BIODIVERSITAS
Volume 23, Number 11, November 2022
Pages: 5585-5592

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231106

Identification and quantification of vitellogenin gene in eyebrow goby (*Oxyurichthys ophthalmonema*)

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Manuscript received: 23 September 2022. Revision accepted: 27 October 2022.

Abstract. *Sa-An AC, Quinitio GF, Anasco NC, Traifalgar RFM, Nillos MGG. 2022. Identification and quantification of vitellogenin gene in eyebrow goby (*Oxyurichthys ophthalmonema*). Biodiversitas 23: 5585-5592.* Vitellogenin (VTG) gene has been used as a biomarker for exposure to estrogenic compounds in marine and freshwater fish species. There was limited information about the detection of VTG mRNA in an estuarine environment using a bioindicator fish species. In this study, a quantitative real-time polymerase chain reaction (qPCR) assay was developed for quantification of VTG mRNA normalized to β-actin in eyebrow goby, *Oxyurichthys ophthalmonema*. VTG mRNA in the liver of reproductively mature female samples was isolated, amplified, and sequenced. There were 430 bp fragments of VTG, corresponding to a 143 amino acid sequence obtained from eyebrow goby. The partial protein sequence exhibited similar identities with the phosvitin motif of VTG in other gobies. The new qPCR assay was developed through the evaluation of hepatic mRNA levels in nonreproductive male eyebrow goby at 0, 6, 12, and 24 h following intraperitoneal injection of 10 mg kg^{-1} 17β-estradiol. Hepatic mRNA levels were detected 6 h after 17β-estradiol injection and the highest VTG expression was noted 24 h after injection. Results of the present study suggest that male eyebrow goby can be used as indicator species for estrogenic contamination. Likewise, the established qPCR assay can be used to assess recent exposure to estrogenic contaminants in the aquatic environment. The characterization of the amplified VTG gene from eyebrow goby and the application of the qPCR assay developed for eyebrow goby in monitoring EDCs in the field may be considered for future investigation.

Keywords: 17β-estradiol, endocrine disrupting chemical, mRNA indicator, *Oxyurichthys ophthalmonema*, qPCR, vitellogenin

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) have been documented to be present in aquatic systems and they have been reported to have adverse effects on both freshwater and marine organisms (Celino-Brady et al. 2019). Endocrine-disrupting chemicals (EDCs) are exogenous substances or mixtures that disrupt the functions of the endocrine system in organisms, their progeny, and/or populations (Celino-Brady et al. 2021). EDCs can elicit responses even at low concentrations and are increasingly concerned about the environment (Dietrich and Krieger 2009). EDCs have been found to alter endocrine function by mimicking, altering, or inhibiting the action of endogenous hormones responsible for endocrine homeostasis (Söffker and Tyler 2012). They are found to occur in industrial wastes, agricultural runoff, and municipal wastewater effluents (Al-Odaini et al. 2012; Barra et al. 2021).

It is well-established that the liver vitellogenin (VTG) of fish can be used as a sensitive biomarker of endocrine disruption (Blanchet-Letrouvé et al. 2013; Zhong et al. 2014). In female fish, VTG is synthesized in the liver and its synthesis is under hormonal control. The presence of VTG mRNA in the liver indicates the sexual maturity of the female fish because it correlates with the maturation of oocytes (Hara et al. 2016). However, VTG in nonreproductive males and adult males is an indication of exposure to estrogenic contaminants (Rawat et al. 2013; von Osten et al. 2019). Hence, VTG mRNA in the male liver can be used as a biomarker for environmental estrogen.

In measuring VTG mRNA in the liver, various methods have been developed using quantitative real-time polymerase chain reaction (qPCR) primers specifically designed for certain fish species to amplify gene fragments for expression studies. The use of qPCR assay for VTG mRNA in several teleosts has been developed in other countries (Ciu et al. 2017; Mushirobira et al. 2020) and they are used as the basis for policy formulation and monitoring aquatic environments. In previous studies, optimization of the qPCR assay is done using sexually mature females (Bowley et al. 2010) or hormonally induced males (Caspillo et al. 2014; Negintaji et al. 2018) to ensure the presence of VTGs and then characterize the isolated VTG, and sequenced. Some fish species used in VTG quantification studies were zebrafish, *Danio rerio* (Humble et al. 2013; Chen et al. 2018), tilapia, *Oreochromis mossambicus* (Celino-Brady et al. 2019; Chen et al. 2020), These studies showed that the presence of VTG in male fish is a good biomarker for contamination of estrogens.

In the Philippines, the assay that has been employed in determining estrogenic contamination in fish was the enzyme-linked immunosorbent assay ELISA (Paraso et al. 2017). ELISA is a protein-based assay that is considered expensive, and time-consuming. VTG proteins have high inter-species variability and antibodies usually show poor inter-species cross-reactivity. This makes it difficult to use protein-based assays in non-model species, hence there is a need to develop a rapid and accurate qPCR assay for the VTG gene in the liver of the eyebrow goby.

For the development of the qPCR assay, eyebrow goby, *Oxyurichthys ophthalmonema* (Bleeker, 1856), was used since it is an easy to catch, abundant, and widely distributed species in the Philippines. In addition, eyebrow goby caught in Lagatik River, part of the New Washington -Batan Estuary, has the characteristics of a sentinel species that are useful in studying EDCs. The collection site was presumed to be not contaminated with EDCs. The New Washington-Batan Estuary borders the municipalities of New Washington, and Altavas, in Aklan Province, Philippines. The Batan estuary is surrounded by mangroves and it is the major source of many fishery products in the area. Recently, little or no available data on the activity of EDCs in the Batan estuary have been reported.

