Original Article

Vitellogenin Gene Expression and Sex Steroid Levels as Biomarkers in Yellowfin Seabream (*Acanthopagrus latus*) Exposed to Bisphenol-A

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Received: 14.12.2018 *Accepted: 30.01.2019*

ABSTRACT

Background: The egg yolk precursor protein vitellogenin (VTG) has proven to be a useful biomarker, used to identify organisms exposed to estrogenic compounds.

Methods: We investigated variations in the VTG gene expression pattern and plasma sex steroid hormones concentrations in the yellowfin Seabream, *Acanthopagrus latus*, (*A. latus*) by various doses of bisphenol-A (BPA) exposure for 7 and 14 days. We developed a quantitative real time polymerase chain reaction (RT-PCR) assay for the expression of VTG gene in *A. latus.* The dose-response pattern of VTG gene expression in *A. latus* exposed to various doses of BPA was characterized. In order to design RT-PCR primers specific to *A. latus* VTG, a partial sequence of the VTG gene was obtained.

Results: The RT-PCR assay was effective in detecting increased VTG gene expression in *A. latus* exposed to BPA. It also demonstrated that the VTG expression was affected by BPA in a dose and time-dependent manner. Plasma testosterone (T) levels were decreased in the treated fish in comparison with those found in the control group, when they were exposed to 100 µg/g of BPA and 2 µg/g of E2. In contrast, the plasma levels of 17β-estradiol (E2) were significantly increased in a dose-dependent manner.

Conclusion: The results suggest that VTG mRNA quantification can provide a sensitive and early signal in the detection of estrogens in marine wildlife. It also indicated that BPA could lead to an imbalance of sex steroid hormones with potentially harmful consequences on sexually immature male *A. latus*.

Keywords: *Acanthoparus Latus*, Bisphenol-A, Gene Expression, Real Time PCR Assay, Sex Steroid Hormones, Vitellogenin.

IJT 2019 (1): 27-33

INTRODUCTION

In recent years, it has become apparent that endocrine disrupting chemicals (EDCs) have become a growing

threat to human and wildlife health. EDCs are exogenous compounds that have the potential to mimick or block the regulation of endogenous hormones, causing adverse effects on the health of sea animals including fish. Among EDCs, Bisphenol A (BPA) has received particular attention because of its large-scale production and environmental widespread applications. BPA is utilized in the manufacturing of polycarbonate plastics and epoxy resins, and is found in food packaging, electronic instruments, fungicides, dental sealants and paper wraps (1-3). Iranian petrochemical plants that make BPA and the products are located on the coast of the Persian Gulf. Due to lack of high-efficiency sewage treatment plants, large amounts of untreated sewage including EDCs are released into the Persian Gulf (4).

Vitellogenin (VTG) is the egg yolk precursor protein that is normally produced in the liver of oviparous fish in response to endogenous estrogen. Although male fish possess the VTG gene, it is not usually expressed in them due to very low levels of plasma estrogen (5). However, exposure to xenoestrogens in the aquatic environment is well known to induce the expression of the VTG gene in immature male fish. Thus, the induction of VTG gene expression in juvenile or mature male fish can be used as a sensitive biomarker for xenoestrogens in oviparous fish (6-9). Obviously, an assay for VTG gene expression in *A. latus* is needed, which does not currently exist. We determined the gene activation by measuring the amount of VTG mRNA expression, using real time polymerase chain reaction (RT-PCR) technique. This required a VTG-specific primer which did not exist either. Although the synthesis of VTG in immature male *A. latus* exposed to EDCs has been reported in several studies (10, 11), VTG mRNA sequence is not currently available.

Sexually immature male *A. latus* were chosen as the marine fish test species in this study. The *A. latus* have become an important marine model species for ecotoxicology studies in the Persian Gulf due to high economic and ecologic values, gender-specific features (i.e., hermaphroditism), easy adaptation to captivity, high resistance and rearing techniques availability. This fish is a native species in the Indian Ocean and inshore waters of Persian Gulf. This sea animal is a hermaphrodite species that first develop as mature male but later transform into female species (12).

