 2018). We have demonstrated expression of VTG in fathead minnow surface mucus is detectable within two days of exposure to ~ 5 ng/L EE2 and remains elevated during a depuration period of at least 14 days. Both plasma and mucus VTG display the sensitivity needed to assess estrogenic exposure in real-world applications (Kostich, Flick, and Martinson, 2013). We have also examined the expression timing and sensitivity of *vtg* in the same experimental system and found it to be a rapid and sensitive measure of estrogenic exposure. The kinetics of mRNA expression differ from that of protein VTG, suggesting the possibility to apply them in concert to discriminate recent or on-going estrogenic exposures from those that occurred within 14 days. The kinetic information could be useful in developing a computational model to link biomarker responses to chemical concentrations similar to one created by (Watanabe et al., 2009). We found the RT-qPCR assay to be the most affordable and reliable method overall and the mucus ELISA to be a viable alternative to the traditional plasma ELISA in terms of sensitivity and ease of use. In small fish species that were otherwise inaccessible using plasma-based methods due to difficulty in obtaining sufficient blood volume, the mucus method may provide a means to monitor VTG. Lastly, because the mucus can be applied to intact organisms, it can be used to evaluate estrogenic exposure in threatened and endangered species or in longitudinal studies.

5. Disclaimer

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CRediT authorship contribution statement

J. Lazorchak conceived of the experiments. All authors designed experiments and collected samples. D. Bencic and R. Flick operated and maintained the diluter system during exposure and coordinated with chemistry contractor for sample collection. M.J. See conducted all molecular assays, statistical analyses and prepared the manuscript. R. Flick checked data. A. Biales edited the manuscript and supervised the project.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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