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Research Article

Vitellogenin Induction in Male *Oreochromis niloticus*: Indication of Estrogenicity in Taal Lake, Philippines

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ABSTRACT

Vitellogenin (VTG) is an egg-yolk protein precursor normally produced by sexually mature female fish but can also be synthesized by males under the influence of endocrine-disrupting chemicals (EDCs). 17β-estradiol (E2), an EDC, has been reported to contaminate aquatic systems and cause the feminization of male fishes. The prevalence of EDCs in Taal Lake threatens its biodiversity. Taal Lake is the habitat of the endemic freshwater sardine, *Sardinella tawilis*. The continuous loading of EDCs might affect the population dynamics of such critical wild species, leading to a decline in the wild fish catch and extinction as a long-term effect. This study determined the contamination of Taal Lake with E2 and the VTG induction in male *Oreochromis niloticus*. Water samples were collected from Taal Lake for E2 analysis through ELISA. Also, sexually mature male *O. niloticus* were obtained, and concentrations of their blood plasma VTG were determined using ELISA. Water samples from Taal Lake had E2 concentrations at 1.22 ± 0.64 µg/L. The collected male *O. niloticus* had VTG concentrations at 478.90 ± 129.98 ng/mL. The results from this study provide evidence of vitellogenin production in male *O. niloticus*. However, the measured VTG induction is not limited to exposure to 17β-estradiol.

Keywords: vitellogenin induction, 17β-estradiol, estrogenicity, Taal Lake, male tilapia

INTRODUCTION

A large group of compounds called endocrine-disrupting chemicals (EDCs) released into the environment may lead to detrimental disorders in living organisms, due to the possibility of the interaction of these compounds with cellular components (Pamplona-Silva et al. 2018). These compounds can interact with estrogen receptors and interfere with the synthesis, secretion, transport, binding, action, and elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes, hence called endocrine disruptors (EPA 2016).

The prevalence of EDCs in water resources threatens aquatic biodiversity, affecting the development and reproduction of wildlife species (Bhandari et al. 2015). Some reported effects include disturbances in the development and expression of sexual characteristics. For example, vitellogenin is an egg-yolk protein precursor normally produced by adult female fish; however, it can also be synthesized by males under the influence of EDCs (Caspillo et al. 2014; Depiereux et al. 2014; Davis et al. 2009; Rodas-Ortiz et al. 2008). Experimental studies revealed that concentrations as low as one ng/L of 17β-estradiol can induce vitellogenin production in exposed male fish (Hansen et al. 1998; Purdom et al. 1994). In other experimental studies, high incidences of feminization and intersex were observed in male fish after chronic exposure to estrogen (Green et al. 2015; Caspillo et al. 2014; Depiereux et al. 2014; Adebiyi et al. 2013; Kosai et al. 2011). Taal Lake has high biological significance because of its unique collection of fauna. The freshwater sardine, *Sardinella tawilis, is* endemic to this lake. The continuous loading of these contaminants might significantly disrupt the reproduction and population dynamics of such critical wild species leading to a decline in the wild fish catch and extinction as a long-term effect.

Many different chemicals have been recognized as EDCs. Classical examples that are frequently found in the environment, include natural and synthetic hormones, BPA, phthalates, alkylphenols (APs), polycyclic aromatic hydrocarbons (PAHs), and agricultural chemicals (Pamplona-Silva et al. 2018). Among the different EDCs, estrogenic compounds have merited the most concern. Paraso and Capitan 2012, have studied the estrogenic contamination of Laguna de Bay and vitellogenin induction has also been observed in male common carp caged in Laguna de Bay, Philippines. The detected VTG in the male common carp established the estrogenic contamination in Laguna de Bay. Williams et al. (2003) identified natural estrogens, such as estrone and 17β-estradiol, and synthetic ethinylestradiol contribute the most significant endocrine disruption. These estrogenic substances are released into the freshwater systems through municipal sewage effluents, livestock waste, agricultural discharge, and industrial effluents (Ying et al. 2002). Human and animal excretion is considered the primary source of estrogen in aquatic ecosystems (Matozzo et al. 2008). These substances are difficult to detect and impossible to remove once they reach the aquatic environment.