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Due to its structural similarity with endogenous 17-β-estradiol, BPA can potentially disrupt the synthesis and homeostasis of sex steroid hormones (13, 14). The disruption of sex steroids homeostasis can affect the sexual differentiation of the fish (15). Hermaphrodite fish, including those used in this study, have a critical and sensitive period of sexual differentiation that may be more endangered by exposure to xenoestrogens than in other fish species.

The objectives of this study were to: *a)* develop a quantitative RT-PCR assay for the expression of VTG gene in *A. latus*; *b)* characterize the dose-response pattern of VTG gene expression in immature male *A. latus after* exposure to BPA, and *c)* investigate the potential adverse effects of BPA on the plasma levels of sex steroid hormones in this fish.

MATERIALS AND METHODS

Fish

Yellowfin seabream, *A. latus*, weighing 60±4.67 grams were caught in Moosa Creek, located in the northwestern part of the Persian Gulf, Iran. They were transported alive to the laboratory and held in fiberglass tanks containing 300 liters of filtered sea water. The water was disinfected by ultraviolet radiation and aerated regularly. During the adaptation and test period, the fish were kept under 12 hours of alternate light and dark cycle at pH 8.1, salinity 45±1% and temperature 25±1°C. The fish were acclimatized to the test condition for 12 days. About 30% of experimental seawater was changed every day. During acclimatization and experimental period, the fish were fed 3% of their body weight, using a commercially available feed (Beyza 21 Feed Mill, Fars, Iran).

Chemicals

Bisphenol A (BPA; CAS 80-05-7) with a purity of 99% and 17β-estradiol (E2; Sigma–Aldrich brand) and 2-Phenoxyethanol (Merck Chemicals) were obtained from the authorized distributors in Tehran, Iran.

BPA Exposure

Ninety one fish were randomly distributed into seven tanks, 13 per tank for two weeks. At the beginning, the fish were anesthetized with 0.1% 2-phenoxyethanol and were injected intraperitoneally with 0, 1, 10, 50 and 100 µg/g/week of BPA, dissolved in olive oil. The injections were made in half doses twice a week. The solvent controls received olive oil only (the vehicle) while the positive controls were injected with 2µg/g/week of E2, but they were not injected with BPA.

Sample Preparation

The fish were sampled on days 1, 7 and 14. At each sampling time, six fish from each tank were caught quickly and anaesthetized with 2-phenoxyethanol. The fish were weighed and blood samples were collected immediately from the caudal artery, using heparinized syringes and transferred in ice-chilled tubes. The blood samples were then centrifuged at 1000×g for 10 min at 4°C. The plasma was separated and stored at -80°C until further analysis. Immediately after blood samples collection, the fish were dissected; the livers removed; and were placed into labeled cryo-tubes. They were then snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation processes.

VTG Gene Expression Analysis

RNA Extraction and cDNA Synthesis

The total RNA was extracted from an individual male liver, using the RNX^{TM}-plus solution according to the manufacturer's recommendations (Sinaclon, Tehran, Iran). Briefly, one ml RNX-plus was added to a tube containing 50mg homogenized tissue and incubated at room temperature for 5 minutes. Then, 200µl chloroform was added to the solution, incubated on ice for 5 min and centrifuged at 12000 rpm for 15 minutes. The aqueous phase was then transferred to another tube, to which an equal volume of isopropanol had been added. The mixture was centrifuged at 12000 rpm for 15 minutes and the resulting pellet was washed with 70% ethanol and dissolved in diethyl pyrocarbonate-treated water (DEPC). The purity and integrity of the extracted RNA were evaluated by optical density measurements and visual observation of sample electrophoresis on 1% agarose gel. The total RNA sample was diluted 1/100 in an Eppendorf tube and quantified at 260 nm and 280 nm in a UV-visible spectrophotometer. The total RNA yield (µg/ml) was calculated based on the absorbance at 260 nm, and was kept at –80ºC for further analyses.

