Cloning and study of vitellogenin expression in wild cyprinid *Petroleuciscus esfahani* as a biomarker of endocrine disruption along the Zayandeh Roud River, Iran

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Abstract

Aquatic environments are the ultimate sink for most of anthropogenic pollutants. The Zayandeh Roud River is the most important river in the central Iranian Plateau, supplying water resources to a large population. In order to determine the potential occurrence and *in vivo* effects of EDCs with estrogenic properties we analyzed the wild populations of an extensively distributed endemic fish species, *Petroleuciscus esfahani*. For this purpose, specimens were caught from two sites upstream and two sites downstream of the expected major anthropogenic pollution sources. *P. esfahani* full length cDNAs for vitellogenin (*vtg*), with 4,177 base pairs (bp) encoding a 1,339 amino acids (aa), and for β -actin (*actb*), with 1,776 bp encoding a 375 aa, were amplified and cloned. Hepatic *vtg* mRNA expression levels were measured by quantitative real-time PCR. Condition factor, gonadosomatic index and sex ratio were calculated and compared with *vtg* expression. Gonad histology was performed to study the possible presence of intersex condition. Detection of *vtg* transcripts in male individuals from the two downstream sampling sites supports the hypothesis of exposure to estrogenic compounds in these regions. Higher *vtg* expression in male individuals, together with reduced gonad size and condition factor, in specimens from the third site, located downstream of the major steel mill plant, suggest a major endocrine disruption in this area.

Keywords: Endocrine disruption, *Petroleuciscus esfahani*, Vitellogenin expression, Zayandeh Roud River

1. Introduction

There is a growing concern about a high variety of environmental pollutants affecting human and wildlife health (Sun et al., 2011). Among them, there is a family of contaminants known generically as endocrine disrupting chemicals (EDCs). One major class of EDCs is formed by compounds with estrogenic properties, also known as xenoestrogens, which mainly affect at the level of the hypothalamus-pituitary-gonad (HPG) axis (WHO/PCS, 2002; WHO/UNEP, 2013). Many of the anthropogenic activities are potential sources of this group of chemicals, which finally find their way to rivers and lakes and affect aquatic organisms (Jeffries et al., 2010).

The Zayandeh Roud River is the only permanent and the most important river in the central Iran, which plays a key role in providing Isfahan and the adjacent provinces with drinking water. Land use along this river includes agricultural fields, cattle farms, municipal waste water treatment plants and heavy industries such as steel mill companies, power plants and oil refinery installations. Therefore, there is a high probability of contamination by EDCs. According to our knowledge, the information about the presence and *in vivo* consequences of EDCs in water ecosystems from Isfahan province or generally in Iran is very scarce. The limited data available for the Zayandeh Roud River, which are dedicated to measuring some heavy metal and a series of compounds belonging to polycyclic aromatic hydrocarbons, affirm their existence in water and/or sediments (Nemati et al., 2009; Abdollahi, 2013).

Since chemical monitoring of the aquatic environment alone is restricted to identification of some of the EDCs with estrogenic effects, and the detection methods are costly and complex, the use of biomarkers in sentinel species has been considered an effective approach to assess the health status of the aquatic ecosystems (Frenzilli et al., 2008; Blazer et al., 2012; Gilroy et al., 2012). Obviously, aquatic species are more exposed to EDCs than terrestrial animals (WHO/PCS, 2002). Among different aquatic species used for EDCs screening, fishes are the prominent model due to their particular features, the most important of which is that the basic aspects of the function and structure of the HPG axis are conserved in all vertebrates. Therefore, the results from their study can be extrapolated to other vertebrates including humans (Ankley et al., 2009). *Petroleuciscus esfahani* is a small cyprinid fish endemic to the Zayandeh Roud basin

(Coad & Bogutskaya, 2010). This species is abundant along the river and is even available in locations with constant input of contaminants. All these features, together with its limited immigration capacity, make this fish a suitable candidate for sentinel species in this ecosystem.