In this study, the vitellogenin induction in male *O. niloticus* is attributed as the observed effect to the presence of E2 and other estrogenic compounds since the induction of vitellogenin in male fish is considered one of the most common biomarkers for the detection of estrogenic compounds in aquatic environments (Synder et al. 2003). Estrogenic compounds are those that have similar chemical properties to the hormone 17β-estradiol.

METHODOLOGY

The study determined the concentration of 17β-estradiol (E2) in the surface water of Taal Lake, in a region where tilapias are produced commercially in floating net cages. It also determined the vitellogenin induction in male tilapias *(O. niloticus) by measuring* the vitellogenin level in blood plasma. In this study, 17β-estradiol served as a model compound for environmental estrogens, while the determination of other environmental estrogens was not included. Tilapia is considered the relevant fish species for this study because they are not known to be hermaphrodites; they are resilient to environmental pollutants and an important food fish that are grown extensively in aquaculture enclosures in Taal Lake and other Philippine freshwater lakes. This study has not included controlled exposure where E2 was used exclusively to induce VTG production in male Tilapias since exposure to estrogens in the environment is never single-chemical.

Site Description

Water and fish samples were collected from Taal Lake. Surface water samples were collected from four sampling sites, and each sampling site has a distance of at least 50 meters from each other (coordinates: 14°02'29.1"N 121°00'08.4"E; 14°02'32.5"N 121°00'24.0"E; 14°02'30.3"N 121°00'27.1"E; 14°02'24.4"N 121°00'24.4"E). The sampling sites were located near the volcano island with high stocking density fish cages and populated land areas along the shoreline. Fish samples were collected in one of these cages.

Figure 1. Sampling points in Taal Lake (Google Maps 2016).

Fish Sampling and Blood Plasma Extraction

Sexually mature male tilapias, grown in floating fish cages for about eight months, were randomly collected on-site during the summer of April 20, 2016, using cast nets. The fish were kept in large, aerated tanks with water collected from the sampling sites and were immediately transported to the laboratory. Eight fish were anesthetized with 100 mg/L tricaine methanesulfonate solution (Himedia® MS222 - ethyl 3-aminobenzoate methanesulfonate salt). Fish were patted dry, and the fork length, total length, and weight of the fish were recorded. Fish fork length ranged from 170 – 190 mm, total length ranged from 245 – 270 mm, and weight went from 300 – 400 g.

One milliliter of the blood sample from each fish was collected from the caudal vasculature using 3-mL heparinized syringes. The blood samples were centrifuged at 3000 x g with a maintained temperature of -10˚C for 10 minutes to separate the blood components. The blood plasma was collected and transferred into sterile 1-mL Eppendorf tubes using sterile pipette tips. The fresh blood plasma samples were immediately stored in the freezer at -40 °C until the assay was performed (Asem-Hiablie et al. 2013; Paraso et al. 2019).

Quantification of Vitellogenin (VTG) in Blood Samples of Male Fish

An immunochemical method, Enzyme-Linked Immunosorbent Assay (ELISA), was used to quantify the concentration of VTG in the blood plasma of male *O. niloticus*. ELISA has been the preferred method in determining VTG and E2, due to its good sensitivity, which is generally in the range of ng/mL. ELISA is a highly sensitive method that uses antibodies specific to VTG and is usually not subject to interference from other proteins (Jones et al. 2000). The technique involves coating the wells with VTG from the samples.

A commercially available VTG ELISA kit – TECO® REACH Perch (Perciformes) Vitellogenin ELISA (TECO® medical Group, Gewerbestrasse, Switzerland) was used to quantify the concentration of VTG present in the previously obtained blood plasma samples. All the reagents needed for the assay, including a set of standardized reagents and specific antibody-coated microplate wells, are in the test kit. Other materials and equipment required for the test were micropipettes, multichannel pipettes, a vortex mixer, an orbital shaker, and a microplate reader capable of measuring at 450 nm.