In this study, a sensitive and effective qPCR assay for the quantification of hepatic VTG mRNA was developed to determine the VTG mRNA expression of eyebrow goby from Lagatik River, New Washington, Aklan. To our knowledge, this is the first description of a methodology for developing qPCR assay for VTG in the Philippines and the first report on the detection of VTG mRNA from an estuarine environment using eyebrow goby as indicator species.

MATERIALS AND METHODS

Experimental animals

Eyebrow goby were collected from Lagatik River, part of the New Washington-Batan Estuary. The New Washington-Batan Estuary borders the municipalities of New Washington, and Altavas, in Aklan Province, Philippines. The Batan estuary is surrounded by mangroves and it is the major source of many fishery products in the area (Kamiyama 2015). Recently, little or no available data on the activity of EDCs in Batan estuary have been reported. The samples were presumed to be not contaminated with EDCs. After collection, fish were brought to the Aklan State University Laboratory, New Washington Campus. It was difficult to distinguish male from female goby through external morphological identification, hence, the sex of the fish was only known after dissection and examination of the gonads using a dissecting microscope. Out of 40 eyebrow goby, there were only 15 female goby and they were measured for body weight (BW) to the nearest 0.1g and total length (TL). The liver of three female goby samples was used for sequencing and optimization for the qPCR assay. The obtained VTG from female fish (positive control) served as a reference for the qPCR amplification products.

Laboratory exposure to 17β-estradiol

After one week of acclimatization, 40 eyebrow goby were individually distributed in a 3-L capacity container covered with black plastic. They were fed with Ocean Free (OF) Super Mineral (complete food for tropical fish) once a day during the acclimation and exposure period. The fish were injected with 10 mg kg^{-1} 17β-estradiol suspended in canola oil. Before intraperitoneal injection, fish were anesthetized with 100 ppm 2-phenoxyethanol. Then, the fish were returned to their respective containers after injection. Salinity (range 29-32 ppt) and temperature (range: 26-28°C) were measured at the beginning of the exposure. After 0, 6, 12, 24, and 48 h of injection, ten fish every sampling hour were euthanized with 100 ppm 2-phenoxyethanol, and their livers and gonads were collected. After dissection, there were only three or four males out of ten fish every sampling hour. These were used for RNA isolation and subsequent analysis.

Total RNA isolation

Total RNA isolation was done using the Trizol RNA Isolation protocol of W.M. Keck Foundation Biotechnology Microarray Resource Laboratory at Yale University (New Haven, Connecticut, USA). The extraction process with 5 major steps, which include homogenization, phase separation, RNA precipitation, RNA washing, and RNA redissolving. Liver tissue samples in each fish were homogenized in 1 mL of TRIZOL reagent using a homogenizer. Around 200 µL chloroform was then added, vortexed vigorously for 15 sec, and incubated at room temperature for 2-3 min. Samples were then centrifuged at 12500 rpm for 15 min at 4°C. After centrifugation, the colorless upper aqueous phase was transferred into another tube. RNA was precipitated from the aqueous phase by mixing with 500 µL isopropyl alcohol. Samples were then incubated at room temperature for 10 min and centrifuged at 12500 rpm for 10 min at 4°C. The RNA pellet was washed twice with 1000 µL 75% ethanol, vortexed, and centrifuged at 7500 rpm for 5 min at 4°C. The remaining alcohol was then removed completely. The RNA pellet was air-dried for 10 min and dissolved in 25 µL of Nuclease-free water.

cDNA synthesis

The cDNA was made using the iScriptTM cDNA Synthesis kit (Bio-Rad). The master mix was prepared on ice with the following components; 2 µL 5x iScript Reverse Transcription Supermix, 5.5 µL Nuclease-free water, and 2.5 µL of Total RNA.

The following conditions were used for PCR: 25°C for 5 min (primer annealing), 46°C for 20 min (reverse transcription) 95°C for 1 min (inactivation), and 4°C holds.

Gel electrophoresis

The Total RNA was run in a 2.0% agarose gel with gel red stain in 1X TBE. The gel was placed on the entire gel cassette in the gel electrophoresis (Enduro Gel XL) rig and a 1XTBE running buffer was added to the rig. The gel was submerged in a running buffer. Using a pipette, 2 µL of the RNA sample was transferred in an Eppendorf tube and 1

µL 6x loading dye was added to the sample. The 2 µL 1kb ladder was also premixed with loading dye. The electrophoresis was performed at 100 volts for 30 min.

Isolation of VTG and PCR

The cDNA of the target VTG of eyebrow goby was amplified using the primers for VTG (Barucca et al. 2006). The PCR was performed in a T100TM thermocycler (Bio-Rad) using 1 µL cDNA template, 3 µL nuclease-free water, 5.0 µL 2x Taq master mix, and 0.5 µL VF1, 0.5 µL VR2 with PCR conditions: 94°C, 2 min (Initial denaturation), 94°C, 30 sec (Denaturation), 55°C, 45 sec. (Annealing), 72°C, 30 sec (Extension), 72°C, 5 min (F.E) for 30 cycles. The amplification products were tested for size on 2% gel containing gel red. Table 1 shows the primers used for the amplification of eyebrow VTG.

Sequencing and sequence alignment

The PCR products were sent and sequenced at the Macrogen Kinovette Scientific Solution (Quezon City, Philippines). The sequencing lasted for two weeks. To align the sequences obtained, MEGA 7.0 software was used. Chromatograms of forward and reverse sequences were trimmed and a consensus sequence of VTG was made. Using Nucleotide BLAST, the 430 bp VTG sequence of eyebrow goby, was compared with other species using percent identity and query cover. Translation of nucleotide sequences into amino acid sequences was done using the Translate tool ExPASy software, while BLAST analysis or computation for the amino acid sequence was performed using the BLAST network service (BLASTP (version 2.10.1+).