Fish exposure to EDCs with estrogenic activity can lead to several consequences, including i) vitellogenin induction in juveniles or males (Blazer et al., 2014; Duffy et al., 2014), ii) delay or absence of secondary sexual characteristics and sexual behavior in males (Goksøyr et al., 2003), iii) intersex condition (Puy-Azurmendi et al., 2013), iv) skewed sex ratio (Jobling et al., 1996), and in extreme cases v) complete extinction of the population (Kidd et al., 2007). Vitellogenin (Vtg) is a female-specific precursor protein for egg yolk, which is normally synthesized in female hepatocytes in response to endogenous estrogens (Goksøyr et al., 2003). Males also possess the vitellogenin genes (*vtg*), which are silenced but can be induced when exposed to xenoestrogens (Hutchinson et al., 2005). Actually, vitellogenin induction in males is the strongest biomarker for fish exposure to estrogenic compounds (Nadzialek et al., 2011). *vtg* mRNA is induced rapidly (within a few hours of exposure) and therefore, are useful for short-term screenings of the ecosystems, as well as it has been already field tested in many ecotoxicological examinations (Frenzilli et al., 2008; Blanchet-Letrouvé et al., 2013; Bizarro et al., 2014).

The aim of this study was to determine the possible exposure of xenoestrogens on *P. esfahani* as a bioindicator in the Zayandeh Roud River ecosystem. For this purpose, full sequence of the cDNA encoding *vtg* and *actb* was cloned for the first time in this species, and hepatic *vtg* mRNA expression was quantified using real-time PCR. Moreover, condition factor, gonadosomatic index and sex ratio were calculated and compared to *vtg* expression. Gonad histology was also performed with the goal of detecting any state of intersex in the specimens.

2. Materials and methods

2.1. Studied area and sampling points

The Zayandeh Roud River, with a drainage area of 41,500 km², is the largest river in the central plateau of Iran. Sampling sites were selected throughout the river having in mind a possible gradient of anthropogenic impacts. Cheshmeh Dimeh (S1) (32°31' N - 50°13' E), and

Khersoonak (S2) (32°31' N - 50°22' E), are located upstream of the Zayandeh Roud Dam and were considered as uncontaminated sites. In these two sites, some extensive and scatter crop and cattle production can be found. On the other hand, Chamgordan (S3) (32°23' N - 51°17' E) and Safaieh Bridge (S4) (32°21' N - 51°26'E) are situated downstream of the Zayandeh Roud Dam and were considered *a priori* as possible contaminated sites. S3 is a lagoon like reservoir which is formed by entering the river into an enclosed area and it receives effluents from the nearby major steel mill plant as well as agricultural fields. S4 is located downstream of the sewage treatment plant discharges from the Zarin Shahr City (population in 2012: 95,326) (Figure 1). During fish collections, temperature, dissolved oxygen, pH, and conductivity were measured on site, and total dissolved solids (TDS), biochemical oxygen demand (BOD), and chemical oxygen demand (COD) were determined in laboratory (Table 1).

Table 1. Water physico-chemical parameters in four sampling sites from the Zayandeh Roud River.

Site	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (μS/cm)	TDS (ppm)	BOD (ppm)	COD (ppm)
S1	6	9.7	8.15	194	388	40	120
S2	4.8	9.6	7.88	215	431	30	110
S3	10.2	9	8.50	1140	570	70	151
S4	6.6	13.6	8.97	456	912	80	162

Figure 1. Sampling locations of *P. esfahani* from the Zayandeh Roud River. Cheshmeh Dimeh (S1), Khersoonak (S2), Chamgordan (S3) and Safaieh Bridge (S4).

2.2. Fish sampling and analyzed parameters

Fish were captured during the non-reproductive period (January to February, 2012) by seine net (8 m × 1 m, 5 mm mesh size) in S1, S2 and S4, and by boat electrofishing in S3 because of higher water depth in this site. After transporting the fish to the laboratory, 10 individuals were aquatically exposed to 17β-estradiol (E₂) (2 µg/L) for 24 hours (Bowman et al., 2000) with the purpose of increasing the chance of cloning the *vgt* cDNA. At the end of this period, male and female liver samples were preserved in RNAlater® (Invitrogen Life