Assay Procedure

The frozen blood plasma samples were thawed in normal tap cold water (15-20˚C) within 10-15 minutes. The prepared blood plasma samples were subjected to Enzyme-Linked Immunosorbent Assay (ELISA). All samples were analyzed in duplicates. The test protocol was done under the protocol set by the manufacturer of the ELISA kit. All blood plasma samples were prepared before the start of the assay by diluting it to a dilution of 1:1000 with a Dilution

Buffer provided in the kit. A 1000 µL dilution buffer was combined with a 1 µL blood plasma sample. The microplate wells were first allocated for NSB/blank (Non-Specific Binding), standards, controls, and blood plasma samples (with unknown VTG concentration).

The 50 µL matrix solution was added to all wells using a multichannel pipette. Then, 50 µL dilution buffer was added to the assigned NSB wells. 50 µL of each prepared standard VTG solution (80 ng/mL, 27 ng/mL, 9 ng/mL, 3 ng/mL, 1ng/mL, and 0 ng/mL), prepared controls (C1 and C2), and pre-diluted male blood plasma samples were added into the corresponding wells. The wells were then covered and incubated for 120 ± 5 min at room temperature (18-30˚C) on a shaker (500 rpm). After incubation, the contents of the wells were aspirated and washed three times using 350 µL diluted wash buffer. After washing, 100µL Biotinylated AB was added to each well. The wells were again incubated for 60 ± 5 min at room temperature (18-30˚C) on a shaker (500rpm). After the incubation, the wells were washed three times using a wash buffer. Following the washing step, 100 µL of SA-HRP Conjugate was added to each well. The wells were again covered and incubated for 30 ± 5 min at room temperature (18-30˚C) on a shaker (500 rpm). After the incubation, the wells were washed five times with wash buffer. A 100 µL of the TMB Substrate Solution was added to each well, and the plate was incubated for 15 – 30 minutes, in the dark, at room temperature (18-30˚C) on a shaker (500 rpm). The microplate was covered with aluminum foil to prevent light exposure. To stop the reaction, 100 µL stop solution was added to the wells. Color development was measured 10 minutes after adding the stop solution using a microplate absorbance reader (iMark™, Bio-Rad Laboratories, Inc.) at 415 nm with the reference filter at 655 nm. The values of absorbance were analyzed by using MyAssays Online Software ("Four-Parametric Logistic Curve" online data tools, MyAssays Ltd., 2016).

Water Collection

Water samples were collected from Taal Lake for 17β-estradiol (E2) analysis. 100 mL grab samples of lake water were collected five centimeters below the water surface in amber glass bottles with polytetrafluoroethylene caps (Asem-Hiablie, 2013; Jurgens, et Al. 1999). The water samples were transferred to the bottles using a glass funnel. The funnel and grab collector were rinsed thoroughly using lake water from each site before the sample was collected. All water samples were contained in an insulated container and were immediately transported to the laboratory. Water samples were then filtered using a 1 µm pore diameter glass fiber filter (Pall®; Pall Corporation, Michigan, Mexico). A minimal volume of methanol (AR, RCI Labscan) was poured on the residue on the filter to maximize the extraction of the analyte. One percent (1%) of formaldehyde was added to the filtered water samples and was stored at 4°C before use.

Pre-treatment of Water Samples

Solid-phase extraction (SPE) of the water samples was performed. The SPE procedure used C18 SPE cartridges (Supelco®, Bellefonte, PA, USA) following the water pre-treatment procedures specified in the cartridge user manual. Each cartridge was preconditioned with 5 mL methanol and 10 mL deionized water. Water samples (100 mL) were loaded into SPE cartridges under gentle vacuum pressure at a flow rate of 20 mL/min. The cartridges were washed with deionized water at a flow rate of 20 mL/min and were then vacuum-dried for one minute. Drying was followed by washing 5 mL of 95% n-hexane (AR, RCI Labscan) at a flow rate of 20 mL/min. The 17β-estradiol was eluted from the C18 cartridge using 5 mL dichloromethane (Baker Analyzed®, USA) at a flow rate of 3-5 mL/min. The solvent was evaporated to dryness under the fume hood. The resulting residue, which contains the 17β-estradiol, was dissolved using 100 µL methanol, and the mixture was stirred using a vortex mixer to dissolve the residue completely. Deionized water (900 µL) was added to achieve a concentration of 10 % (v/v) methanol.