Phylogenetic analysis

To determine the phylogenetic position of the samples, the VTG gene sequences were aligned with those of other species available in the GenBank (www.ncbi.nih.gov) using MEGA 7.0 software: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequences Alignment (Kumar and Tamura 2016).

The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) in the units of the number of base substitutions per site. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 215 positions in the final data set. The tree was inferred through Minimum Evolution with 500 replicates. Evolutionary analyses were conducted in MEGA version 7 (Kumar and Tamura 2016).

Quantitative real-time PCR

Optimization of the qPCR assay was done using mature female eyebrow goby. The obtained VTG from the mature female was used as a positive control in this study. Isolation of eyebrow goby VTG was performed using cDNA prepared from a total RNA sample isolated from the liver of a female. For VTG isolation, the primer sequences used were designed for various species (Barucca et al. 2006): VF1: 5´-CAGGTNTTRGCWCARGAYTG-3´ and VR2 5´-AGRMASMACCCAGGARTGVGC-3´. The β-actin primers used were originally designed for *Sparus aurata* (Goncalves et al. 2007): Forward 5'-GATCATGTTCGAGACCTTCAA--3' and Reverse 5'-TCCAATCCAGACAGAGTATTTACG-3' (Table 1).

All qPCR reactions were done on a CFX Connect Real-Time PCR (Bio-Rad) using the IQTM SYBR Green Supermix qPCR kit (Bio-Rad). The components for qPCR for VTG were: 5.0 µL IQ SYBR green, 0.5 µL VF1, 0.5 µL VR2, 3.4 µL nuclease-free water, 1.0 µL cDNA, while for the housekeeping gene (β-actin): 5.0 µL IQ SYBR green, 0.5 µL β-actin F, 0.5 µL β-actin R, 3.4 µL nuclease-free water, 1.0 µL cDNA. The qPCR conditions were: 94°C, 2 min (Initial denaturation), 94°C, 30 sec (Denaturation), 55°C, 45 sec. (Annealing), 72°C, 30 sec (Extension), 72°C, 5 min (F.E) for 30 cycles. Triplicate qPCR reactions were performed for each sample, including β-actin and VTG gene. The VTG single melting temperature peak was 84.0±1°C while β-actin had 86.50±1°C. The qualities of the PCR amplifications were validated by comparing them to the positive control. Expression levels of VTG for individual fish were normalized using β-actin (housekeeping genes) expression levels and fold changes were calculated based on the expression levels in the laboratory exposure control group.

Table 1. The forward and reverse primers used to amplify the vitellogenin (Barucca et al. 2006) and β-actin (Goncalves et al. 2007) in eyebrow goby

Primer	Sequence	Monomers
VTG		
VFI (forward)	5´-CAGGTNTTRGCWCARGAYTG-3´	20
VR2 (reverse)	5´-AGRMASMACCCAGGARTGVGC-3´	21
β-actin		
B-actin (forward)	5´- GATCATGTTCGAGACCTTCAA-3'	21
B-actin (reverse)	5´- TCCAATCCAGACAGAGTATTTACG-3'	24

RESULTS AND DISCUSSION

Identity of VTG gene from eyebrow goby

Out of the 15 female samples collected from Lagatik River, New Washington, Aklan, central Philippines, only 3 samples were processed for RNA extraction. Table 2 shows the mean BW of 10.4 g, 128.1 mm total TL, GSI of 1.56%, and 5.0% HSI. The amplified products using the VF1/VR2 primers combination (Barucca et al. 2006) and β-actin primers formed distinct bands at around 500 bp and 660 bp, respectively (Figure 1). Sequencing of the amplified partial VTG gene revealed 430 bp nucleotide fragments corresponding to 143 amino sequences (Figure 2). The VTG partial nucleotide sequences obtained were processed at BLAST (www.ncbi.com) and compared with other VTG genes available at GenBank. BLAST results (Table 3) showed that the partial nucleotide sequence of VTG gene in eyebrow goby was 71-75% identical with *Boleophthalmus pectinirostris*, 70% similar to yellowfin goby, *Acanthogobius flavimanus* and yellowfin goby, *Acanthogobius hasta*, 62.27% similar identity to black goby, *Gobius niger*, and 65.83% yellowstripe goby, *Mugilogobius chulae*. The amino acid sequences obtained in this work was aligned with other fish VTG sequences used in the study of Barucca et al. (2006) (Table 4) using the MEGA version 7.0 software set at the default parameters. The translated partial nucleotide sequence of eyebrow goby revealed similarities with the partial protein sequence of the phosvitin (Pv) domain of VTG in other fishes (Table 5).

Comparison of eyebrow goby partial protein sequence with other gobies such as *A. flavimanus* (Q8JIF9), *A. hasta* (A7E1Q7), mudskipper, *Periophthalmus magnuspinnatus* (A0A3B4AF19), *M. chulae* (A0A0HJLA6), *G. niger* (Q3ZUB4) exhibited 80/143 (5%), 80/143 (56%), 79/143 (55%), 72/144 (50%), 56 /132 (42%) sequence identities. The phylogenetic tree shows the affiliation of eyebrow goby VTG with other fish species (Figure 3). The tree was inferred through Minimum Evolution with 500 replicates. Eyebrow goby VTG rooted together with bootstrap support of 100% and nested 100% with *G. niger*.