Technologies) separately. On the other hand, fish from all the sampling sites were kept in aerated tanks with water from the corresponding sampling site until processed (around 1 hour). Fish were anaesthetized by immersion in 100 ppm MS₂₂₂. Length (standard length $\pm$ 1 mm) and body weight (body weight $\pm$ 0.01 g) were measured. Gonads were excised to assign sex and weight (gonad weight $\pm$ 0.01 g). After that, the condition factor (CF) and gonadosomatic index (GSI) were calculated by $((\text{body weight} - \text{gonad weight}) \times \text{length}^{-3} \times 10^5)$ and $(\text{gonad weight} \times \text{body weight}^{-1} \times 100)$ formulas, respectively. The middle section of gonads (and in small individuals the whole organ) were fixed in 10 % formalin, dehydrated in a graded ethanol series and embedded in paraffin blocks. 7-8 micrometer sections were mounted on microscope slides stained with haematoxylin and eosin (Presnell & Schreibman, 1997). Slides were examined by light microscopy in order to detect any intersex condition. From each sampling site, around 30 mg of liver from 20 specimens (around 10 individuals from each sex) were selected randomly, preserved individually in RNAlater[®], and stored at -20 °C till processed.

2.3. Cloning *vtg* and β -actin (*actb*) in *Petroleuciscus esfahani*

Total RNA extraction from *P. esfahani* preserved liver samples (30 mg), and measurement of RNA quality and quantity was carried out according to the procedure explained in Baldisserotto et al., 2014. Total RNA was extracted on one side from the liver sample of a female captured from S1 and on the other side from the pooled liver samples of E₂ treated males. In order to increase the chance of *vtg* cloning, the mentioned two total RNA samples were mixed prior to cDNA synthesis. This pooled total RNA sample, was reverse-transcribed using the qScript[™] cDNA synthesis kit (Quanta BioSciences).

Degenerated primers for *vtg* were designed from conserved regions along the full length cDNA sequences from *Cyprinus carpio vtgb1* (AB331884), *Carassius auratus vtg* (DQ641252), *Cirrhinus molitorella vtgb1* (GU324313), *Catla catla vtgb1* (EF190987), *Gobiocypris rarus vtgaol* (EU623080), *Pimephales promelas vtg* (AF130354), *Phoxinus oxycephalus vtg1* (EF639845). For *actb*, primers available at the laboratory, used for the cloning of *Rhamdia quelen actb* (KC195970) partial sequence (Baldisserotto et al., 2014), were also used (see Supplementary material, Table 1). Afterwards, PCR reactions were performed with BIOTAQ[™] DNA polymerase (Bioline) and samples were cycled (95 °C, 10 min; [95 °C, 30 s; 60.4 °C, 30 s;

72 °C, 30 s] × 35 cycles; 72 °C, 10 min). The PCR products were run in agarose gel. Bands with expected size were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Cloning and sequencing were performed according to Baldisserotto et al., 2014. The intermediate *vtg* and *actb* products were 1,278 bp and 334 bp long, respectively.

Since the distance from the intermediate fragment of *vtg* cDNA to the 3' end was expected to be very long, an elongation strategy was followed. For that purpose, several PCR reactions were performed using 3 nested forward specific oligonucleotides, designed from the 3' end of the intermediate *vtg* fragment (overlapping a minimum of 470 bp), and 3 reverse degenerate primers (shown in Supplementary material, Table 1), designed from the conserved areas of other cyprinids. The rest of the procedure was as described above. With this approach, *vtg* was extended 1,724 additional base pairs toward the 3' end.

Using total RNA as template, the 3' and 5' ends of *vtg* or *actb* mRNAs were amplified using the FirstChoice® RLM-RACE Kit (Ambion, Life Technologies). To amplify the 3'-ends, specific forward primers were designed in the 3'-end of the previously obtained fragments (see above) at 4 (for *vtg*) or 2 (for *actb*) different positions and were used in combination with the 3'-RACE Outer or Inner primers supplied in the kit. For 5'-RACE amplifications, specific reverse primers were designed in the 5'-end of the previously cloned fragments at 2 different positions and were used in combination with the 5'-RACE Outer or Inner primers from the kit (Supplementary material, Table 1). The primers were designed to obtain a minimum overlapping of 164 bp for *vtg* and 45 bp for *actb* between the RACE clones and the previously achieved partial cDNAs. The cloning and sequencing of PCR products were performed as described above. Finally, the obtained cDNA sequences were assembled using the algorithm merger available in EMBOSS explorer (<http://pro.genomics.purdue.edu/emboss/>).

