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Assessment of Endocrine Disrupting Chemicals Exposure in Sea Bass *(Lates calcarifer)* and Wild Fishes Using Vitellogenin as a Biomarker

Witchuda Prasatkaew[1], Phochit Nanthanawat[2*] and Chutima Thanomsit[3]

[1]*Environmental Science Program, Faculty of Science, Burapha University,Thailand*
[2]*Department of Biotechnology, Faculty of Science, Burapha University, Thailand*
[3]*Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Thailand*

*Corresponding author : phochit@buu.ac.th
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Abstract

Endocrine disrupting chemicals (EDCs) are widely used in Southeast Asia and have been found to contaminate aquatic environments. However, study of EDCs and their effects on aquatic animals are deficient. Induction of vitellogenin in males and juveniles is a useful biomarker for exposure to EDCs. In this study, the induction of vitellogenin was evaluated after exposure to single and a mixture of EDCs. After that, vitellogenin was applied as a biomarker of EDCs in wild fish from areas suspected to be contaminated with high concentrations of EDCs in Chon Buri province, Thailand. The exposure of EDCs in juvenile sea bass showed that both 4-nonylphenol and $CdCl_2$ significantly induced vitellogenin, while a combination of multiple EDCs had reduced vitellogenin expression compared to single EDCs exposure. The assessment of vitellogenin in the field study identified industrial and agricultural practices as potential sources of EDCs. These data suggest that EDCs are present in these aquatic environments and validate vitellogenin as a biomarker of EDCs.

Keywords: Vitellogenin; Endocrine Disrupting Chemicals; Biomarker; Sea bass

1. Introduction

Exposure to endocrine disrupting chemicals (EDCs) and xenoestrogens may interfere with normal endocrine function, which can mimic or modify the action of endogenous hormones and affect physiological functions such as reproduction and development in several different species of animals as well as humans (Combalbert and Hernandez-Raquet, 2010). These chemicals are wildly used in the formulation of plasticizers, paints, detergents, personal care products, pharmaceuticals, and pesticides (Matozzo *et al*., 2008).

The presence of EDCs in aquatic systems have been investigated in many countries of Southeast Asia (Duong *et al*., 2010). However, the study of EDCs and their effects in these region are deficient. The assessment of environmental contamination by EDCs and their effects are critical for environmental conservation. Previous studies have identified that nonylphenol, benzo[a]pyrene and cadmium chloride are chemicals with high

accumu lation in the surface water and sediment bed of Thailand (Boonyatumanond *et al*., 2006; Chaiyara *et al*., 2013; Duong *et al*., 2010). Study on the effects of these EDCs both as single and mixed exposure are important to understand the relationship of EDCs and their effects on aquatic animals. Detection of biomarkers can be an effective way to evaluate whether EDCs in aquatic environments affect the surrounding animals (Jasinska *et al*., 2015).

Vitellogenin (VTG) is a lipo-phosphoglycoprotein that is normally produced in sexually mature female oviparous vertebrates in response to endogenous estrogen (17β-estradiol; E_2). After being synthesized in the liver, VTG is transported via the bloodstream and cleaved to form egg-yolk proteins in the ovary. However, the VTG gene and estrogen receptor are also present in males and juveniles and can be stimulated by exogenous estrogens. As a result, induction of VTG in males or juveniles is an acceptable biomarker of EDCs exposure (Booth and Skene, 2006; Canapa *et al*., 2007; Chang *et al*., 2014).

In the present study, induction of VTG was evaluated in juvenile sea bass after exposure to single and a mixture of EDCs. Then, VTG in the wild fish from areas suspected to be contaminated with high concentrations of EDCs in Chon Buri province were determined. Sampling was performed in industrial, residential and agricultural areas. Assessment of VTG induction in wild fishes were determined in both quality and quantity using Western blot and competitive ELISA. These data verified the occurrence of EDCs in aquatic environments and raise the concern of EDCs affecting the wild populations.

