VITELLOGENIN INDUCTION AND GONAD ABNORMALITIES IN MALE COMMON CARP (*Cyprinus carpio* Linnaeus) INTRODUCED TO LAGUNA DE BAY, PHILIPPINES

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ABSTRACT

Experiments were conducted to determine contamination of Laguna de Bay in the Philippines with estrogenic endocrine disrupting compounds and to examine their impact on introduced fish. Results revealed 17β-estradiol (E_2) levels in water samples ranging from 0.29±0.07 µg/L to 0.40±0.16 µg/L. Caging of adult male common carp (*Cyprinus carpio* Linnaeus) in the east and west bays of the lake induced production of the egg-yolk protein precursor vitellogenin (8.33±0.40 to 8.77±0.60 µg/ml) and mild to moderate lesions in the testis that are consistent with estrogenic exposure. In general, the results validate pollution of the lake with estrogenic substances. Whether or not the obtained E_2 levels could elicit biologic effects that can compromise reproduction and population dynamics in fish and in higher vertebrates should be a subject of future research endeavors. Given the current weight of evidence on the adverse effects of endocrine disruption by environmental estrogens, the implementation of measures that could prevent the discharge of these compounds in the lake watershed is recommended.

Keywords: 17β-estradiol, endocrine disruptors, fish, Laguna de Bay, vitellogenin

INTRODUCTION

Among various endocrine disrupting compounds (EDCs), those with estrogenic activity have merited the most concern. This could be attributed to the significant role of estrogen in the development, growth, maintenance and adult function of reproductive tracts of vertebrates (Filby *et al*., 2006). Estrogenic EDCs encompass a wide range of chemical classes, including natural and manmade steroidal and non-steroidal hormones, plant constituents (phytoestrogens), pesticides, pharmaceuticals, plasticizers and surfactants (Damstra *et al*., 2002; Filby *et al*., 2006; Örn *et al*., 2006). Most are released into the environment via municipal sewage discharges and industrial effluent, which account for most of the reported cases of endocrine disruption, including disturbances in the development and

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expression of sexual characteristics in fish, amphibians and reptiles (Allen *et al*., 1999; Hutchinson *et al*., 2006; Toft *et al*., 2003). The natural estrogen 17β-estradiol (E_2) is considered as the most potent among estrogenic EDCs (Gross-Sorokin *et al*., 2006). Although excreted mainly by humans and livestock in inactive form, these compounds are believed to be reactivated through the process of deconjugation in surface waters and during sewage treatment (Allen *et al*., 1999).The feminization of wild fish populations in Japan, US and in Europe has been attributed to the presence of these compounds in sewage effluent.

As the largest lake in the Philippines, Laguna de Bay is a vital freshwater resource in Metro Manila and the nearby provinces of Cavite, Laguna, Batangas, Rizal and Quezon. Aside from being the habitat of various biological resources such as fish, mollusks and crustaceans, the lake has significant economic uses to various municipalities and to the following sectors: business, transport, electricity, industrial cooling, agriculture and recreation (LLDA, 2006). Unfortunately, the lake also serves as a sink for domestic, agricultural, industrial and municipal effluents that are largely untreated prior to discharge (Zimmer and Bendoricchio, 2001). Because many anthropogenic activities account for a large percentage of land use in the Laguna de Bay watershed, its contamination with estrogenic EDCs is a definite possibility but remains largely unstudied. Moreover, endpoints of endocrine disruption among fish populations in the lake have not yet been assessed locally.

MATERIALS AND METHODS

17β-estradiol levels

Water collection

The west and east bays were targeted for water sampling from August 19-23, 2010 and from September 27-30, 2010. Two (2) locations in each bay were designated as sampling sites because of their proximity to densely populated areas (east bay: Paete and Sta. Cruz; west bay: Calamba, and Sta. Rosa City). Four (4) sampling points per site were then identified with each site having a distance of at least 100 m from the next. A grab sample of surface water (1L) in each sampling point was collected with a portable water sampler and transferred into pre-cleaned amber borosilicate glass with polytetrafluoroethene caps. These were transported on ice to the Environmental Chemistry Laboratory, Institute of Chemistry, UPLB , and were processed within 24 h after collection.

Pretreatment of samples.