2.4. *vtg* phylogenetic and evolutionary analysis

The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987) and was conducted in MEGA5 (Tamura et al., 2011). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein, 1985). Branches corresponding to less than 50 % of the replicates were collapsed. The evolutionary distances were computed using the Poisson

correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances of the tree. The analysis involved 57 amino acid sequences retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov/pubmed, accessed in January 2013). All positions containing gaps and missing data were eliminated.

2.5. Real-time PCR (QPCR)

Total RNA isolation, quantification and the assessment of quality were performed as described above. Quantification was made in a Mastercycler[®]epgradient S Realplex². Results were normalized to *actb* as an internal control and a calibrator sample, a female captured from S1, was measured on every QPCR plate to correct for inter-assay differences. Optimization of QPCR conditions was done using different primer combinations (3 pairs for *actb* and 12 pairs for *vtg*), on a temperature gradient for annealing (50 to 60 °C), primers concentrations (100 nM, 200 nM and 400 nM) and template concentration (five 1:10 dilution series from 10 ng to 1 pg of input ARN). The resulting curves had amplification efficiencies and r^2 of 0.86 and 0.999 for *vtg* and of 0.84 and 0.998 for *actb*, respectively. Amplicon sizes were 128 bp for *vtg* and 131 bp for *actb*. QPCR reactions (10 µL) were performed with 10 ng of cDNA (assumed from RNA input), sense and antisense primers (200 nM each; indicated in Supplementary material, Table 1) and PerfeCTa[™] SYBR[®] Green FastMix[™] (Quanta BioSciences). Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001). The PCR thermal profile was as follows: 95 °C, 5 min; [95 °C, 15 s; 60 °C, 30 s] × 40 cycles; melting curve [60–95°C, 20 min]. PCR products were visualized in agarose gel, cloned and sequenced, and sequences matched the genes of interest.

2.6. Statistics

Data were analyzed for possible outliers using GraphPad tools available at <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>. Statistical analyses were performed with SPSS v. 22. Differences in length, weight, condition factor, GSI and *vtg* expression levels between sites were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan test. All statistics were run separately for males and females from each sampling

site. Sex ratio differences from the expected 1:1 ratio at each site were checked by chi-square tests. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Assessed parameters in studied sites

For length and weight, higher values were observed S1 and S2. In females, weight did not show significant difference between S3 and S4. CF had significant differences in males between all the studied sites except for S1 and S4. Maximum and minimum of CF were in S2 and S3, respectively. In females, S2 and S3 had the highest and lowest CF, respectively, whereas S1 did not show any significant difference to S3 and S4 (Table 2). Histological assessment revealed no evidence of presence of oocytes in testis tissue in the four investigated stations (data are not shown). In S1, S2 and S3, sex ratio was skewed from 1:1 in favor of females, forming 80 %, 62 % and 76 % of the total capture, respectively (Figure 2). For GSI, there was no significant difference between the four sites for females, while for males it was significantly lower in S3 (Figure 3).

Table 2. Morphometric comparisons of *P. esfahani* collected from different sampling sites in the Zayandeh Roud River.

Site	Length (mm)		Weight (g)		Condition factor	
	Females	Males	Females	Males	Females	Males
S1	108.39 $\pm$ 3.07 ^b	101.33 $\pm$ 5.08 ^b	18.23 $\pm$ 1.41 ^b	14.75 $\pm$ 2.19 ^b	1.25 $\pm$ 0.03 ^{bc}	1.31 $\pm$ 0.05 ^b
S2	120.70 $\pm$ 3.03 ^a	114.83 $\pm$ 2.64 ^a	28.43 $\pm$ 2.03 ^a	22.56 $\pm$ 1.45 ^a	1.52 $\pm$ 0.05 ^a	1.49 $\pm$ 0.03 ^a
S3	73.64 $\pm$ 5.74 ^d	57.30 $\pm$ 1.74 ^d	6.66 $\pm$ 1.87 ^c	2.36 $\pm$ 0.29 ^d	1.19 $\pm$ 0.04 ^c	1.20 $\pm$ 0.05 ^c
S4	91.13 $\pm$ 2.31 ^c	90.92 $\pm$ 1.61 ^c	10.73 $\pm$ 0.88 ^c	10.79 $\pm$ 0.65 ^c	1.32 $\pm$ 0.03 ^b	1.38 $\pm$ 0.02 ^b