A sample of the total RNA (3µl) was used as a template in a 10µl volume RNA-primer mixture, containing 1µl oligo dT and the DEPC-treated water up to 10µl. This solution was first denatured at 65ºC for 5 minutes and chilled on ice immediately for 2 minutes according to the recommended protocol (CinnaGen First Strand cDNA synthesis kit; Tehran, Iran). The cDNA synthesis solution was prepared, using a mixture of 20 units ribonuclease inhibitor, 10mM dNTPs, 10x buffer MMuLV and the DEPC-treated water up to 10µl in each tube. The mixture for cDNA synthesis (10µl) was added to each tube, containing RNA-primer mixture and incubated in a BioRad thermocycler (USA) at 42ºC for 60 minutes and then at 85ºC for 5 minutes. Lastly, two tubes representing negative controls containing all of the components except for RNA and MMuLV accompanied each reaction.

Identification and Sequencing of A. latus VTG

First, a partial sequencing of the VTG gene was obtained from *A. latus* by amplifying a segment of the gene; using primers from the conserved regions of the VTG gene in other fish and then sequenced the amplified DNA. The obtained partial VTG sequence was used to design specific primers for a quantitative real time PCR analysis. In order to determine the relative rate of expression of the gene, RT-PCR analysis was performed on the VTG gene and a housekeeping gene, *β-actin*. The ratio between the mRNA levels of the two genes was used to quantify the VTG gene expression.

To isolate a VTG cDNA fragment, primers were designed from conserved region by alignment of available VTG mRNA sequences from Sparus aurata (HG794236.1), Lithognathus mormyrus (AY271255.1) and Pagrus major (AB181839.1). Primers used for amplification were: *forward*, 5´TGCAGGCAAGATTCAGATCC3´and *reverse*, 5´ TGTGCTGTACTCCTGTCTGA3´.

Real time PCR was performed, using 2.5µl of synthesized cDNA with 0.3µl Taq polymerase (Cinnagen, Tehran, Iran), 0.8µl $MgCl_2$, 0.5µl dNTPs, 0.5µl of each 10µM primer, 2.5µl 10x buffer and deionized distilled water in a 25µl total reaction volume. All of the common components were added into the master mix and aliquoted into the tubes. The cycling conditions were as follows: *a)* initial denaturing step at 94ºC for 5 min,

b) 35 cycles at 94ºC for 30 sec., *c)* at 60ºC for 15 sec., *d)* at 72ºC for 30 sec., and *e)* a final cycle at 72ºC for 5 min. Each experimental step was repeated at least three times in order to ensure the reproducibility of the experiment. The molecular size of the digested products was checked on 1% agarose gel electrophoresis.

Quantitative Real Time PCR

Real-time PCR was performed on a *Step One* real time PCR system (Applied Biosystems) in a 48-well PCR plate. A PCR mixture for one reaction contained 12.5µl of SYBR Green master mix (RealQ Plus 2x Master Mix Green), 1µl of each gene specific primer and 10.5µl of cDNA that was diluted in RNase free water. The sequences of specific primers for the genes are presented in Table 1. A negative template control (water only) was analyzed on each plate to determine whether nonspecific products formed during the PCR amplification. Both primer pairs were optimized for annealing temperature, and melting curve analyses were performed to ensure amplification of a single product. All samples and negative controls were analyzed in duplicate, using a two-step protocol with an initial denaturation at 94°C for 2 minutes. The thermal cycle profile was performed as follows: denaturation at 94°C for 30 sec., annealing at 62°C for 20 sec., extension at 72°C for 5 sec. for a total of 45 PCR cycles, and a dissociation cycle to generate a melt curve. At the completion of amplification process, melt curves were generated to evaluate the amplification of a single product, and the threshold cycle (Ct) was determined for each reaction. The values of Ct for the gene of interest were normalized to the endogenous control gene β-actin, using the ΔΔCt method (16). The PCR products were visualized on an agarose gel, then were cloned and sequenced to match the genes of interest.