2. Materials and Methods

2.1 Materials and Chemicals

17β-Estradiol (E_2), nonylphenol, benzo[a] pyrene and cadmium chloride were purchased from Sigma-Aldrich (Thailand), with a purity of 97.0%, 98.0%, 96.0% and 99.0%, respectively. Other reagents for SDS-PAGE and Western blot, including molecular weight standards, nitrocellulose membranes, Bradford reagent, bovine serum albumin (BSA), ammonium persulfate, 3,3 diaminobenzidine (DAB), dimethyl sulfoxide (DMSO), coomassie brilliant blue R-250 (CBR-250), and goat anti mouse IgG horseradish peroxidase conjugate (GAM-HRP) were obtained from Bio-Rad (Thailand). Tween-20 was obtained from Amresco and skim milk was purchased from Anline, Bangkok Thailand. All other chemicals used were of analytical grade and obtained from TTK Science Co., Ltd. (Thailand).

The monoclonal antibody anti VTG used for this assay (MAb-sea bass VTG 23 and 41) was generated by fusion of myeloma and spleen of BALB/ cMlac mice immunized with purified sea bass VTG as described previously in Prasatkaew *et al*. (2019).

2.2 VTG induction in juvenile sea bass

Juvenile sea bass (*Lates calcarifer* body weight of 13.5 ± 1.0 g) were obtained from Talaythong farm, Chon Buri, Thailand. Fish were maintained in 50 L tanks, with aerated freshwater at 25-28 °C. After being held in the laboratory for 7 days, fish were separated into 10 groups (n=10 per group) : three control groups including (i) non-treatment control, (ii) vehicle control, and (iii) positive control; three single EDCs treated groups including (iv) nonylphenol; NP 25 mg/kg body weight (BW), (v) cadmium chloride; $CdCl_2$ 0.5 mg/kg BW and (vi) benzo[a]pyrene; BaP 0.5 mg/kg BW; four EDCs mixture treated groups including (vii) NP 25 mg/kg and BaP 0.5 mg/kg, (viii) NP 25 mg/kg and $CdCl_2$ 0.5 mg/kg, (ix) BaP 0.5 mg/kg and $CdCl_2$ 0.5 mg/kg and (x) mixture of NP 25 mg/kg, BaP 0.5 mg/kg and $CdCl_2$ 0.5 mg/kg. All chemicals were dissolved in DMSO (0.7%, W/V) and 0.15 mol/L PBS, pH 7.4. Sea bass were injected intra-peritoneal with each chemical and intramuscular for E_2 2 mg/kg BW as positive control. These amounts of chemical used were based on previous studies (Prasatkaew *et al*., 2019). After exposure for 3, 6 and 9 days, plasma were collected from caudal vein and immediately centrifuged at 1500×g for 5 min. Then, VTG levels were determined by competitive ELISA.

2.3 VTG determination by competitive ELISA

The assay was based on the protocol by Lomax *et al*. (1998) and its optimization by Prasatkaew *et al*. (2019). Purified VTG (40 µg/mL; 100 µl) diluted in blocking solution (5% skim milk in 0.15 M PBS pH 7.2) was added in each well of a 96-well plate and incubated at 4 °C overnight. Then, the dilution of VTG (4 - 100,000 ng/mL) or samples were pre-incubate with MAb-sea bass VTG 41 (1:500) in 1.5 mL microtube at 4°C overnight in parallel. After incubation, the plate was washed three times with 0.15 M PBS containing 1% Tween-20 (PBST). Then, each pre-incubated solution (100 µL) was dispensed in duplicate into the plate and the plate was incubated for 2 hours at room temperature. Following a wash, the secondary antibody (GAM-HRP dilution 1:10,000) was added and incubated again. Then, the plate was washed with PBST three times and 100 µL of chromogenic substrate solution (0.025% TMB, 2.5% DMSO and 0.35% H_2O_2 in 0.1 M citrate buffer pH 4.5) was added and incubated in the dark for 15 min. After that, the reactions were stopped with 100 µl of 1 M H_2SO_4. Absorbance value was measured at 450 nm using a microtiterplate reader (Versamax, USA) and the VTG level was calculated from a standard VTG linear regression equation.