Table 2. Body weight (BW), total length (TL), gonadosomatic index (GSI), and hepatosomatic index (HSI) of female eyebrow goby caught at Lagatik River, New Washington, Aklan, central Philippines that were used for the PCR Assay Determination

Number	BW (g)	TL (mm)	GSI (%)	HSI (%)
1	13.83	132.6	1.01	4.77
2	9.45	127.8	2.01	2.65
3	7.92	124.0	1.67	7.58
MEAN	10.4	128.1	1.56	5.0
SD	3.07	4.31	0.51	2.47

Table 4. List of partial VTG gene of other fish species from the GenBank

Species	GenBank Acc. no.
Anguilla anguilla partial vtg gene for vtg exons 1-4	AMO76792.1
Anguilla Anguilla partial mRNA for vtg (vtg gene)	AMO76793.1
Thunnus thynnus partial mRNA for vtg 1 (vtg 1 gene)	AMO76794.1
Thunnus thynnus partial mRNA for vtg 2 (vtg 2 gene)	AMO76795.1
Mullus barbatus partial mRNA for vtg (vtg gene)	AMO76796.1
Mugil cephalus partial mRNA for vtg (vtg gene)	AMO76797.1
Gobius niger partial mRNA for vtg (vtg gene)	AMO76798.1
Gobius niger partial mRNA for vtg (vtg gene)	DQ073804.1

Table 3. BLAST result of the eyebrow goby VTG sequences as compared to VTG- like of other fish species from the GenBank

Species	Percent Identity (%)	GenBank accession numbers
PREDICTED: : *Boleophthalmus pectinirostris* vitellogenin-1-like (LOC110156390), mRNA	71.09	XM_020920594.1
Acanthogobius flavimanus Vg- 530 mRNA for vitellogenin, complete cds	70.85	AB088473.1
Acanthogobius hasta vitellogenin mRNA, complete cds	70.38	AY677084.1
PREDICTED: : *Boleophthalmus pectinirostris* vitellogenin-1-like (LOC110160031), mRNA	75.47	XM_020924633.1
Gobius niger vitellogenin-like mRNA, partial sequence	65.27	DQ073804.1
Mugilogobius chulae vitellogenin (Vg), mRNA, complete cds	65.83	KP162167.1

Table 5. BLASTP result of the eyebrow goby VTG partial protein sequence as compared to other fish species from the GenBank

Description of goby species	Score	E-value	Accession number
Phosvitin OS: *Acanthogobius flavimanus*	181 bits (458)	7e-50	Q8JIF9
Phosvitin OS: *Acanthogobius hasta*	180 bits (456)	2e-49	A7E1Q7
Phosvitin OS: *Periophthalmus magnuspinnatus*	176 bits (447)	6e-49	A0A3B4AF19
Phosvitin OS: *Mugilogobius chulae*	153 bits (384)	7e-40	A0A0HJLA6
VTG fragment: *Gobius niger*	114 (285)	9e-30	Q3ZUB4

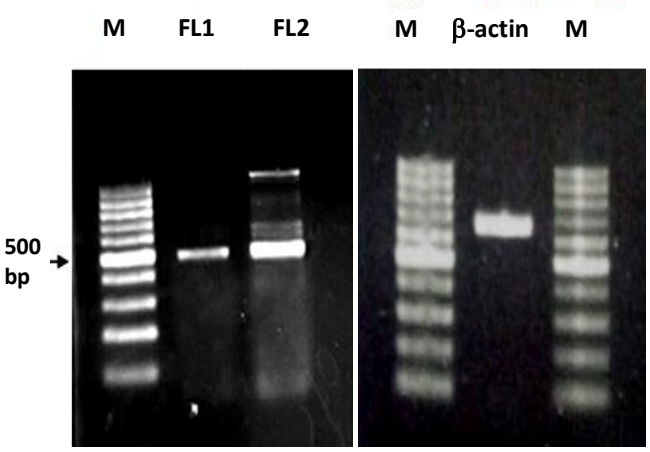

Figure 1. A. Vitellogenin fragments obtained by RT-PCR and β-actin from liver total RNA of eyebrow goby. (M) molecular marker (100-1000 bp ladder), FL1 and FL2 female goby

The use of VTG mRNA expression as a marker for EDCs through conventional (Barucca et al. 2006) or qPCR has been reported in many studies (Bowley et al. 2010; Ferreira et al. 2013; Caspillo et al. 2014; Duffy et al. 2014; Ishibashi et al. 2016; Chen et al. 2018; Chen et al. 2020) or by advanced macroarray technology (Hirakawa et al. 2012; Zare et al. 2018). The use of these assays has more advantages compared to the early assay systems, such as the use of immunological methods like immunodiffusion or immunoelectrophoresis, radioimmunoassay (RIA), and ELISA. The use of qPCR has been proven to be a reliable and sensitive approach to demonstrate endocrine disruption in laboratory hormone-induced medaka, *Oryzias latipes* (Isibashi et al. 2016), Atlantic salmon, *Salmo salar* (Duffy et al. 2014,) zebrafish, *Danio rerio* (Humble et al. 2013; Chen et al. 2018), tilapia, *Oreochromis mossambicus* (Celino-Brady et al. 2019; Chen et al. 2020), as well as in fishes caught from the field or wild populations (Barucca et al. 2006; Bowley et al. 2010).

```
1   CAGGTTTTGGCTCAGGATTGTAGTGATGAGCTTCAATTCGTCATTTTGCTGAGGAAAGAC   60
      Q  V  L  A  Q  D  C  S  D  E  L  Q  F  V  I  L  L  R  K  D_20

61  AGTGCTGGCCGTGACAAGATCTATGTTAGACTTGGTGAAAAGGAAATCGAGATGTACTTT  120
      S  A  G  R  D  K  I  Y  V  R  L  G  E  K  E  I  E  M  Y  F_40

121 AATGGAGACAGACCATTTGTTACGATTGATAAAAAGGGCATTTCCATCGACAACCTGCCT  180
      N  G  D  R  P  F  V  T  I  D  K  K  G  I  S  I  D  N  L  P  Y-60

181 TACAACAAAGATTCAGTGAAGATTGAGTTGATTGAGAAGGAGGACTACAGGGGGCTGGCT  240
      Y  N  K  D  S  V  K  I  E  L  I  E  K  E  D  Y  R  G  L  A_80

241 ATGAAAGCTCTGCAGTATGGCATCGCTGACCTCTTCTTCAGTGCTAATGATGTGGAGCTC  300
      M  K  A  L  Q  Y  G  I  A  D  L  F  F  S  A  N  D  V  E  L_100

301 TACATGCCAGAAACTTTCAGGAACAAAGTGTGTGGTCTGTGTGGTCAAGCAAATGGGGAC  360
      Y  M  P  E  T  F  R  N  K  V  C  G  L  C  G  Q  A  N  G  D-120

361 AGAAAAAACGACTCCCGCATGCCCGACGGCCGTGTGACCAATAACCCTATCAGCTTTGCC  420
      R  K  N  D  S  R  M  P  D  G  R  V  T  N  N  P  I  S  F  A_140

421 CACTCCTGGG  460
      H  S  W_143
```