Raw water samples (1L) were filtered and subjected to solid phase extraction (SPE) to concentrate the E_2 content to amounts detectable by the assay. C18 SPE cartridges (Supelco, Bellefonte, PA, USA) were preconditioned with MeOH (5 ml) and distilled H_2O (10 ml). The water samples were then loaded into the cartridge under gentle vacuum pressure. The cartridge was washed with distilled H_2O followed by n-hexane (Merck ACSÒ) and finally by dichloromethane (AR ScharlauÒ) to elute the analyte, which was then blown to dryness with the use of nitrogen gas. Methanol (100%) was added to the residue and the mixture was then

stirred with a vortex mixer. Distilled water was added to adjust the content at 10% (v/v) MeOH.

17β-estradiol analysis

The resulting samples were subjected to Enzyme-Linked Immunosorbent Assay (ELISA) with the use of a test kit (EcologienaÒ, Tokiwa Chemical Industries Co., Ltd., Japan). Each sample was analyzed in duplicate in accordance to the manufacturer's directions.

Vitellogenin (VTG) levels

Caging study

A total of 33 sexually mature male common carp with maximum total length of 150-290 mm were obtained from a local supplier. A caging study was then conducted in July 2010 where fish were kept for 21 days in four 2m x 2m cages made of fishnet and bamboo, and with each cage housing a minimum of five individuals. Two cages were placed in the east bay (Paete and Sta. Cruz) whereas the other two were situated in the west bay (Calamba and Sta. Rosa City). All cages were positioned approximately 300 m from the shore and within the vicinity of fish pens. The geographical coordinates of fish cages were established using Global Positioning System (GPS):

West Bay: Calamba: N14°11.438'; E121°12.213'; Sta. Rosa: N14°20.108'; E121°06.675'

East Bay: Sta. Cruz: N14°17.900'; 121°25.129'; Paete: N14°21.435'; E121° 28.238

A reference group of fish (n=12) was kept in a roofed concrete outdoor pond of the UPLB Limnological Research Station. Dechlorinated tap water was used in the pond, of which approximately 3/4 was replaced on a daily basis. Feeding was done twice daily with commercially available bloodworm.

Vitellogenin quantification

Each fish was anesthetized with tricaine methane sulfonate (MS222) (Sigma -AldrichÒ). With the use of a heparinized syringe, 0.5 ml of blood was drawn from the caudal vein and immediately transferred into microcentrifuge tubes. Aprotinin was added to approximately 2 TIU/ml. The tubes were then centrifuged at 3000 x *g* for 10 min, and placed on ice thereafter. The supernatant was transferred into sterile microcentrifuge tubes and frozen at 80°C until analysis**.** Quantification of vitellogenin (VTG) was done with the use of a commercially available carp VTG ELISA kit (Biosense Laboratories AS, Bergen, Norway). All samples were analyzed in duplicate following the manufacturer's directions.

Gonadosomatic index (GSI) and histopathological alterations of the testis

Caging study

Thirty-five (35) sexually mature male common carp with maximum total length of 150-290 mm were obtained from a local supplier. Using the same locations

described previously, two cages with six individuals each was placed in the west bay, whereas another two was kept in the east bay. A reference group of fish (n=11) was raised in the outdoor pond of the UPLB Limnological Research Station and fed and maintained as described earlier. At the end of the caging period, each fish was sacrificed by overdose with MS222 (Sigma-Aldrich). Both testes were removed and the GSI was computed using the formula:

Gonadosomatic Index = [testis weight (g)/ (total wt (g)] x 100)

Tissue preparation

The testes were labeled and fixed immediately in phosphate buffered formalin for at least 24 h. These were cut into three equal portions (anterior, middle, and posterior), dehydrated through graded alcohol concentrations, cleared and embedded in paraffin following standard histological procedures. Five (5) µm-thick transverse sections were cut from each portion creating a total of six (6) testis sections per fish. These were stained with H and E prior to mounting in glass slides. Digitized images of the organs were obtained with a Nikon DS-L2 camera control unit connected to a Nikon Eclipse E-200 microscope.

Testicular analysis

Sections were analyzed based on the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (Johnson *et al*., 2009) using a set of selected primary and secondary diagnostic criteria. The gonadal stage of the testis and severity of the lesions were also determined using the morphologic criteria established by the same authors.

