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Estrogenic effects of water from the Yangtze River (Nanjing section) on goldfish (*Carassius auratus*) after an early life stage exposure

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Abstract

Effects of river water from the Yangtze River (Nanjing section) on fish development, vitellogenin (VTG) induction, gonado-somatic index (GSI) and sex ratio were investigated by exposing goldfish (*Carassius auratus*) in the early life stage (from fertilization to 28 days post-hatch) to water samples (25%, 50% and 100%) collected from three representative sections. The results showed that there was no significant effect on hatching success for any of the exposure groups, but survival was significantly reduced when compared with the control ($P < 0.05$). Body lengths, weights of all treated fish did not differ significantly from those of the control. Condition factors (CF) of larval fish exposed to 50% and 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section were significantly lower than that of the control ($P< 0.05$). VTG inductions were significant in larval fish exposed to all the dilution series of river water. No significant difference in CF value was observed in any exposure group after 150 days of depuration. VTG was fully eliminated after 75 days of depuration. For both female and male, GSI did not significantly differ between exposure groups and the control after 150 days of convalescence. The highest female:male ratios were observed in response to the treatment with 50% or 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section (53:47, 56:44 and 54:46, respectively), but no significant difference in sex ratio was observed in any treated group when compared to the control. The results showed that early life stage exposure of river water from the Yangtze River (Nanjing section) had adverse effects on goldfish development and reproductive health, and the effects on CF and VTG were reversible after depuration in clean water.

Key words: the Yangtze River; estrogenic effects; goldfish; early life stage exposure

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Introduction

Recently, feminization of fish due to long term exposure to low concentrations of estrogens in aquatic environments have caused great concern throughout the world. Simultaneous development of male and female tissue in the gonads and testicular atrophy were observed in wild carp (*Cyprinus carpio*) sampled in a section of the Anoia River (northeastern Spain) (Solé et al., 2003). Higher levels of plasma vitellogenin (VTG) were found in male brown trout (*Salmo trutta*) sampled from six Danish streams impacted by sewage effluent, compared to males from unaffected reference sites (Bjerregaard et al., 2006). Male wild goldfish (*Carassius auratus*) sampled in the Young-San River in Korea, showed high concentrations of VTG and lowered gonado-somatic index (GSI) (Li et al., 2009). Feminized males are most often observed near wastewater outfalls or in areas receiving large amount of domestic or industrial wastewater.

The Yangtze River is the largest river in China. Nanjing is a highly urbanized city in the Yangtze River Delta, which is the most developed area in China. In Nanjing, a great deal of effluent from wastewater treatment plants (WWTPs), untreated sewage and upstream wastewater enters the Yangtze River. Six important environmental estrogens including estrone (E_1), 17β-estradiol (E_2), estriol (E_3), 4-*tert*-octylphenol (4-*t*-OP), nonylphenol (NP) and bisphenol A (BPA) have been detected in the Yangtze River (Nanjing section) (Lu et al., 2010).

The undifferentiated gonads are susceptible to the organizational effects of steroids (Devlin and Nagahama, 2002). Fish can be induced to phenotypic males or females, independent of genotype, when exposed to xenoestrogens prior to or during sexual differentiation (Nimrod and Benson, 1998). The critical and labile period is early life stage, covering from the embryo/hatching stage until the juvenile stage (Piferrer, 2001). Furthermore, exposure of environmental estrogens in early life stage can affect the

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development of fish (Jiang et al., 2008).

The objective of this study is to investigate estrogenic effects of the water samples taken from the Yangtze River (Nanjing section) on development and sex differentiation of goldfish following an early life stage exposure. It would be a scientific reference to protect and recover important fish resource in the Yangtze River (Nanjing section). The impact of river water collected from the Yangtze River on fish was evaluated using hatching rate, survival, length, weight, condition factor (CF), VTG induction, GSI and sex ratio as endpoints.