Figure 2. Eyebrow goby vitellogenin partial sequence of 430 bp and the corresponding 143 partial protein sequence

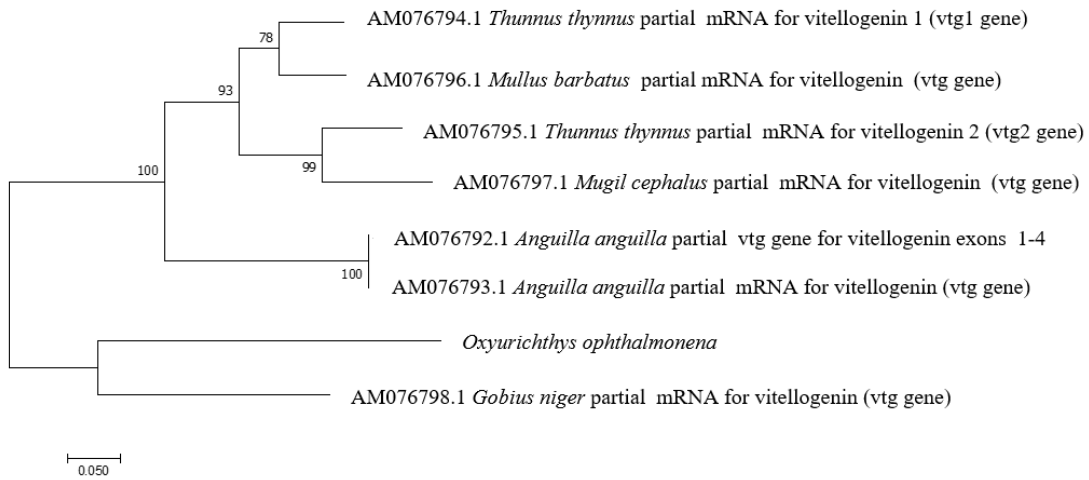

Figure 3. Phylogenetic tree inferred from the combined vtg genes

In this study, the qPCR assay developed for eyebrow goby was optimized for the VTG gene. The products were tested by melt-curve analysis to conform to 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 84.0±1°C for VTG and 86.50±1°C for β-actin (housekeeping gene). In the negative control (no cDNA template) no amplification products were observed indicating that primer dimerization or nontarget amplification was not the issue for quantification. For other species like shanny, *Lipophrys pholis*, the melting temperature for 167bp VTG I and 161 VTG II amplicons were 83.5°C and 83°C respectively (Ferreira et al. 2013). In round goby, melting temperature varies with 77.3°C, 83.8°C, and 82.3°C for VTG II and VTG III, and 18SrRNA. These findings show that each VTG mRNA type differs in the melting temperature and it should form a single peak in every reaction during qPCR assay (Barucca et al. 2006).

The present study used female eyebrow goby to determine the partial VTG genes. The amplified product using the VF1/VR2 primer combination (Barucca et al. 2006) formed distinct bands at around 500 bp. After sequencing these amplified fragments, the total VTG nucleotide fragments were 430 bp. The obtained VTG sequence was compared to other VTG genes in the GenBank. The partial VTG genes obtained from the liver samples of eyebrow goby are in agreement with other studies that demonstrated the presence of VTG genes in the liver (Barucca et al. 2006; Bowley et al. 2010; Ferreira et al. 2013). The obtained partial VTG sequence corresponds to 143 amino acids. The partial protein sequence of eyebrow goby, when compared with other gobies such as *A. flavimanus* (Q8JIF9), *A. hasta* (A7E1Q7), *P. magnuspinnatus* (A0A3B4AF19), *M. chulae* (A0A0HJLA6), *G. niger* (Q3ZUB4), exhibited high sequence identities (Figure 4).

The phylogenetic analysis confirmed that eyebrow goby nested 100% with *G. niger*. Barucca et al. (2006) reported that the *G. niger* VTG is intermediate between VTG type I and VTG type II indicating that the obtained sequence is not identified as type I or type II. In the present study, it was not categorized as type I or type II VTG. Results of the BLASTP analysis confirmed that the obtained partial nucleotide sequences code for partial protein sequence in eyebrow VTG, particularly Pv motif (Table 5). This Pv region in the C-terminus is highly conserved, such that many studies in fishes designed VTG degenerate primers based on the Pv region (Bowman et al. 2000).

The present study successfully isolated and amplified the target VTG genes in eyebrow goby using primers which were designed for various fish species (Barucca et al. 2006). Since this is the first report on the partial VTG gene of eyebrow goby, there is still a need to design qPCR primers to better understand the nature and type of VTG genes as well as the mechanism of vitellogenesis in the eyebrow goby.

Quantification of VTG gene from eyebrow goby using qPCR

When the fish were injected with E2, results showed elevated levels of hepatic mRNA VTG transcripts in fish with 10 mg kg^{-1} E2 after 6 h (0.22 fold changes over the control), but a decrease in the number of mRNA transcripts at 12 h (0.05 fold changes over control) injection was also noted (Table 6). The VTG mRNA transcripts in the treated groups were induced 6 h after injection with E2 and the highest induction was observed after 24 h (25.65 fold changes over control) exposure. VTG mRNA level in females (positive control, 81.34 fold changes over control) showed 3-500 times higher than the treated groups. The findings showed no time-dependent increase in VTG mRNAExposure for 48 h (4.34 fold changes over control) resulting in a noticeable decrease in VTG mRNA level.