Statistical analysis

Test results were compared among groups (east bay, west bay and reference) and between the east and west bay groups. For the former, a one-way analysis of variance (ANOVA) for a completely randomized design was used to analyze for significant differences in GSI. VTG levels were compared using the nonparametric Kruskal-Wallis test where each fish in a group was regarded as a replicate. To examine treatment effects between east and west bay groups, the Student's t-test was used. This test was also used to compare measured concentrations of E_2 in water samples. All statistics were calculated using SAS version 6.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

17β-estradiol levels in water samples

Water samples revealed highly detectable levels of E_2 during both sampling periods (Table 1). This indicates that hormonal excretions of human and/or animal origin have reached the lake. Although E_2 values did not differ significantly in the east and west bays, it can be noticed that the former consistently had slightly higher levels of steroid hormones than the latter. The E_2 levels in this study were markedly higher than those obtained from surface waters in other Asian countries such as

Table 1. Levels of 17β-estradiol in water samples from the east and west sites of Laguna de Bay (mean ±SD).

Sampling site	Measured concentration (µg/l)	
	August	September
East Bay (n=8)	0.29 ± 0.07	0.39 ± 0.15
West Bay (n=7)	0.37 ± 0.12	0.40 ± 0.16

Korea, Laos, Cambodia, Vietnam, China, Indonesia, Thailand, Malaysia and Taiwan where values ranged from 2.3±0.1 ng/l (Malaysia) to <15 ng/l (Taiwan) (Duong *et al*., 2010; Chen *et al*., 2007). Water samples from Vietnam, China, Laos, Cambodia and Taiwan were obtained from sites adjacent to crowded municipal areas while those from Indonesia, Thailand and Malaysia were collected from relatively clean surface waters (Duong *et al*., 2010). In more developed societies like Japan, the United States and in Europe, domestic sewage effluents had E_2 concentrations (<1 to 48 ng/l) (Jobling *et al*., 2006) that are way below the levels observed in this study.

Unlike some of the countries mentioned previously, centralized municipal sewage treatment plants are virtually non-existent in the Philippines. The absence of sewage treatment in many areas and the sheer human population of 13.6 M inhabiting the Laguna Lake watershed (NSO, 2007) contaminate the lake with largely untreated effluent that could have led to the high E_2 levels observed in this study. The practice of releasing untreated effluent into waterways by swine and poultry farmers in Laguna province, particularly among smallholders (Paraso *et al*., 2010), could have also contributed its share into the estrogenic load of the lake.

Vitellogenin levels

All fish had detectable VTG levels; however, the mean value for the caged groups did not differ significantly from each other (Table 2). Nevertheless, differences between the caged and reference groups were clearly apparent at $p<0.01$ level. This suggests a biologically active estrogenic input in the lake as vitellogenin, which is normally produced in female fish, can also be synthesized by males under the influence of both natural estrogens and estrogen-mimics. Although E_2 was a highly probable cause of this phenomenon, VTG production cannot be solely attributed to this compound as there could be a myriad of estrogenic EDCs in the lake. These include industrial compounds with estrogen-like properties as well as commercial feeds used in fish cages that are potential sources of phytoestrogens. The VTG concentrations in this study were comparable to those obtained in male teleosts collected downstream from wastewater treatment plants (WWTPs) such as the rainbow trout (*Oncorhynchus mykiss*) (8-11 mg/ml) (Aerni *et al*., 2007), gudgeon (*Gobio gobio*) (8 mg/ml) (Van Aerle *et al*., 2001) and salmon (*Salmo trutta*) (Vermeirssen *et al*., 2005).

Table 2. Plasma vitellogenin levels of reference fish and fish caged in Laguna de Bay (means±SD).

Sampling site	Number of fish	VTG levels (µg/ml)
West Bay	9	8.33 ± 0.40^{a}
East Bay	12	8.77 ± 0.60^{a}
Reference	12	3.53 ± 4.85^{c}

Values within a column with different superscripts are different at $p<0.01$ using Kruskal-Wallis test.