Note: Data are reported as mean $\pm$ standard error of the mean (SEM). Values not sharing a common letter within a given parameter inside same sex are statistically different ($p < 0.05$)

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Figure 2. Analysis of sex ratio in *P. esfahani* collected from four different sites in the Zayandeh Roud River. Asterisk (*) indicates statistically significant differences from the expected 1:1 ratio. The number inside each bar indicates the number of analyzed individuals.

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Figure 3. GSI for females and males of *P. esfahani* from four different sites in Zayandeh Roud River. Different letters indicate statistically significant differences.

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3.2. Cloning vtg and actb full length cDNAs in *Petroleuciscus esfahani*

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P. esfahani amplified and cloned full length cDNA for *vtg* was 4,177 base pairs (bp) long, encoding for a protein of 1,339 amino acids (aa) (Supplementary material, Figure 1). The cDNA sequence showed 96 % identity to *P. promelas vtg* (AF130354), 94 % to *P. oxycephalus vtg1*

(EF639845), 91% to *C. carpio vtgb1* (AB331884), and 90% to *C. molitorella vtgb1* (GU324313), but slightly lower to *C. carpio vtgb2* (AB106873) and *Danio rerio vtg2* (NM_001044913), 88 % and 85 %, respectively. Similarly, the deduced aa sequence showed a 93 % identity to *P. promelas* Vtg (AAD23878), 88 % to *P. oxycephalus* Vtg1 (ABR27689), *C. carpio* Vtgb1 (BAF73406), and *C. molitorella* Vtgb1 (ADB77954), while the identity was 84 % and 74 % to *C. carpio* Vtgb2 (BAD51933) and *D. rerio* Vtg2 (NP_001038378), respectively. *P. esfahani* full length cDNA for *actb* was 1,776 bp long, encoding for a 375 aa protein (Supplementary material, Figure 2). The nucleotide sequence for this gene showed 97 % identity of to *D. rerio actb2* (NM_181601) and *Hemibarbus mylodon actb1* (EF554924), and 96 % identity to *actb* from *Elopichthys bambusa* (JN102135), *Cirrhinus molitorella* (DQ007446), and *Rhodeus uyekii* (KJ867513). The deduced amino acid sequence showed 99 % identity to β -actin in *C. molitorella* (AAY25518), *E. bambusa* (AEK69350), *Labeo calbasu* (AAL57317), *Pargus major* (BAD88412), and *Platichthys flesus* (AAF63665). Both *vtg* and *actb* sequences were sent to GenBank with acc. nos. KF766534 and KF766533, respectively.

3.3. vtg phylogenetic and evolutionary analysis

Phylogenetic analysis of vertebrates vitellogenin amino acid sequences demonstrates that Vtg of *P. esfahani* is clearly clustered within the Cyprinidae family, and more closely related to Vtg1 from *P. oxycephalus* (ABR27689), and Vtg from *P. promelas* (AAD23878) and *Tanichthys alburnoides* (ABN13867) (Figure 4).

Figure 4. Phylogenetic tree of vitellogenin protein sequences from vertebrates. Numbers next to branch points are the percentage of replicate trees in which the associated taxa clustered together. The accession numbers for the sequences extracted from GenBank are indicated in the figure.

3.4. *vtg* mRNA quantification

Quantification of *vtg* mRNA showed that in females, the highest *vtg* expression was detected in S3 which is statistically similar to S2 samples. Besides, the differences between S1, S2 and S4 were non-significant. In male specimens, no *vtg* mRNA transcription was detected in S1 and S2. While, in S3 and S4, *vtg* expression was detected in 5 out of 9 and 2 out of 8 male samples, respectively. S3 had the highest *vtg* expression level, while in S4 it was not significantly different from zero (Figure 5).