Table 1. Specific Primer sequences used for RT-PCR of an *A.latus* VTG gene transcript.

Primer	Sequence
Forward VTG	AACAGAGCGGTGAGGGCATC
Reverse VTG	TGCAGATTCCACAGGTCTGTCC
Forward β-actin	ATGGGCCAGAAAGACAGCTACGTG
Reverse β-actin	CTTCTCCATGTCGTCCCAGTTGGT

Hormone Assays

Plasma concentrations of testosterone (T) and 17β-estradiol (E2) were measured, using a commercially available radioimmunoassay kit (RIA; Immunotech; Paris, France).

Statistical Analysis

All data are expressed as means ±standard error. Two-way analysis of variance (ANOVA) was used to examine the significance of dose, time and interaction. Duncan's post hoc test was used to determine the significant differences of data among the groups (17). The levels of VTG mRNA in each sample were normalized against the level of β-actin in the same samples, based on standard curves. The normalized levels of the VTG transcripts in each BPA or E2 treated group, was then compared to those of the control groups, using ANOVA followed by Duncan's tests.

RESULTS

In this study, there were no mortalities among the fish in any treatment group during the adaptation and experimental period.

Characterization of A. latus VTG cDNA

The partial cDNA of the *A. latus* VTG gene was characterized from the PCR product amplified, using primers based on sequences retrieved from a gene bank (Accession No. LC269106.1). We obtained a 152 bp nucleotide sequence of *A. latus* VTG gene that shared a 94% identity with Sparidae VTG sequences (Fig. 1).

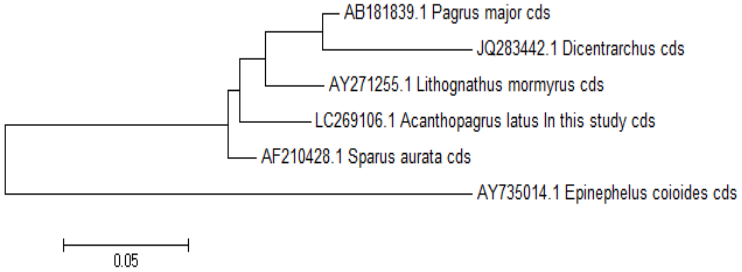

Figure 1. Phylogenetic tree of the VTG gene constructed via neighbor-joining analysis (bootstrap value 500).

Expression of A. latus VTG mRNA after BPA exposure

We investigated variations in the VTG expression pattern in *A. latus* by BPA and E2 exposure for 7 and 14 days. The expression of VTG transcript in *A. latus* in response to BPA and E2 exposure is shown in Figure 2. The VTG gene expression significantly increased in treated fish after 7 days compared to those in the control groups, except for the treatment with 1µg/g of BPA (Fig.2). On day 14, the VTG gene expression was increased significantly in response to exposure to all concentrations of BPA. The response of VTG mRNA to BPA exposure was found to occur in a dose and time dependent manner. A significantly higher increase

($p<0.05$) was observed in the fish treated with BPA after 14 days compared to those after 7 days of exposure to the same treatment. As seen in Figure 2, the greatest increase in VTG gene expression was observed for the exposure of fish to 100µg/g of BPA ($p< 0.01$).