Despite the difference in VTG levels between caged and reference groups, the values obtained in the latter cannot go unnoticed. VTG production in the reference group could have been due to inadvertent exposure to estrogenic compounds in the water or in the food although a detailed study is needed to confirm this. The common carp has been found to be more sensitive to EDCs physiologically, thus exhibiting much higher VTG responses at even low exposures to estrogenic compounds (Desforges *et al*., 2010). Values comparable to the VTG in the reference group had been recorded in male salmonids (Tyler *et al*., 1996).

Gonadosomatic indices and histopathological alterations of the testis

Many studies have confirmed that exposure to potent estrogens leads to inhibition of testicular growth in fish (Hassanin *et al*., 2002). Experimental exposure to E_2 has led to pathological alterations in gonads that include arrested spermatogenesis and degenerative changes with more severe lesions at higher E_2 concentrations (Miles-Richarson *et al*., 1999). These observations led to the use of GSI as a biomarker for environmental estrogen exposure studies (Hassanin *et al*., 2002). Comparison of GSI between caged fish and the reference group in this study did not yield differences consistent with estrogenic effect (Table 3). A similar result was obtained by Thorpe *et al*. (2000) in juvenile male rainbow trout exposed to 244 ng/L of E_2. Although no significant differences were recorded in this study, it is worthwhile to take notice of the decreasing trend of GSI in exposed fish compared to the reference group.

Testes from the reference group showed lobules that contain an

Table 3. Morphometric data and gonadosomatic index (GSI) of reference fish and fish caged in Laguna de Bay (means±SD).

Sampling site	Length (cm)	Weight (g)	GSI
West Bay (n=12)	21.96±4.65	250.58±21.35	3.27±2.65
East Bay (n=12)	20.21±1.67	242.5±8.66	4.78±1.76
Reference (n=11)	19.55±5.57	222.91±27.68	5.83±2.56

Values with different superscripts within a column are significantly different at $p<0.05$ using one-way ANOVA.

accumulation of spermatozoa in the testis lumina. Gonad development within the group was noted to be synchronous. All were in late stage spermatogenesis, which is characterized by the presence of predominantly free spermatozoa in seminiferous tubules. Thin interlobular tissue was also observed. Most of the caged fish had testes in the same developmental stage as that of reference fish; however, there were some that had testis in the early (n=2) and mid-spermatogenic stages (n=1).

Testicular changes that have been associated with estrogenic exposure were observed in caged fish (Figure, Table 4). A few showed increased proportion of spermatogonia concurrent with a reduction in germ cell population and a lesser amount of free spermatozoa, which suggests a possible delay in the maturation of germ cells. Similarly, testis samples from walleye (*Stizostedion vitreum*) collected from an effluent channel also exhibited earlier stage of spermatogenesis (Levitt *et al.,* 2001). The proportional distribution of gonadal cell types may be altered by exposure to estrogenic EDC, which has been attributed to the suppression of the follicle stimulating hormone (FSH) and luteinizing hormone (LH) release, thus leading to an arrest in normal spermatogenesis (Johnson *et al*., 2009; Miles-Richardson *et al*., 1999).