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M.   QVLAQDCTSSLNFIVLLKKDSSNRYEINAKIGRDWDIDMRYNEERPTVTINGQEIARESL
G.   -------SDKPEFLVLLKKESEQSYAVSVKIG-TDDIDMFIKEGRKAVKVNEQEVAMANL
A.   QLLAQDCTDELKFIVLLKKDSANRYMVNVKIG-TKDIDMFLDGERPAVMINGKEIARESL
A1.  QLLAQDCTDELKFIVLLKKDSANRYMVNVKIG-TKDIDMFLDGERPAVVINGKEIARQSL
P.   QLMAQDCTDDLKFIVLMKKDSADRHMVNVKIG-TNDIDMLLNGEIPTVTMNGKEIARDSL
O.   QVLAQDCSDELQFVILLRKDSAGRDKIYVRLG-EKEIEMYFNGDRPFVTIDKKGISIDNL
            :.. :*::*::*:*     : .::*   :*:*  .     * :: : ::  .*

M.   PYNKDSFKIELKNSNK---LVLYAKEFGITELNFSNMEVELHILNDYRNRVCGLCGQANG
G.   PYVKDSIKIELKDDK----LVLYAPRSGISEIFFNNQEVTVHVAGTMRNKVCGLCGQGNG
A.   PYNKDSVSIRLTESNM---LVLSAQRFGITELHLSSTGVEIHVPEYLRNRVCGLCGQANG
A1.  PYNKDSVSIRLTESNT---LVLSAQRFGITELHLSNTGVEIHVPEYLRNRVCGLCGQANG
P.   PYNKDSVKIEMKAGNK---IVLYAQTFGITELHFSASDVSIRVPEYMRNRVCGLCGQGNG
O.   PYNKDSVKIELIEKEDYRGLAMKALQYGIADLFFSANDVELYMPETFRNKVCGLCGQANG
     ** ***..*.:   :    :.: *   **::: :.   * : :    **:*******.**

M.   DKRNDLRMPNGNLNDNPVSFVSSW
G.   DDQSDYRTPKGRVIDSPVSF----
A.   DRKNDSRMPDGRVTNNPISFAHSW
A1.  DKRNDFRLPNGRVTDNPISFAHSW
P.   DRRNDYRMPSGRVTDNPISFAHSW
O.   DRKNDSRMPDGRVTNNPISFAHSW
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Figure 4. Comparison of the partial protein sequence of eyebrow goby with other gobies. M: *Mugilogobius chulae*; G: *Gobius niger*; A: *Acanthogobius flavimanus*; A1: *Acanthogobius hasta*; P: *Periophthalmus magnuspinnatus*; O: *Oxyurichthys ophthalmonena*

Table 6. Expression of VTG gene in the liver of male eyebrow goby exposed to 10 mg kg^{-1} E2 after 6 h, 12 h, 24 h, 48 h. Values were normalized against β-actin as a housekeeping gene and represent relative mean RNA expression value (n: 3) to the 0 h as the negative control. Expression in positive control served as the reference for the qPCR amplification products

Hours after injection	mRNA level (fold gene expression)	SD
0	0	0
6	0.22	0.24
12	0.05	0.03
24	25.65	29.04
48	4.34	4.34
Standard (mature female)	81.34	27.00

In this present study, a qPCR assay was developed to detect VTG mRNA in male eyebrow goby. Using the established qPCR assay, a considerable amount of VTG genes was noted after 6 h injection with E2, indicating that E2 was able to induce expression of VTG gene on male goby. The highest expression of VTG mRNA was recorded after 24 h and a decrease in the quantity at 48 h. Previous studies reported that the qPCR assay could detect recent exposure to estrogenic contamination either by injection (Negintaji et al. 2018; Azad and Al-Jandal 2020) or water exposure (Hirakawa et al. 2012; Liang et al. 2012; Lei et al. 2013,). The mud carp, *Cirrhinus molitorella* juvenile fish in the three E2-exposed groups (5, 50, and 500 ng l-1) had significantly higher levels of VTG expression in their livers than the control group, and these levels were time-dose dependent on days 2, 4, and 6 (Liang et al. 2012).

Previous research showed the induction of VTG mRNA transcripts in immature male fish after injection of E2 (Chen et al. 2020). The male eyebrow goby collected for this study were all immature. It should be noted that there was considerable variation in the VTG mRNA expression level in individual eyebrow goby samples. As reported in other studies, the fish-to-fish variation in VTG mRNA might be due to the kinetics of induction and clearance of mRNA and the inaccuracy during delivery of the different compounds due to the different sizes of rainbow trout, *Oncorhynchus mykiss)* (Celcius et al. 2000). Furthermore, Celcius et al. (2000) reported that repeated doses using larger fish showed a lower variability within a treatment group. In this study, it was presumed that the Lagatik River was not contaminated by EDCs. However, there is a need to confirm this in future investigations of the possibility of exposure to EDCs prior to laboratory experiments by measuring hormone content in both water and sediment samples.

In conclusion, a newly developed qPCR assay was used in this study to quickly determine estrogenic effects in male eyebrow goby exposed to E2. VTG was effectively induced in the liver of male goby after short-term exposure to 10 mg kg^{-1} E2 and could be used as a biomarker for estrogenicity determination. These findings could be used as a starting point for future research into the long-term effects of exogenous estrogenic EDC exposure on testicular development and other reproductive capacities in eyebrow goby and other fishes. The findings of this study also suggest that male eyebrow goby could be used to monitor estrogenic effects in the shortest time possible.