2.4 Cross-reactivity testing of MAb-sea bass VTG

All freshwater fish were obtained from the department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin Campus in Surin province. They were male nile tilapia (*Oreochromis niloticus* (206.75 ± 24.78 g), silver barbs (*Barbonymus gonionotus*) (55.77 ± 11.15 g), hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) (250.77 ± 6.93 g), nile tilapia (*O. niloticus-mossambicus*) (206.75 ± 24.78 g), striped snakehead (*Channa striata*) (506.75 ± 21.33 g), snake skin gourami (*Trichogaster pectoralis*) (46.75 ± 4.01 g), and moonbeam gourami (*T. microlepis*) (42.77 ± 4.40 g). The marine fish was coral grouper (*Epinephelus corallicola*) (92.00 ± 7.81 g) and was obtained from Trat coastal fisheries research and development center. Fish were maintained in 500-L tanks, with aerating freshwater or seawater at room temperature. Then all fish (n = 3 per tank/group) were injected with 17β-estradiol (E_2; 2 mg/kg BW) to induce vitellogenesis. Non-treatment group fish were injected with the same volumes of DMSO and 0.15 mol/L PBS without E_2 (vehicle control). Fish received three intra-peritoneal injections every three days. Then, three days following the last injection, blood was sampled from each fish.

2.5 Cross-reactivity testing

Western blot was used for testing cross-reactivity of MAb-sea bass VTG 23. Plasma from control and E_2-treated fish were separated by 7.5% SDS-PAGE. Then, proteins were transferred to nitrocellulose membrane and run on a Trans-Blot® SD Semi-Dry Transfer cell apparatus (Bio-Rad) at 15 V for 15 min in transfer buffer (0.025 M Tris, 0.192 M Glycine, 20% methanol). Nonspecific binding sites were blocked by immersing the membrane in blocking solution for 1 hour at room temperature. The membrane was incubated with MAb-sea bass VTG 23 (1:1,000) or MAb-sea bass VTG 41 (1:10,000) at 4°C overnight. After three washes, the membrane was incubated in GAM-HRP (1:3,000) as a secondary antibody for 2 hours at room temperature. Then, the membrane was washed again with PBST for 5 min 3 times and then developed with a chromogenic substrate solution (0.03% DAB, 0.06% H_2O_2, and 0.05% $CoCl_2$ in 0.15 M PBS pH 7.2).

2.6 Field study

In a field study, fish which were positive to MAb-sea bass VTG cross-reactivity testing were selected. Fish were captured by fishing or net from industrial, residential and agricultural areas in Chon Buri province plus Bang Phra reservoir as a reference area for 20 fish in each area. For the industrial area, fish were caught downstream of Saha group industrial park-Sriracha. The residential area was downstream of Chon Buri central wastewater treatment plant of Chon Buri provincial administrative organization and the agricultural area was Phanat Nikhom district. Fish blood was sampled from the caudal vein and stored in an

icebox and transferred to the laboratory. Plasma was examined for VTG by Western blot and competitive ELISA as described above.

2.7 Statistical analysis

Plasma VTG levels were calculated from the linear part of the standard curve. VTG was presented as the mean ± standard deviation. Differences between the sampling area and the reference area were assessed using one-way analysis of variance followed by Tukey's test with minitab® 17 software. Values were determined to be significant when $p < 0.05$.

3. Results and Discussion

3.1 VTG induction in juvenile sea bass

The induction of VTG in sea bass after exposure to single or a combination of NP, BaP and $CdCl_2$, plus the negative control (non-treated control, vehicle control) was determined by competitive ELISA using MAb-sea bass VTG 41. In pilot studies, these clones were verified as highly sensitive in sea bass with a detection limit of VTG at 40 ng/mL. The assay performance was similar to the performance in other species: 10-22 ng/mL in Smooth flounder (*Pleuronectes putnami*), cod (*G. morhua*) and California halibut (*P. californicus*) (Roy *et al*., 2004; Scott *et al*., 2006; Palumbo *et al*., 2009).