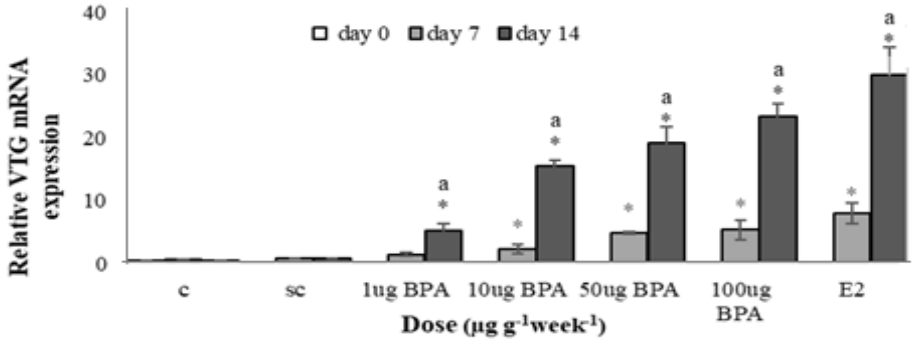

Figure 2. Expressions of *A. latus* VTG mRNA after exposure for 7 and 14 days to various concentrations of BPA and E2 (positive control). mRNA expression in liver tissue is shown relative to β-actin expression after normalization. An asterisk indicates a statistically significant difference, $p<0.05$ compared with the controls (C: control, SC: solvent control). [a]Significant difference from day 7 in the same concentration.

Plasma Steroid Hormones

To elucidate whether BPA could affect sex steroid hormone levels in immature male *A. Latus*, we measured the plasma levels of T and E2 (Figures 3 & 4). The plasma levels of steroid hormones in these fish were affected by varying doses of BPA after 7 and 14 days of treatment. The plasma T levels showed a significant reduction ($p < 0.05$) on day 14 in the fish treated with 100µg/g of BPA and 2µg/g of E2, compared with those in the control groups (Fig. 3). Moreover, a significant reduction was also found in the fish treated with E2 after 14 days, compared with those for 7 days of exposure to the same treatment ($p < 0.05$). After 1 week of exposure, E2 plasma level was significantly increased in the fish exposed to 50 and 100µg/g BPA, and 2µg/g E2 (Fig. 4). Also, a significant increase ($p < 0.05$) in E2 plasma levels was observed for 10µg/g of BPA after 2 weeks. No difference in plasma E2 level was noted in fish exposed to 1µg/g BPA (Fig. 4).

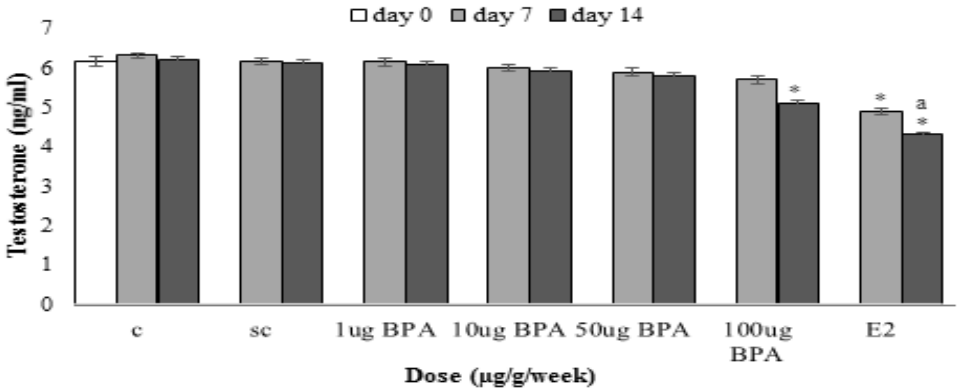

Figure 3. Plasma levels of testosterone in *A. latus* after 7 and 14 days treatment with different doses of BPA (The data are expressed as the mean ±SE). *Denotes significant differences from the control group (C: control, SC: solvent control), ($p<0.05$). [a]Significant difference from day 7 in the same concentration.