ACKNOWLEDGEMENTS

This study was sponsored in part by the Department of Science and Technology-Accelerated Science and Technology Human Resource Development Program (DOST- ASTHRDP) and the Office of the Vice Chancellor for Research and Extension (OVCRE) University of the Philippines Visayas, Miagao, Iloilo. The authors would like to thank the assistance of Ms. Rowena Cadiz of UPV Miagao, Iloilo, and Dr. Casiano H. Choresca and his staff at Fisheries Biotechnology Center, Science City of Muñoz, Nueva Ecija. The authors are also grateful to Dr. Yasmin Primavera-Tirol and Prof. Ronald Maliao of Aklan State University, New Washington Campus for the use of facilities during the laboratory experiments. There is no conflict of interest.

REFERENCES

Al-Odaini NA, Zakaria MP, Yaziz MI, Surif S, Kannan N. 2012. Occurrence of synthetic hormones in sewage effluents and Langat River and its tributaries, Malaysia. Intl J Environ Anal Chem 1-13. DOI: 10.1080/03067319.2012.727810.

Azad IS, Al-Jandal N. 2020. Induction of vitellogenin in cultured fish: Vitellogenin gene expression response of blue fin and yellow fin breams to injected 17β-estradiol. J Environ Chem Ecotoxicol 12 (1): 8-16. DOI: 10. 5897/jece2019.0448.

Barra RO, Chiang G, Saavedra MF, Orrego R, Servos MR, Hewitt LM, McMaster ME, Bahamonde P, Tucca F, Munkittrick KR. 2021 Endocrine disruptor impacts on fish from Chile: The influence of wastewaters. Front Endocrinol 12: 611281. DOI: 10.3389/fendo.2021.611281.

Barucca M, Canapa A, Olmo E, Regoli F. 2006. Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterranean fish species. Environ Res 101: 68-73. DOI: 10.1016/j.envres.2005.07.002.

Blanchet-Letrouvé I, Lafont AG, Poirier L, Baloche S, Zalouk-Vergnoux A, Dufour S, Mouneyrac C. 2013. Vg mRNA induction in an endangered fish species (*Anguilla anguilla*) from the Loire estuary France. Ecotoxicol Environ Saf 97: 103-113. DOI: 10.1016/j.ecoenv.2013.07.019.

Bowley LA, Alam F, Marentette JR, Balshine S, Wilson JY. 2010. Characterization of vitellogenin gene expression in round goby (*Neogobius melanostomus)* using quantitative polymerase chain reaction. Environ Toxicol Chem 29: 2751-2760. DOI: 10.1002/etc.324.

Bowman CJ, Kroll KJ, Hemmer MJ, Folmar LC, Denslow ND. 2000. Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). Gen Comp Endocrinol 120: 300-313. DOI: 10.1006/gcen.2000.7565.

Caspillo NR, Volkova K, Hallgren S, Olsson PE, Porsch-Hällström I. 2014. Short-term treatment of adult male zebrafish (*Danio rerio*) with 17α-ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation. Comp Biochem Physiol Part-C: Toxicol Pharmacol 164: 35-42. DOI: 10.1016/j.cbpc.2014.04.003.

Celius T, Matthews JB, Giesy JP, Zacharewski TR. 2000. Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiate and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17 beat or alpha-zearalenol. J Steroid Biochem Mol Biol 75 (2-3): 109-119. DOI: 10.1016/S0960-0760(00)00165-5.

Celino-Brady FT, Petro-Sakuma CK, Breves JP, Lerner DT, Seale AP. 2019. Early-life exposure to 17β-estradiol and 4-nonylphenol impacts

the growth hormone/insulin-like growth-factor system and estrogen receptors in Mozambique tilapia, *Oreochromis mossambicus*. Aquat Toxicol 217: 105336. DOI: 10.1016/j.aquatox.2019.105336.

Celino-Brady FT, Lerner DT, Seale AP. 2021. Experimental approaches for characterizing the endocrine-disrupting effects of environmental chemicals in fish. Front Endocrinol 11: 1-21. DOI: 10.1016/j.cbpc.2017.11.010.

Chen M, Zhang J, Pang S, Wang C, Wang L, Sun Y, Song M, Liang Y. 2018. Evaluating estrogenic and anti-estrogenic effect of endocrine disrupting chemicals (EDCs) by zebra fish (*Danio rerio*) embryo-based vitellogenin 1 (vtg1) mRNA expression. Comp Biochem Physiol C Toxicol Pharmacol 204: 45-50. DOI: 10.1016/j.cbpc.2017.11.010.

Chen J-R, Wu SM, Tsai SC, Hsien FC, Huang CT. 2020. Changes in vitellogenin and estrogen receptor expression and 17β-estradiol concentration in male juvenile tilapia can be used to evaluate endocrine-disrupting chemicals. Comp Biochem Physiol C Toxicol Pharmacol 229: 108682. DOI: 10.1016/j.cbpc.2019.108682.229.

Cui XF, Zhao Y, Chen HP, Deng SP, Jiang DN, Wu TL, Zhu CH, Li GL. 2017. Cloning, expression and functional characterization on vitellogenesis of estrogen receptors in *Scatophagus argus*. Gen Comp Endocrinol 246: 237-245. DOI: 10.1016/j.ygcen.2017.03.002.

Dietrich DR, Krieger HO. 2009. Histological analysis of endocrine disruptive effects in small laboratory fish. Vol 1. John Wiley & Sons Inc, New Jersey.

Duffy T A, Iwanowicz LR, Mccormick SD. 2014. Comparative responses to endocrine disrupting compounds in early life stages of atlantic salmon, *Salmo salar*. Aquat Toxicol 152: 1-10. DOI: 10.1016/j.aquatox.2014.03.015.