In the present study, a significant increase of VTG levels ($p<0.05$) was found in the positive control (E_2-treatment) at 2,380 to 2,547 ng/mL (Fig. 1). This data demonstrates that 2 mg/kg of E_2 can up-regulate plasma VTG in juvenile sea bass 3 days after exposure. This is consistent with similar reports, largemouth bass (*Micropterus salmoides*) showed an increase of plasma VTG at 48 hours post injection with 0.05, 0.5, 5.0 mg/kg of E_2 (Bowman *et al*., 2002) and in male goldfish (*Carassius auratus*), plasma VTG was induced by E_2 (10 mg/kg BW) at 7 days after injection. In addition, VTG level in exposed males was significantly higher than that in females, 7,860 ± 2,346 and 4,779 ± 814 µg/mL, respectively (Wang *et al*., 2015).

A single exposure to NP caused a significant increase of VTG levels approximately 7 times higher than the negative control groups (302 ± 3 ng/mL) at 3 days post-injection. Similar to juvenile Atlantic salmon (*Salmo salar*), 25 mg/kg BW of NP treatment induced VTG expression from day 2 with a maximum at day 7 post-injection (Arukwe *et al*., 2001). Also in male European sea bass (*Dicentrarchus labrax*), the 50 mg/kg NP-treated fish showed an induction of plasma VTG since day 4 with a maximum at day 14 (Vaccaro *et al*., 2005). The additional treatments of NP and BaP showed no significant increase in VTG level compared to

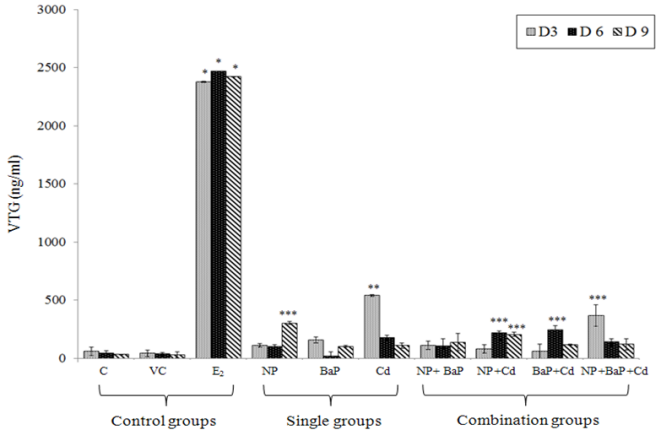

Figure 1. Plasma VTG concentration in juvenile sea bass from each group by competitive ELISA using MAb- sea bass VTG 41. Asterisks indicate a significant difference compared to the negative control (non-treatment and vehicle control); a group that shares the same asterisks are not significantly different ($p > 0.05$).

those treated with NP alone. This may be from an interaction between NP to estrogen receptor (ER) and BaP to arylhy-drocarbon receptor (AhR) signaling pathways. Previous studies reported the anti-estrogenic properties of AhR modulators such as BaP and PCB77 (Chang *et al*., 2014; Mortensen and Arukwe, 2007; Zheng *et al*., 2006). Treatment with TBT and BaP resulted in a decrease of VTG, as measured by alkali labile phosphorus assay (ALP), when compared with the control female false kelpfish (*Sebastiscus marmoratus*) (Zheng *et al*., 2006). In Atlantic salmon (*S. salar*), exposure of hepatocytes to NP in combination with 0.01 μM of PCB-77 showed a decrease of NP-induced VTG expression and inhibition of VTG mRNA expression (Mortensen and Arukwe, 2007).

VTG was significantly induced in $CdCl_2$ treatment groups at 540 ± 25 ng/mL, at day 3 (Fig. 1). A significant up-regulation of VTG mRNA was also reported after exposure to 0.5 mg/kg of $CdCl_2$ at 1 day/hour post-exposure and the maximum level could be observed 3 days after exposure (Huang *et al*., 2014).