1 Materials and methods

1.1 Reagents

Purified goldfish vitellogenin and primary antibody (rabbit anti-goldfish vitellogenin) were obtained from the College of Marine Life Sciences, Ocean University of China (Qingdao, China). Phosphate-buffered saline, non-fat milk, and goat anti-rabbit IgG labeled with alkaline phosphatase (AP) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Tween-20 (with purities >99%) was purchased from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). *p*-Nitrophenylphosphate (pNPP) was purchased from Shanghai Boyun Biotech Co., Ltd. (Shanghai, China). Diethanolamine and $MgCl_2 \cdot 6H_2O$ were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Coomassie Brilliant Blue G-250 (Ultra Pure Grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (with purity > 98%) was purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

1.2 Water sampling

Based on the hydrology of the studied area and the effluent discharge points from WWTPs in Nanjing, three representative river sections were assigned near to the main inlets of the Yangtze River and principle WWTP outlets. They were the Jiangxinzhou section, Sanchahe section and Daqiao section. The location of the three representative sections is shown in Fig. 1. Water samples were collected during April–May 2009. All of the sampling equipment was disinfected with a weak bleach solution, after which it was rinsed with tap water and then distilled water. In order to prevent bacterial growth, methanol (5‰) was added to each water sample immediately after collection.

1.3 Fertilized eggs

Healthy fertilized goldfish eggs were obtained from Nanjing Fuzimiao Flower and Bird Market, China. Their average diameter was 0.11 cm.

1.4 Exposure experiment

Test conditions and duration in this study were referred to that for Common carp (*Cyprinus carpio*) recommended

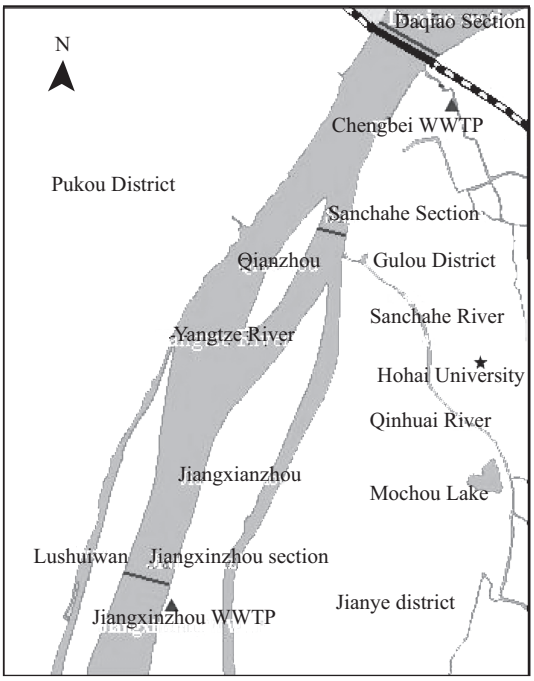

Fig. 1 Location of the three representative sections.

in OECD TG 210, fish early life stage toxicity test (OECD, 1992). The experimental temperature was kept at (22 ± 1)°C. The duration of exposure was from fertilization to 28 days post-hatch (dph). In common carps, metamorphosis from larva to juvenile occurs at 15 mm total length, which corresponds to 2–4 weeks after hatching (Hirai et al., 2006). Therefore, fish during river water exposure were called larvae in this work. Experimental solutions were prepared by serial dilution of the river water (25%, 50% and 100%) in dechlorinated municipal water. Randomly assigned separated 100 eggs were placed uniformly in 100 mm petri dishes containing 50 mL of various experimental solutions. All experiments were performed with three replicates of each treatment. A control, using fish in dechlorinated municipal water, was performed during period of treatment. Dead eggs were removed and the experimental solutions were changed daily. The hatched fish were immediately transferred to a 2-L of glass tank containing the same concentration of experimental solutions under constant aeration. A semi-static test was conducted by replacing 2/3 of experimental solutions once a day. Feces and uneaten food were removed every other day by suction. Dead fish were recorded and removed immediately. Hatching rate was obtained when egg hatch finished. Larvae of 1–3 dph lay on the wall of the glass tank and needed no food. Larvae of 4–10 dph were fed with yolk thrice a day. Then live worms were fed to the fish until the whole experiment finished. Survival was calculated as (live fish/hatched fish) × 100% after the early life stage exposure to the river water. Then randomly assigned 3 fish were taken out from each tank. Length, weight were measured and CF values were calculated. The fish were stored at –80°C for measurement of VTG. The rest fish were transferred to clean water and 3 fish were taken out from each tank after 30, 75 and 150 days of depuration. Length, weight, CF and VTG were

measured. Gonads can be developed to II–III phase after 150 days of depuration. According to the standard of gonad development of cyprinid by Liu (1993), spermaries and ovaries during II–III phase are different in color, shape and transparence etc. Fish can be identified female or male at this time. For the same exposure concentration groups, the gonads of randomly assigned three males or three females were removed and weighed and GSI were calculated. Length, weight and CF values of the anatomized fish were obtained. Randomly assigned 15 fish were taken out from each test group. Their gonads were removed and sex ratio was determined by identification of the gonadal sex.