Determination of 17β-estradiol Concentration

Detection and quantification of 17β-estradiol were accomplished using an immunochemical method, Enzyme-Linked Immunosorbent Assay (ELISA). This was done using a commercially available ELISA kit (EcologienaÒ, Tokiwa Chemical Industries Co., Ltd, Japan). The 17β-estradiol assay standards were prepared. All water samples were analyzed in duplicates following the protocol set by the manufacturer of the 17β-estradiol ELISA kit.

Assay Procedure

The microplate wells were first allocated for blank, standard, and water samples (unknown concentration). The conjugate solution, 17β-estradiol assay standards (0 µg/L, 0.05 µg/L, 0.15 µg/L, 0.40 µg/L, 1.00 µg/L), and wash solution were prepared before the start of the assay. Conjugate solution (100 µL) was mixed with 100 µL of each 17β-estradiol standard and water samples to form a 200 µL conjugate-standard solution or conjugate-sample solution. 100 µL aliquots of the mixture were dispensed into each allocated microplate well. The microplate was covered with a film to prevent contamination and evaporation and was incubated for 60 minutes at room temperature (18-25 ˚C). After the incubation, the microplate was rinsed three times with 300 µL of the wash solution. Color solution (100 µL) was dispensed into each microplate well and was then incubated for 30 minutes at room temperature (18-25 ˚C). Then, 100 µL stop solution was added into each microplate well to terminate the reaction. Water samples and standards absorbance were read at 450 nm wavelength

using a microplate absorbance reader (iMarkTM, Bio-Rad Laboratories, Inc.). The values of absorbance were then analyzed using MyAssays Online Software ("Four-Parametric Logistic Curve" online data tools, MyAssays Ltd., 2016).

RESULTS AND DISCUSSION

Plasma Vitellogenin (VTG) Concentrations

The male *O. niloticus* samples had detectable concentrations of plasma VTG. The fish collected from Taal Lake had an average VTG concentration of 478.90 ± 129.98 ng/mL.

Table 1. Vitellogenin concentration of Male tilapia (*O. niloticus*) from Taal Lake

Fish Number	VTG Concentration in Fish Sample (ng/mL)	Average ± SD VTG (ng/mL)
1	386.9	478.90 ± 129.98
2	449.9	
3	448.5	
4	318.9	
5	17.8	
6	643.6	
7	625.6	
8	1980.0	

**Data were analyzed using software capable of generating a 4-Parameter Logistic curve – "MyAssays".*

The detected vitellogenin in male *O. niloticus* establishes the contamination with estrogen in Taal Lake. Under normal circumstances, male fish may have little or undetectable VTG levels in their blood plasma (Ankley and Johnson 2004) and VTG induction by 17β-estradiol has been reported to be dose-dependent (Berg et al. 2004). The detected VTG concentration in males indicates an unnatural response to estrogens present in Taal Lake. The measured VTG concentration in male tilapia in this study, which is 478.90 ± 129.98 ng/mL shown in Table 1, is similar to those detected in the adult male tilapia cultured in the lakes of San Pablo City, Laguna with extensive aquaculture activities, which ranges from 40.68 ± 40.97 µg/mL to 57.22 ± 52.71 µg/mL (Mabansag et al. 2019). The observed vitellogenin induction can be attributed to the presence of E2 as well as to other estrogenic compounds.