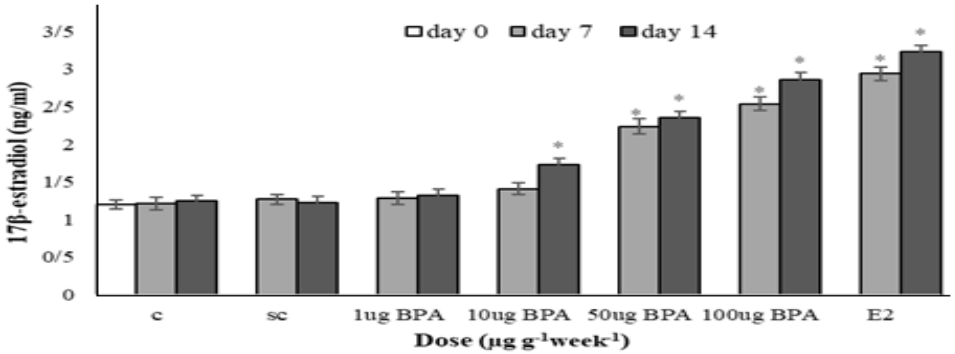

Figure 4. Plasma levels of 17β-estradiol in *A. latus* after 7 and 14 days treatment with different doses of BPA (The data are expressed as the mean ±SE). *Denotes significant differences from the control group ($p<0.05$), (C: control, SC: solvent control).

DISCUSSION

This study was conducted to evaluate the effects of BPA and E2 on the VTG gene expression and the levels of sex steroid hormones in immature male *A. latus* for 14 days. We characterized and analyzed the cDNA encoding VTG in *A. latus*. The identity of this cDNA was confirmed by comparing it with the nucleotide sequences of other known teleost VTGs. The molecular phylogenetic analysis revealed that the *A. latus* VTG gene had a higher sequence homology to *Sparidae* than to other species (Fig. 1). The sequence of *A. latus* VTG mRNA may be used as a useful molecular marker for the monitoring of fish reproductive health in the marine environment. This is a particularly important issue in Iran, since this fish has been a popular and commercially available species in the Persian Gulf marine farms.

The study of changes in gene expression, as a molecular marker, has become the method of choice for toxicological research in fish (9, 18-20). Gene expression profile can provide valuable information regarding EDCs exposure and potential effects on higher biological levels (21). Thus, gene expression techniques provide a sensitive and measurable endpoint for ecotoxicity and, therefore, can serve as an early warning of a specific biological outcome. In this study, *A. latus* were used to examine the effect of BPA on the estrogenic gene expression. Although some studies have used VTG as a biomarker of estrogen exposure in marine species, further studies should be conducted in order to elucidate the threats posed by EDCs to specific species. In this study, we developed a real time PCR assay for VTG gene expression in *A. latus*. It has been demonstrated that E2, as a natural estrogen, can induce the expression of hepatic VTG gene in various fish (9,22,23).

We examined changes in the VTG gene and the effect of E2 in *A. latus* following exposure to varying concentrations of BPA. We carried out real time PCR analyses, which revealed upregulated expression of VTG gene in the liver of immature male *A. latus* that responded to E2 exposure in a dose-dependent manner after 7 and 14 days. The results of our gene expression assays clearly demonstrated that the injection of varying doses of BPA caused a marked increase in VTG gene expression in immature male *A. latus*. This finding indicates the estrogenic effect of BPA. The gene expression increased significantly in treated fish after 7 days of exposure compared to those in the control groups. On day 14, the VTG gene expression increased significantly higher in response to exposure to BPA and E2 at all concentrations used except for BPA treatment at 1µg/g. Throughout this experiment, the response of VTG mRNA to BPA exposure occurred in a dose and time dependent manner.

Previous studies have demonstrated that exposure to some EDCs induces upregulation of VTG gene expression in male fish. For instance, long term exposure to different water concentrations of BPA (10, 200, or 400 µg/L) increases the expression of VTG genes in F1 male zebrafish in a dose-dependent manner (24). In addition, exposure to 1 mg/L Bisphenol AF for 28 days also significantly increased VTG gene expression in male zebrafish (25). Moreover, the injection of adult male Indian freshwater murrel fish (*Channa punctatus*) with E2 (1, 10 and 40 µg/100g/body wt.) showed a dose-dependent response with respect to VTG genes expression (22). Similarly, Arukwe *et al*. (26) observed a significant increase in VTG gene expression in a dose and time dependent pattern in rainbow trout fish (*Oncorhynchus mykiss*) when treated with varying doses of nonylphenol.