1.5 Measurement of whole-body VTG, CF and GSI

Fish was unfrozen and added to a certain phosphate-buffered saline (PBS, pH 7.15). The homogenate was obtained by a homogenizer. The sample was immediately centrifuged for 10 min (4000 $\times g$) at 4°C. The supernate was removed for measurement of VTG. Whole-body VTG levels were measured following a competitive enzyme-linked immunosorbent assay (ELISA), as described by Rempel et al. (2006). VTG levels were normalized to total protein per larval homogenate to control for potential survivorship differences between treatments and among replicates (Todorov et al., 2002). The total protein concentrations of the larval homogenates were determined using a Coomassie Protein Assay Kit (Bradford, 1976), with bovine serum albumin as the standard. Measurements were conducted on a microplate reader at 595 nm.

The CF was calculated for each fish according to Eq. (1):

$$\mathrm{CF} = \frac{W}{L^3} \times 100 \quad (1)$$

where, W (g) is the weight of each fish, L (cm) is the length of each fish.

Gonads were removed and weighed and the GSI (%) was calculated as Eq. (2):

$$\mathrm{GSI} = \frac{W_\mathrm{g}}{W_\mathrm{f}} \times 100\% \quad (2)$$

where, W_g (g) is the wet weight of gonad, W_f (g) is the wet weight of fish.

1.6 Statistical analysis

For each endpoint, data were expressed as mean ± standard deviation (SD). All data from different treatments were checked for normality and equal variance. Data were compared by one-way analysis of variance (ANOVA) and statistically different treatments were identified by Dunnett's t test. All differences were considered significant at $p < 0.05$. All statistical analyses were performed using the SPSS statistical package (ver. 13.0, SPSS Company, Chicago, USA).

2 Results

2.1 Effects of water samples on larval goldfish development and VTG induction

Effects of water samples on hatching rate, survival, length, weight and CF following an early life stage exposure are shown in Table 1.

For all control and treatment groups, eye points occurred at day 2 after hatching, larvae were hatched from day 3 to day 7. There were no significant effects of exposure to river water on the time taken for embryos to hatch. As seen in Table 1, the highest hatching rate and survival were observed in response to the control (79.0% and 75.6%, respectively). The lowest hatching rate was observed in response to treatment with 100% river water from the Daqiao section (65.7%) and the lowest survival was observed in response to treatment with 100% river water from the Jiangxinzhou section (34.8%). Exposure to all dilutions of river water from the three sampled sections resulted in reduced hatching rate in fertilized eggs. But no significant hatching rate decrease was observed in any treatment, compared with the control. Exposure to all the dilution series of river water from the three sampled sections resulted in significant decreases in larva survival when compared to the control ($P < 0.05$). The experimental solutions induced clear concentration-dependent decreases of hatching rate and survival for each section. No significant difference in length or weight was observed in fish exposed to all the experimental solutions when compared with the control. Lower CF values were observed in all exposure groups with the exception of those treated by low concentrations of river water (25% river water from the Sanchahe section and 25% or 50% river water from the Daqiao section) when compared to the control. Additionally, CF values of fish exposed to 50% or 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section were significantly lower than that of the control ($P < 0.05$).