Figure 5. *P. esfahani* *vtg* mRNA expression in females and males, from four different sites in the Zayandeh Roud River. Different letters indicate statistically significant differences.

4. Discussion

Vitellogenin induction is being used extensively as a biomarker of estrogenic disruption in many research programs around the world (Blanchet-Letrouvé et al., 2013). Study of the gene transcriptional responses can be fundamental for understanding the possible pathways in which chemicals can affect the organisms, because they are the primary interaction points between the chemicals available in the environment and the organism (Moens et al., 2007). In this study exposure to xenoestrogens in the Zayandeh Roud River was assessed by *vtg* expression in *P. esfahani*. Several *vtg* genes have been identified in teleosts, including cyprinids (Finn et al.,

2009). The identity of the cDNA cloned and the deduced aa sequences compared to other cyprinids was higher for *vtg1* than *vtg2* form, suggesting that our cDNA probably encodes *vtg1*. In this study, we did not find any other cDNA encoding other types of Vtg. To date, a single *vtg* form has been also found in another cyprinid species, *C. molitorella* (Liang & Fang, 2012). Miracle et al. (2006) found that *vtg1* induction levels in male fathead minnow (*P. promelas*) were 4 folds higher than *vtg3* when exposed to 17 α -ethynylestradiol (EE₂), which suggests that *vtg1* is an appropriate indicator for estrogenic exposure.

Not detecting *vtg* transcripts in male individuals in the first two upstream sites, confirms our hypothesis that in these regions, estrogenic compounds do not exist or the quantity is not enough to provoke *vtg* expression. On the other hand, quantifiable levels of *vtg* transcripts in the two downstream sites, demonstrate the fish biological response to xenoestrogens. However, significantly greater *vtg* expression levels in males from S3, indicate that this site is more affected by these compounds. This finding is consistent with the land use and topological properties of this region. Characteristics of effluents and hydrology of the area are important site-specific factors that should be considered in assessment of an ecosystem (Frenzilli et al., 2008). In this site, extremely poor water flow leads to accumulation of sediments, and therefore, an increase in bioavailability of these contaminants. According to the studies on the Zayandeh Roud River, concentrations of manganese, zinc (Zn), nickel, lead and cadmium (Cd) in sediments of this area are significantly higher than other parts of the river (Nemati et al., 2009; Tabatabaei, 2010). Many metals are endocrine disruptors and can affect at different levels including steroid receptor pathways and thyroid hormone. Studies on *Aphanius fasciatus* from eastern coast of Tunisia showed that maximum *vtg* mRNA induction in males occurred in sampling site near an industrialized region with high accumulations of copper, Cd and Zn (Annabi & Messaoudi, 2013).

Surprisingly, in S4 *vtg* expression levels were not statistically different from the non contaminated sites. This can be explained in two ways. First, the fast water flow in this region dilutes the xenoestrogens. Second, it can be justified by the presence of compounds having antagonistic effects to estrogenic chemicals (Blazer et al., 2012). For example, the co-exposure of anti-estrogenic compounds semicarbazide and E₂ and in zebrafish (*D. rerio*) or letrozole and

EE₂ in Japanese medaka (*Oryzias latipes*) showed inhibitory effects on *vtg1* mRNA induction (Sun et al., 2011; Gao et al. 2014).

There is a correspondence between potency of estrogenic compounds and inhibition of male gonad growth (Hassanin et al., 2002; Zhaobin & Jianying, 2008). Therefore, lower GSI in male from S3 is another proof for the existence of estrogenic compounds in this area. Male's gonadal development is impeded by exposure to xenoestrogens, because they are not adapted to metabolize elevated concentrations of estrogens. GSI value for females, in S3 was slightly, although not significantly, higher than in the other locations. The high values of GSI and *vtg* expression in this site can be due to higher water temperature in this site which can cause an early development of gonads. Higher water temperature and conductivity in this site is the consequence of using the water for cooling the steel mill plant's installations. However, EDCs with estrogenic activity can also affect the ovarian system development. For example, in fathead minnow (*P. promelas*) exposed to high concentrations of progesterone, females showed higher GSI than the control group; although, the spawning success was significantly lower (DeQuattro et al., 2012).