Ferreira F, Monteiro NM, Vieira MN, Reis-Henriques MA, Castro LF, Santos MM. 2013. A real-time PCR assay for differential expression of vitellogenin I and II genes in the liver of the sentinel fish species *Lipophrys pholis*. Toxicol Mech Methods 23 (8): 591-597. DOI: 10.3109/15376516.2013.809620.

Gonçalves AF, Castro LF, Pereira-Wilson C, Coimbra J, Wilson JM. 2007. Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish?. Comp Biochem Physiol D Genom Proteom 2 (4): 345-355. DOI: 10.1016/j.cbd.2007.08.002.

Hara A, Hiramatsu N, Fujita T. 2016. Vitellogenesis and choriogenesis in fishes. Fish Sci 82 (2): 187-202. DOI: 10.1007/s12562-015-0957-5.

Hirakawa I, Miyagawa S, Katsu Y, Kagami Y, Tatarazako N, Kobayashi T, Kusano T, Mizutani T, Ogino Y, Takeuchi T, Ohta Y. 2012. Gene expression profiles in the testis associated with testis-ova in adult Japanese medaka (*Oryzias latipes*) exposed to 17α-ethinylestradiol. Chemosphere 87 (7): 668-674. DOI: 10.1016/j.chemosphere.2011.12.047.

Humble JL, Hands E, Saaristo M, Lindström K, Lehtonen KK, de Cerio OD, Cancio I, Wilson G, Craft JA. 2013. Characterisation of genes transcriptionally upregulated in the liver of sand goby (*Pomatoschistus minutus*) by 17α -ethinyloestradiol: Identification of distinct vitellogenin and zona radiata protein transcripts. Chemosphere 90: 2722-2729. DOI: 10.1016/j.chemosphere.2012.11.053.

Ishibashi H, Uchida M, Koyanagi A, Kagami Y, Kusano T, Nakao A, Yamamoto R, Ichikawa N, Tominaga N, Ishibashi, Y, Arizono K. 2016. Gene Expression analyses of vitellogenin, choriogenin, and estrogen receptor subtypes in the livers of male medaka (*Oryzias latipes*) exposed to equine estrogens. J Appl Toxicol 36 (11): 1392-1400. DOI: 10.1002/jat.3292.

Kamiyama R, Miyata T, Kurokura H, Ishikawa S. 2015. The impact of distribution change on fisheries in Southeast Asia: a case study in the Batan Estuary, Aklan, Central Philippines. Fish Sci 81 (2): 401-408. DOI: 10.1007/s12562-015-0855-x.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16 (2): 111-120. DOI: 10.1007/BF01731581.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33 (7): 1870-1874. DOI: 10.1093/molbev/msw054.

Lei B, Wen Y, Wang X, Zha J, Li W, Wang Z, Sun Y, Kang J, Wang Y. 2013. Effects of estrone on the early life stages and expression of vitellogenin and estrogen receptor genes of Japanese medaka (*Oryzias latipes*). Chemosphere 93 (6): 1104-1110. DOI: 10.1016/j.chemosphere.2013.06.025.

Liang Y, Fang Z. 2012. Molecular cloning and mRNA expression of the vitellogenin and nuclear receptor gene induced by 17β-estradiol in the mud carp, *Cirrhinus molitorella*. Ecotoxicology 21 (3): 719-729. DOI: 10.1007/s10646-011-0831-x.

Mushirobira Y, Niida M, Hotta T, Fujinami Y, Soyano K. 2020. Hepatic expression profiles of three subtypes of vitellogenin and estrogen receptor during vitellogenesis in cultured female yellowtail. Gen Comp Endocrinol 299: 113612. DOI: 10.1016/j.ygcen.2020.113612.

Negintaji A, Safahieh A, Zolgharnein H, Matroodi S. 2018. Short-term induction of vitellogenesis in the immature male yellowfin seabream (*Acanthopagrus latus*) exposed to bisphenol A and 17 β-estradiol. Toxicol Ind Health 34 (2): 119-127. DOI: 10.1177/0748233717748099.

Paraso MG, Morales JK, Clavecillas AA, Lola M, Epifania S. 2017. Estrogenic effects in feral male common carp (*Cyprinus carpio*) from Laguna de Bay, Philippines. Bull Environ Contam Toxicol 98 (5): 638-642. DOI: 10.1007/s00128-017-2060-3.

Rawat VS, Rani KV, Phartyal R, Sehgal N. 2013. Vitellogenin genes in fish: differential expression on exposure to estradiol. Fish Physiol Biochem 39 (1): 39-46. DOI: 10.1007/s10695-012-9626-0.

Söffker M, Tyler CR. 2012. Endocrine disrupting chemicals and sexual behaviors in fish-a critical review on effects and possible consequences. Crit Rev Toxicol 42 (8): 653-668. DOI: 10.3109/10408444.2012.692114.

von Osten J, Aguayo-Dione G, Dzul-Caamal R, Lara-Flores M. 2019. Expression of estrogenic response genes to different concentration of 17ß-estradiol in male mosquitofish (*Gambusia yucatana*). Iran J Fish Sci 18 (2): 272-282. DOI: 10.22092/IJFS.2018.117433.

Zare A, Henry D, Chua G, Gordon P, Habibi HR. 2018. Differential hepatic gene expression profile of male fathead minnows exposed to daily varying dose of environmental contaminants individually and in mixture. Front Endocrinol 9: 749. DOI: 10.3389/fendo.2018.00749.

Zhong L, Yuan L, Rao Y, Li Z, Zhang X, Liao T, Xu Y, Dai H. 2014. Distribution of vitellogenin in zebrafish (*Danio rerio*) tissues for biomarker analysis. Aquat Toxicol 149: 1-7. DOI: 10.1016/j.aquatox.2014.01.022.