Testes from the majority of caged fish contained macrophage aggregates

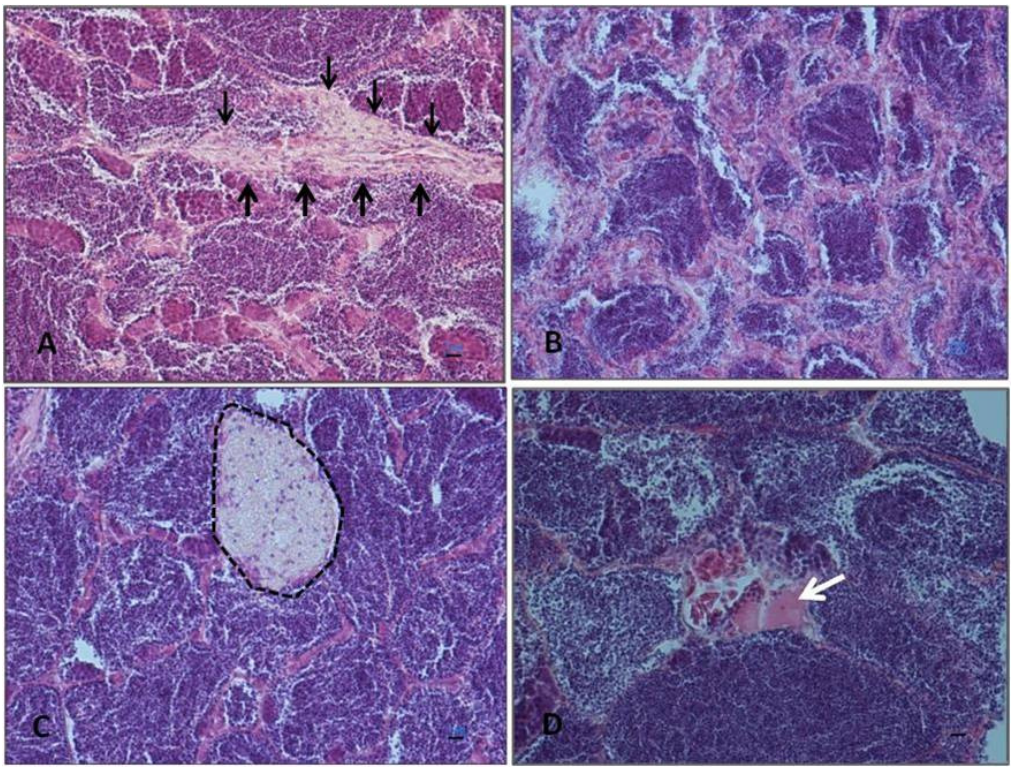

Figure. Photomicrographs of testicular lesions in a common carp caged in the east and west sites of Laguna de Bay: (A) interstitial fibrosis (black arrows); (B) increased spermatogonia; (C) macrophage aggregate (dotted lines) (D) vitellogenin (white arrow). H and E. Bar: 1mm.

Table 4. Frequency of testicular changes in the reference group (n=11) and in fish caged in the east (n=12) and west sites (n=12) of Laguna de Bay.

Diagnostic criteria	Reference group	West bay	East bay
Primary			
Presence of testis-ova	--	--	--
Increased proportion of spermatogonia	--	3	3
Testicular degeneration	--	--	--
Secondary			
Decreased proportion of spermatogonia	--	--	--
Interstitial fibrosis	--	1	--
Increased vascular or interstitial proteinaceous fluid	--	3	4
Altered proportions of spermatozoa or spermatocytes	--	1	2
Macrophage aggregates	--	8	8

Values are presented as number of observations.
(--) means not detected.

ranging from mild (3-5 occurrences per tissue section) to moderate (6-8 occurrences per tissue section) severity. Feral carp captured downstream of a sewage treatment plant (STP) in Spain also presented this lesion (Lavado *et al*., 2004). Macrophage aggregates are initiated by exposure to infectious or non-infectious agents (e.g. toxicants). These could attain larger size and may occur in greater number depending on the quality and quantity of the instigating agent (Johnson *et al*., 2009). The presence of intravascular proteinaceous fluid (presumed to be VTG) in the testes of some of the caged fish further supports the hypothesis that they were exposed to estrogenic compound. Sequestration of VTG in peripheral tissues occurs because of its large volume of distribution and because of the absence of a natural repository in males, which is the ovary.

CONCLUSION

This study was able to establish estrogenic contamination in Laguna de Bay that could be a contributing factor to its deteriorating water quality. Whether or not the existing E_2 levels can elicit biologic effects that could compromise fish reproduction and population dynamics should be a subject of future research endeavors. The few and probably incipient histopathologic changes in the testis may

be attributed to the limited caging period. This necessitates the conduct of a full life cycle test to better assess if reproductive function in fish is being adversely affected in the lake and if estrogenic contamination could be correlated with the declining wild fish catch in the area. Future research directions should also include an investigation of the impact of exposure on other organ-systems in fish, the identification of other estrogenic EDCs in the water and the establishment of E_2 level in the lake for a longer time period. The implications of consuming fish tainted with environmental estrogens may be a cause of concern. Although reports have demonstrated the adverse effects of exposure to very low environmental levels of these compounds in fish-eating wildlife vertebrates and in laboratory animals, the paucity of related studies in humans precludes the establishment of a strong link between the two. However, given the available scientific data on the causes and consequences of endocrine disruption, concerned government agencies should endeavor in limiting the exposure of aquatic ecosystems to these substances.

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