Growth suppression was clearly observed in the Zayandeh Roud River downstream sites, especially in S3. Decreased body and gonad size in males could be due to the *vtg* expression in these areas. Since *vtg* does not have any biological role in males, it imposes energetic expenses which could have been used in body or testis growth (Hashimoto et al., 2000; Jeffries et al., 2008; 2010). Besides, there are evidences that EDCs can affect other physiological systems such as hypothalamus-pituitary-thyroid, and somatotrophic axes and consequently cause deficiencies in growth and development (Jiao & Cheng, 2010; Reinecke, 2010; Arukwe et al., 2014; Li et al., 2014).). Additionally, we found a high prevalence of *Ligula intestinalis* and anemia in the population of *P. esfahani* in S3. Various studies revealed that chemicals such as polychlorinated biphenyls may produce immunosuppressive effects which facilitate infection by parasites or make them more prone to diseases (Sures, 2006; Schwacke et al., 2012). This suggests that, in S3, the general welfare and health of the population is affected. All these factors can be correlated to the substantial increase in the effort that was made to capture sufficient number of individuals in this region.

In the three first sampling sites, sex ratio was female biased. Although EDCs can influence fish sexual development and alter the sex ratio from 1:1 (Cheek et al., 2001; Blazer et al., 2012), other factor should be in mind when surveying wild populations. For species in which sex ratios do not deviate from 1:1 among families, it is very likely that the sex-determination system is chromosomal-like. However, in other species, including some Cyprinids, in which sex ratio differs from 1:1, sex determination is polyfactorial or controlled by environmental conditions such as difference in habitat use or mortality rate between genders (Devlin & Nagahama, 2002; Mckellar et al., 2009; Konečná & Reichard, 2011). Therefore, considering that no *vgt* mRNA was detected in the males from S1 and S2, existence of high concentrations of xenoestrogens that can change the sex determination pathways is implausible. Accordingly, it is more probable that in this species, natural sex ratio is female biased and the 1:1 sex ratio observed in S4, is due to exposure anti-estrogenic or androgenic chemicals. Hence, we suggest that in species in which mechanism of sex determination is not defined precisely, sex ratio should be used with caution as an index for EDC exposure.

No testis-ova or any other abnormal conditions were detected in any of the histological samples even in males with *vgt* expression. This result is in accordance with some other experimental assays and field studies in which estrogenic contaminants induced male *vgt* expression but did not cause intersexual gonads (Rey Vázquez et al., 2009; Allner et al., 2010). Changing the gonad structure depends on the potency and concentration of the exposed estrogenic compounds (Hassanin et al., 2002). Therefore, it seems that in spite of presence of xenoestrogens in the two downstream sites, their concentration and/or composition were not sufficient to cross the threshold necessary for causing histological abnormalities.

5. Conclusion

In summary, the two upstream sites did not demonstrate signs of disruption by estrogenic chemicals. However, detection of *vgt* transcripts in male individuals from the two downstream locations proved the existence of these compounds. Highest *vgt* expression levels, along with other biological evidences such as growth impairment, reduced gonad size in males, high prevalence of parasites and disease in fish from S3, suggest that in this area the fish are affected by the presence of environmental contaminates released by the nearby steel mill plant. Therefore,

359 considering the important role of the Zayandeh Roud River in different human needs, we
360 recommend that future research involving characterization and measurement of different groups
361 of EDCs and determination of potential effects of these contaminants on human health should be
362 addressed.

363 **Acknowledgements**

364 The present work has been partially financed by the Center for International Scientific Studies &
365 Collaboration (CISSC), Ministry of Science, Research and Technology of Iran on January 2011
366 with project contract number 894/1. We acknowledge Ahmad Ghasemi for his valuable help and
367 Ebrahim Motaghi, Neda Shojaee and Salar Sohrabi for their assistance in field work and
368 specimen collection.

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