17β-estradiol (E2) Concentrations in Water Samples

Environmental pollution by estrogenic compounds can have a potential risk of endocrine disruption to exposed aquatic organisms. The concentration of 17β-estradiol (E2) was measured in the surface water samples from selected sites of the lake to verify the presence of estrogens. Results showed that water samples from Taal Lake had an E2 concentration of 1.22 ± 0.64 µg/L, as shown in Table 2. This measured E2 concentration in Taal Lake was higher than those detected in Laguna de Bay. Water samples collected from the East Bay and West Bay of Laguna de Bay had E2 concentrations of 0.39 ± 0.15 µg/L and 0.40 ± 0.16 µg/L, respectively (Paraso and Capitan 2012). The difference between the surface area of the two lakes could explain the higher E2 concentrations observed in Taal Lake. Laguna de Bay is the largest lake in the Philippines covering a surface area of 911.7 km^2 while Taal Lake has a surface area of 234.2 km^2. Taal Lake is four times smaller than Laguna de Bay, which could concentrate the estrogens on the surface water. It is important to note that estrogenic compounds are mostly nonpolar and have lower densities, which tend to float on the surface of the water.

Table 2. Concentrations of 17β-estradiol in water samples from Taal Lake

Sampling Station Number	17β-estradiol in Water Samples (µg/L)	Average ± SD 17β-estradiol (µg/L)
1	0.840692	1.216441 ± 0.64
2	2.09528	
3	0.6551	
4	1.27469	

**Data were analyzed using software capable of generating a 4-Parameter Logistic curve – "MyAssays".*

There are many pathways for estrogenic contaminants to enter the lake by natural and/or anthropogenic origins. The presence of E2 in aquatic environments is highly associated with wastewater effluents, direct discharges, and run-offs from domestic, agricultural, and industrial sources (Praveena et al. 2016; Rocha et al. 2015; Studer 2011). In this study, the incidence of E2 detection can be related to the distribution characteristics of residential and agricultural areas established around the lake as indicated by the color-coded land use in the map (Figure 2).

The selected fish cage in Taal Lake was located near the volcano island, in an area close to households. According to ADB (2014), only three to five percent of households in the Philippines have access to a sewage system network. On an island with no access to a municipal sewage system, individual or communal septic tanks are utilized, these are usually below the standards set by the Department of Environment and Natural Resources. Consequently, sewage leaching into the water systems could emit considerable amounts of E2 into the lake. It has been studied that the primary source of E2 in municipal wastewater is urine, which contains 67 – 80% of estrogens excreted daily (Maurer et al. 2006). The other primary source of hormone steroids is livestock waste (Ying et al. 2002). Non-point sources, like run-offs loaded with animal waste from agricultural lands and facilities surrounding the lake, could have reached the surface water.