However, VTG levels in the combination groups of $CdCl_2$ were relatively lower than $CdCl_2$ alone. Some researchers have argued that induction of VTG is not altered by metal (Chang *et al*., 2011; Gerbron *et al*., 2015; Hwang *et al*., 2000). Cd showed inhibitory effects on VTG production when the amount of Cd added exceeded the ability of hepatocytes to synthesize metallothioneins. In conclusion, Cd has been found to interact both directly with ER and indirectly to affect ER mediated protein levels. Low concentrations of Cd have been found to activate gene expression with high affinity for the hormone-binding domain in a manner that is inhibited by ER antagonists, further suggesting specificity in the time course of the ER response to natural estrogens or xenoestrogens in fish (Hwang *et al*., 2000; Tilton *et al*., 2003). NP, BaP and $CdCl_2$ are chemicals that accumulate in the surface water and sediment of Thailand (Chaiyara *et al*., 2013; Duongetal *et al*., 2010; Isobe *et al*., 2007). Organisms may be exposed to these chemicals and others from various sources that involve multicomponent mixtures in their ambient environmental media and/or food. Therefore, the combination effects of a mixture of chemicals should be addressed for understanding real effects in the environment.

3.2 Cross reactivity testing

In previous studies, MAb-sea bass VTG 41 showed cross-reaction to marine fish VTGs (*E. corallicola*) and no reactivity with VTG from any of the freshwater fish examined (Prasatkaew *et al*., 2019). As a result, in this present study the performance of other clones of MAb were evaluated for further detection and monitoring of EDCs in wild fishes.

For MAb-sea bass VTG 23, this clone had lower sensitivity than MAb-sea bass VTG 41 (detection limit = 0.4 μg/mL). However, MAb-sea bass VTG 23 can detect VTG in both laboratory and field studies. The range of VTG concentrations in wild fish was large < 0.01 μg/mL to > 50 $\times 10^3$ μg/mL based on a previous study (Scott *et al*., 2007). Therefore, the competitive ELISA developed using MAb-sea bass 23 is appropriate and could be used to monitor EDCs in the field.

In the present study, MAb-sea bass VTG 23 showed cross-reactivity with plasma VTG from E_2-treatment of *O. niloticus*, *B. gonionotus*, *O. niloticus-mossambicus*, *C. striata, T. pectoralis*, and *T. microlepis* and non-reactivity with *C. Microcephalus* × *C. gariepinus* and all control fishes (Fig. 2). It was no surprise that the MAb against VTG from the Latidae family could cross-react with VTG from fish in a different family since previous studies have shown cross-reactivity of antibodies against VTG between non-related species, even with a monoclonal antibody. For example, a universal monoclonal antibody to vertebrate (2D8) generated against purified rainbow trout (*O. mykiss*) VTG showed cross-reactivity with purified VTG of gag (*Mycteroperca microlepis*) VTG, nassau grouper (*E. striatus*) and red hind (*E. guttatus*) (Heppell and Sullivan, 1999). An *et al*. (2007) determined the cross-reactivity of monoclonal antibody against the VTG of crucian carp (*Carassisus carassius*) with VTG from the other teleost fish by sandwich ELISA and found that MAb-crucian carp VTG can detect VTG of the Cyprinoidae family (carp,

rare minnow, and zebrafish) with no cross-reactivity to others. These results confirm that the general properties of fish VTG are similar despite considerable differences in their molecular weights, electrophoretic patterns and post-translational modifications (Garnayak *et al*., 2013).

The present study also showed that VTG from sea bass has a different molecular weight to VTG from other freshwater fish species. The molecular weight of *O. niloticus* VTG was 210 and 140 kDa, similar molecular mass has also been found in *O. niloticus-mossambicus*. Two molecular weight forms have been identified in *C. Striata* (186, 89 kDa) and *T. pectoralis* (186, 97 kDa) while VTG in *E. corallicola* was 152, 133, 96, 81 and 72 kDa and *T. microlepis* was 164 kDa (Fig. 2). Numerous studies show that teleost VTG are high molecular mass phospholipoglycoproteins ranging from 300 - 600 kDa, while the monomeric from of VTG was 150-250 kDa (Booth and Skene, 2006; Garnayak *et al*., 2013; Maltais and Roy, 2009). In stone flounder (*Kareius bicolouratus*), two major bands with molecular mass of 165 and 106 kDa and two faint bands of lower molecular weights were observed in SDS-PAGE (Pan *et al*., 2012). Some polypeptide bands were below 150 kDa, it is likely that the protein we detected is a monomer given that reducing SDS-PAGE analysis was used as dimeric molecules would have been separated during processing of the sample (Booth and Skene, 2006). Purified VTG of goldfish (*C. auratus*) showed three major polypeptide bands corresponding to approximately 130, 106, and 81 kDa (Wang *et al*., 2015).