Table 1 Development of larval goldfish after an early life stage exposure (from fertilization to 28 dph) to water samples

Representative sections	Experimental solutions	Hatching rate (%)	Survival (%)	Length (cm)	Weight (g)	CF (g/cm^3)
Control	Dechlorinated municipal water	79.0 ± 4.6	75.6 ± 1.6	1.71 ± 0.07	0.07 ± 0.01	1.29 ± 0.02
Jiangxinzhou section	25%	78.0 ± 1.0	48.7 ± 1.9*	1.70 ± 0.13	0.06 ± 0.02	1.20 ± 0.20
	50%	77.0 ± 1.0	42.4 ± 2.6*	1.69 ± 0.09	0.05 ± 0.01	1.09 ± 0.03*
	100%	70.3 ± 6.0	34.8 ± 5.3*	1.64 ± 0.10	0.05 ± 0.01	1.06 ± 0.04*
Sanchahe section	25%	77.0 ± 2.6	49.8 ± 4.4*	1.77 ± 0.04	0.07 ± 0.00	1.32 ± 0.06
	50%	75.7 ± 2.1	48.4 ± 4.5*	1.69 ± 0.06	0.06 ± 0.01	1.27 ± 0.04
	100%	68.7 ± 11.4	37.3 ± 1.6*	1.58 ± 0.16	0.05 ± 0.02	1.16 ± 0.10
Daqiao section	25%	78.7 ± 3.5	50.1 ± 3.6*	1.73 ± 0.11	0.07 ± 0.01	1.38 ± 0.04
	50%	76.0 ± 7.0	50.0 ± 4.6*	1.60 ± 0.11	0.05 ± 0.01	1.30 ± 0.07
	100%	65.7 ± 4.2	38.2 ± 5.0*	1.73 ± 0.13	0.06 ± 0.01	1.08 ± 0.04*

Each value represents the mean ± SD ($n = 9$). * Values that are significantly different from control values ($P < 0.05$). CF: condition factor.

Figure 2 illustrated the induction of VTG in larval goldfish after treatments with the dilution series of river water from the three sampled sections. No detectable VTG induction was observed in any of the larval goldfish in the dechlorinated control. Significant inductions of various levels of VTG were observed in fish exposure to all the dilution series of river water sampled from the three representative sections. The highest VTG induction was observed in response to treatment with 100% river water from the Jiangxinzhou section (0.16 ng/μg protein) and the lowest VTG induction was observed in response to treatment with 25% river water from the Sanchahe section (0.04 ng/μg protein). For each section, there was a concentration-dependent induction of VTG in the experimental fish. The strength of estrogenic responses in the three representative sections was in the order of: Jiangxinzhou section > Daqiao section > Sanchahe section.

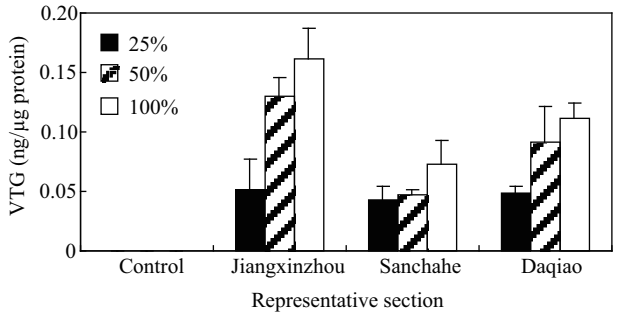

Fig. 2 Whole-body VTG levels in larval goldfish after exposure (from fertilization to 28 dph) to water samples collected from the three representative sections (n = 9).

2.2 Development of juvenile goldfish after convalescence in clean water

To minimize the effect of density on development, fish in the control group were divided into two parts and water in each tank was adjusted according to the fish density throughout the experiment. No mortalities were observed in any of the test groups after depuration in clean water. Length, weight and CF of juvenile fish after 30 or 75 days of depuration (Table 2). Female sex and male sex can be determined by gonad after 150 days of depuration. Length, weight, CF and GSI of female and male fish after 150 days of depuration are shown in Table 3.

From Table 2, the greatest length and weight were observed in fish in the dechlorinated control after 30 days of depuration in clean water (2.49 cm and 0.27 g, respectively), but no significant differences in length and weight were observed in fish exposed to experimental solutions when compared with the control. The greatest CF value was also observed in fish in the dechlorinated control (1.75 g/cm^3). Significant CF decreases were observed in fish exposed to 50% or 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section (P < 0.05). The greatest length, weight and CF were no more observed in fish in the dechlorinated control after 75 days of depuration in clean water and significant CF decreases were only observed in fish exposed to 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section (P< 0.05). From Table 3, length, weight, CF and GSI of female were greater than those of male. No significant differences in length, weight, CF and