We measured the plasma concentrations of T and E2 to see whether BPA could affect sex steroid hormone levels in the immature male *A. latus*. The results clearly demonstrated that BPA could affect the concentration of plasma sex steroid hormone in this fish. Previous studies on mammalians and fish have indicated that BPA could act as a potent anti-androgen compound (27, 28). Moreover, it has been previously shown that BPA (0, 20, 100 and 200 µg/g/day) and E2 (10 and 100 µg/g/day) exposure significantly decreased plasma T levels in male rats in a dose dependent manner (14). Our results also revealed a dose-dependent reduction in plasma T level in *A. latus* after being exposed to various BPA concentrations and a single dose of E2.

Numerous studies have shown that bisphenols can induce E2 synthesis and disturb the balance of T and E2 hormones in fish (13, 29, 30). Yang *et al*. (29) investigated the effect of Bisphenol B (BPB) on the steroid hormone homeostasis in zebrafish. This study reported that the T levels in that species decreased in a concentration dependent manner and the E2 level significantly increased when the fish was exposed to 0.01, 0.1, and 1 mg/l of BPB. In another study, the intraperitoneal injection of 4-nonylphenol given to juvenile rainbow trout caused a significant elevation of plasma E2 in male fish (31). It is documented that sex steroid hormones (T and E2) and their homeostasis play a key role in sexual differentiation and maturation of teleosts. In this context, *A. latus* is considered a hermaphrodite species with protandric gonads, i.e., maturing first as male fish then transforming into female fish. Therefore, alterations in sex steroids levels obviously influence sexual development and differentiation in this fish. It has previously been observed that Bisphenol F (BPF) inhibits the conversion of progesterone to testosterone by downregulating the expression of cytochrome P450 17-alpha-hydroxylase (cyp17) and 17-beta-hydroxysteroid dehydrogenase (17β-HSD) genes and upregulating cytochrome P450 family 19 (CYP19) gene that are responsible for the biosynthesis of estrogen from testosterone in male fish (32). We have assumed that BPA reduces plasma T while increasing plasma E2 levels in *A. latus* in a similar manner. The impairment of steroid levels could result either from interactions in the hypothalamus-pituitary-gonadal axis or through direct action of BPA on enzymes involved in the steroidogensis processes (14, 32).

CONCLUSION

We developed a partial VTG gene sequence and quantitative RT-PCR method for the expression of VTG gene in *A. latus.* This method is effective in detecting changes in the VTG gene expression due to exposure to BPA and E2. The use of VTG mRNA induction in *A. latus* was found to be a sensitive biomarker of exposure to EDCs and was helpful in elucidating and monitoring the adverse effects in marine species. Based on the results, we conclude that VTG mRNA quantification provides a sensitive and valuable method in the detection of environmental estrogens that affect the health of marine species.

The sex steroid hormones levels were altered in immature male fish exposed to BPA. Since steroid hormones play an important role in the normal sexual development and differentiation, the alterations suggest that BPA could lead to adverse effects on the reproduction and the health of *A. latus*. Therefore, BPA exposure could be considered as a major threat to the reproductive health of *A. latus*. Our methodology has the potential for monitoring the aquatic hazards, such as BPA and other EDCs, facing fish and other marine life with high economic and ecologic impact especially in the Persian Gulf.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Mr. Ali Zadi and Dr. Mohammad Hossein Gharacheh for providing technical assistance in support of the study.

CONFLICT OF INTERESTS

Authors declare that there was no conflict of interests in the course of conducting this research.

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