3.3 VTG in wild fish

Selected fish species were chosen after cross-reactivity testing was done. For industrial area, three wild fish species: moonbeam gourami (*T. microlepis*), striped snake-head (*C. striata*), and climbing perch (*A. testudineus*) were found. In the residential and agricultural areas only one wild fish species was obtained in each area, three spotted tilapia (*O. mossambicus*), and nile tilapia (*O. niloticus*), respectively. Two wild fish species were found in the reference area, silver barbs (*B. gonionotus*), and nile tilapia (*O. niloticus*). The sex ratio (male:female) in each area was similar, it was 30:70 in the industrial area, 36:64, 33:67, and 29:71 in the residential, agricultural and reference areas, respectively.

The presence of VTG was different in each sampling site. Induction of VTG was highest at the industrial area at 55.00% (11 from 20), where 33.33% are male (Fig. 3). The residential are had no detectable VTG expression in any sample (n=20) (Fig. 3). While, induction of

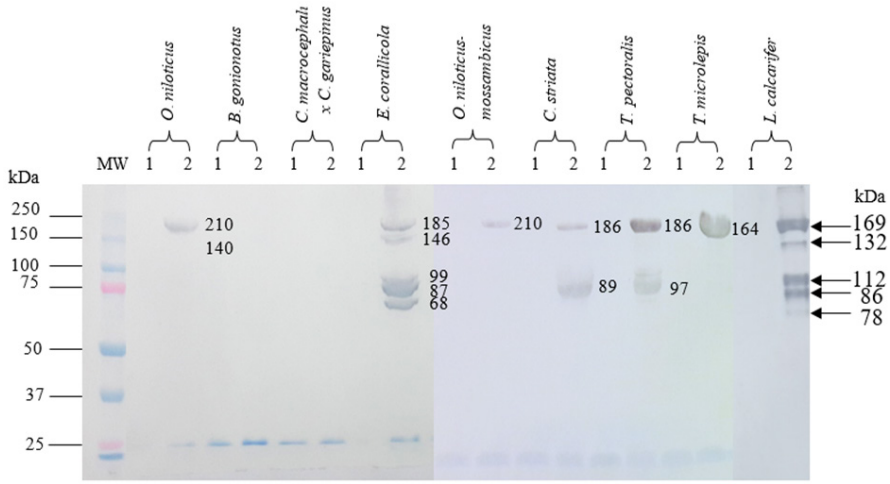

Figure 2. Cross-reactivity testing of MAb-sea bass VTG 23 to plasma VTG from non-E_2 treated (1) and E_2-treated fishes (2) by Western blot analysis. 0.4 μg/μL of total proteins were loaded in each lane (10 μL).

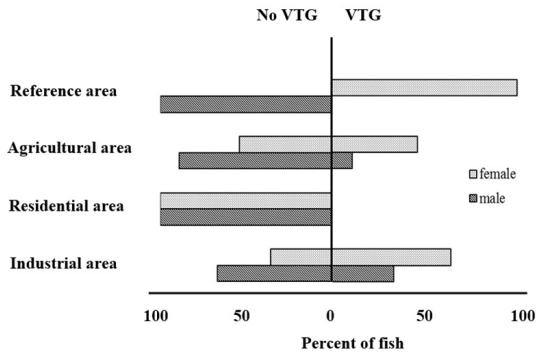

Figure 3. Wild fish exhibiting VTG presence in blood plasma in each of four areas for both males and females by Western blot using MAb sea bass VTG 23.