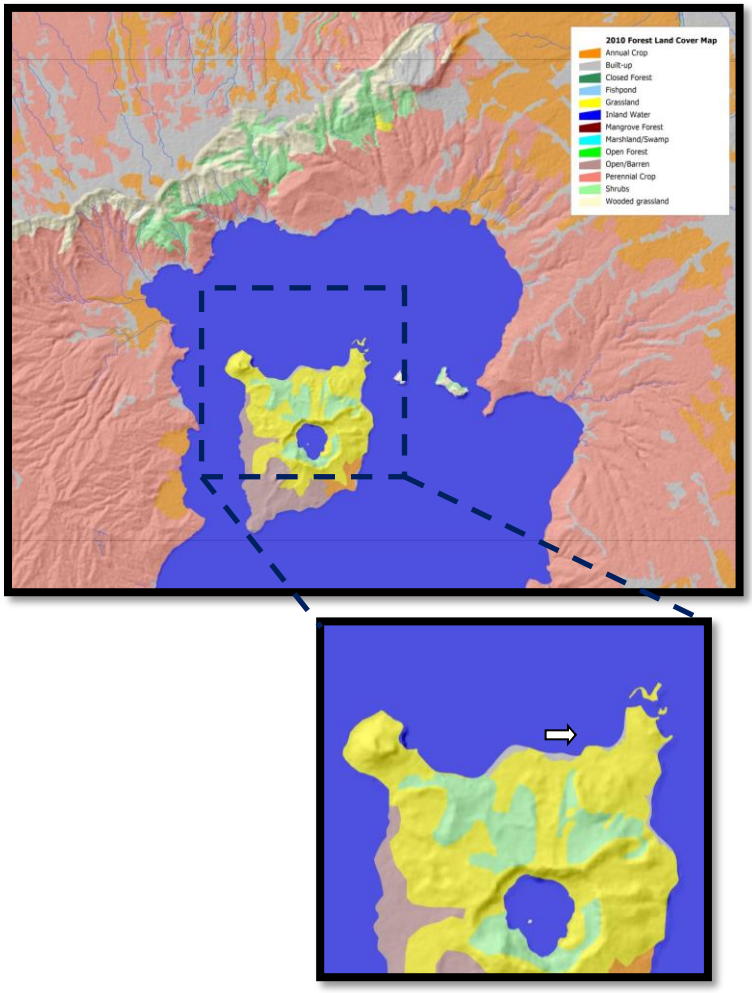

Figure 2. Land-use map of Taal Lake. (Ecosystem Research Development Bureau, ERDB-UPLB, generated, 2010). Indicated is the limnological sampling site (white arrow).

Fish in cages or fences set up in lakes are exposed to a wide range of contaminants under field conditions, some of which may have the same effect as EDCs. Unknown contaminants that mimic estrogen may have feminizing effects. EDCs encompass a variety of substances, aside from hormones synthesized by vertebrates; it includes phytoestrogens, mycotoxins, synthetic hormones, pesticides, detergent components, and persistent environmental pollutants such as PCBs, PCDDs, PCDFs (Cargouet et al. 2007).

The selected sampling site in Taal Lake is surrounded by mainly agricultural lands characterized as an annual crop in the land-use map. The use of pesticides in cultivated lands could have contributed to the presence of other EDCs. In a study by Rodas-Ortiz et al.(2008), they correlated the VTG induction and sexual steroid alterations in male tilapias with the organochlorine pesticide concentrations in the liver and polycyclic aromatic hydrocarbon (PAH) metabolites in bile. Their study revealed significant correlations between plasma VTG and E2 levels with the pesticide hexachlorobenzene (HCB) in the liver and BaP metabolites in bile.

CONCLUSION AND RECOMMENDATION

The collected male *O. niloticus* from Taal Lake had detectable concentrations of plasma vitellogenin. The male tilapia samples had an average vitellogenin concentration of 478.90 ± 129.98 ng/mL. Under normal circumstances, male fish may have minor or undetectable vitellogenin. Thus, the detectable concentrations of plasma vitellogenin in the male tilapia samples indicate induction from estrogenic compounds.

17β-estradiol concentration of 1.22 ± 0.64 µg/L was detected in Taal Lake. The results suggest the detected vitellogenin in male *O. niloticus* is a biological response to estrogen exposure. The results from this study provide evidence of vitellogenin production in male *O. niloticus*. However, the measured vitellogenin is not limited to exposure to 17β-estradiol. Fishes and other animals are exposed to various pollutants under field conditions. Other environmental pollutants may increase or inhibit vitellogenin production in fish.

Continuing research on the detection, quantification, and monitoring of EDCs in the environment is needed to provide a further understanding of environmental concerns. Further research should consider the change in seasons since it could be a factor in the varying concentrations of EDCs in water. Further research may include a whole-lake field study, investigating other endocrine disruptors released into the environment and assessing whether VTG induction is responsive to estrogenic EDCs alone.

Author Contributions: Ailene H. Calamlam and Blesshe L. Querijero conceptualized the study, designed the theoretical framework, performed field data collection and laboratory analysis, and discussed the results and contributed to the final manuscript. Both authors have read and agreed to the published version of the manuscript.

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Appendix (The data supporting the findings of this study are available within the article.)

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