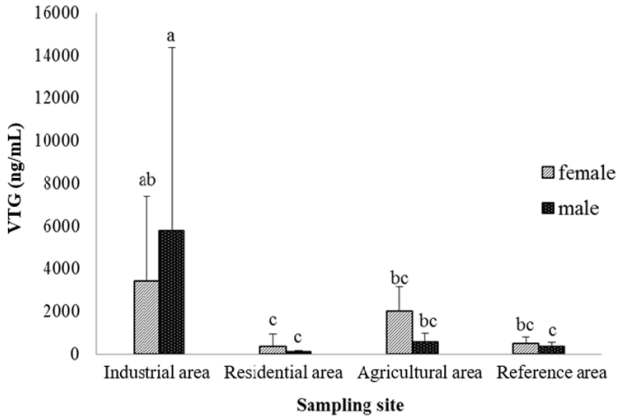

Figure 4. Plasma VTG level from wild fishes by competitive ELISA; Statistical significance for pairwise comparisons between all groups is indicated by letters (a, b, c) where groups with no letters in common show a difference that is statistically significant ($p>0.05$).

VTG in wild fishes from the agricultural area was found in 7 from 20 samples (35.00%), where 11% are male. In the reference area, VTG induction was observed in female only (Fig. 3). The explanation for VTG levels in female is reproductive maturation. The concentration of VTG in plasma increases rapidly during the reproductive period before spawning in females and decreases afterwards (Fazielawanie *et al*., 2011). However, there are different levels of VTG between fish species, for example English sole (*P. vetulus*) has > 20 mg/mL (Lomax *et al*.,1998), while sea bass (*D. labrax*) has 3 mg/mL (Mananos *et al*., 1994) and only 2 mg/mL in Asian catfish, (*Clarias batrachus*) (Garnayak *et al*., 2013). The variance between the species with respect to the levels of plasma VTG and reproductive cycle are probably a consequence of different reproductive strategies, duration of the vitellogenic process and size reached by the oocytes during the growing phase (Garnayak *et al*., 2013).

Competitive ELISA were also used for quantifying plasma VTG in wild fish collected from the industrial, residential and agricultural areas and compared with the reference area. The males in the industrial area exhibited the highest levels of VTG compared to the residential and reference areas. The VTG concentrations in males from the industrial area ranged from 0.2 to 17 μg/mL with a mean concentration of 6 μg/mL while it was 0.04 to 14 μg/mL with a mean concentration of 3 μg/mL in female (Fig. 4). There was no

difference found in the levels of plasma VTG between females and males in the residential area which were 49 to 1,646 and 55 to 303 ng/mL, respectively. The VTG expression in the agricultural area ranged from 257 to 3,433 ng/mL in female and 340 to 1,617 ng/mL in male. In the reference area, it was 184 to 1,076 ng/mL with a mean concentration of 438 ± 273 ng/mL in female and 223 to 742 ng/mL with a mean concentration of 370 ± 184 ng/mL in males (Fig. 4). The evidence in this study strongly suggests that EDCs are present in aquatic environments and affect VTG induction in wild male fish. A similar study of effect of EDCs on the local sanitary sewer system also found high levels of contamination at a variety of domestic, commercial, and industrial locations in a typical urban area (Jackson and Sutton, 2008). Also the reported VTG concentration in male fish downstream and upstream of the Gaobeidian wastewater treatment plant, the largest of the municipal and industrial wastewater treatment plants in Beijing, China, was 88.63 mg/mL in downstream, while that from a control site was undetectable (An *et al*., 2007). Similarly, male toadfish (*T. glaber*) at downstream sites from sewage treatment plants, and subject to urban runoff around Sydney, Australia, exhibited VTG in their blood, while those from sites without a nearby sewage treatment plant did not (Booth and Skene, 2006). These data confirm that both industrial and municipal activites are potential sources of EDCs contamination. However, in the present study only industrial and agricultural ares were found to be a source of EDCs.

4. Conclusion

VTG induction in juvenile sea bass after exposure to EDCs is a valuable detection method regarding the effect of EDCs in aquatic animals for environmental monitoring. The results suggest that the inhibition of VTG production by a combination of EDCs is complicated concerning AhR or ER mediated pathways and their possible mechanisms need further investigation. However, the cross reactivity of MAb-sea bass VTG on other species of freshwater fish was validated as a rapid assessment method for environmental monitoring. Besides, levels of VTG induction in the wild fish from industrial and agricultural areas indicate that there is EDCs contamination in the aquatic environments that affect the normal endocrine function.

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