Table 2 Length, weight and CF of juvenile goldfish after 30 or 75 days of depuration in clean water

Experimental solutions		Depuration in clean water for 30 days			Depuration in clean water for 75 days		
		Length (cm)	Weight (g)	CF (g/cm^3)	Length (cm)	Weight (g)	CF (g/cm^3)
Control		2.49 ± 0.10	0.27 ± 0.04	1.75 ± 0.03	3.85 ± 0.22	0.86 ± 0.16	1.49 ± 0.03
Jiangxinzhou section	25%	2.42 ± 0.40	0.20 ± 0.08	1.38 ± 0.17	4.30 ± 0.57	1.16 ± 0.48	1.41 ± 0.05
	50%	2.43 ± 0.22	0.20 ± 0.05	1.35 ± 0.06*	3.72 ± 0.36	0.78 ± 0.19	1.50 ± 0.09
	100%	2.26 ± 0.14	0.16 ± 0.03	1.35 ± 0.04*	3.36 ± 0.30	0.50 ± 0.13	1.31 ± 0.02*
Sanchahe section	25%	2.31 ± 0.37	0.21 ± 0.08	1.67 ± 0.10	3.77 ± 0.21	0.79 ± 0.14	1.46 ± 0.06
	50%	2.32 ± 0.18	0.22 ± 0.04	1.72 ± 0.08	3.57 ± 0.21	0.67 ± 0.11	1.45 ± 0.03
	100%	2.33 ± 0.18	0.22 ± 0.06	1.73 ± 0.06	4.06 ± 0.26	0.95 ± 0.16	1.41 ± 0.06
Daqiao section	25%	2.46 ± 0.12	0.24 ± 0.02	1.56 ± 0.10	3.72 ± 0.23	0.72 ± 0.16	1.37 ± 0.04
	50%	2.38 ± 0.14	0.20 ± 0.03	1.50 ± 0.06	3.67 ± 0.22	0.73 ± 0.18	1.45 ± 0.13
	100%	2.18 ± 0.20	0.14 ± 0.04	1.37 ± 0.03*	4.15 ± 0.56	0.95 ± 0.38	1.27 ± 0.03*

Each value represents the mean±SD (n = 9).
* Values that are significantly different from control values (P < 0.05).

Table 3 Length, weight, CF and GSI of female and male after 150 days of depuration in clean water

Experimental solutions		Female				Male			
		Length (cm)	Weight (g)	CF (g/cm^3)	GSI (%)	Length (cm)	Weight (g)	CF (g/cm^3)	GSI (%)
Control		5.26 ± 0.19	1.82 ± 0.20	1.24 ± 0.04	0.94 ± 0.16	5.09 ± 0.17	1.64 ± 0.08	1.20 ± 0.05	0.29 ± 0.04
Jiangxinzhou section	25%	5.58 ± 0.26	2.49 ± 0.34	1.33 ± 0.03	0.79 ± 0.23	5.27 ± 0.22	1.97 ± 0.35	1.28 ± 0.04	0.27 ± 0.03
	50%	5.18 ± 0.13	2.24 ± 0.34	1.39 ± 0.08	0.62 ± 0.06	5.19 ± 0.28	1.65 ± 0.26	1.17 ± 0.03	0.23 ± 0.04
	100%	5.13 ± 0.47	1.67 ± 0.22	1.24 ± 0.24	0.74 ± 0.10	5.16 ± 0.14	1.57 ± 0.18	1.11 ± 0.03	0.23 ± 0.09
Sanchahe section	25%	5.26 ± 0.31	1.87 ± 0.25	1.26 ± 0.06	0.97 ± 0.11	5.19 ± 0.21	1.69 ± 0.13	1.19 ± 0.05	0.28 ± 0.07
	50%	5.08 ± 0.05	1.61 ± 0.06	1.21 ± 0.07	0.89 ± 0.07	4.86 ± 0.36	1.43 ± 0.35	1.21 ± 0.05	0.29 ± 0.06
	100%	5.48 ± 0.02	2.37 ± 0.02	1.42 ± 0.05	1.25 ± 0.09	4.96 ± 0.20	1.67 ± 0.23	1.30 ± 0.03	0.28 ± 0.03
Daqiao section	25%	5.49 ± 0.30	1.98 ± 0.31	1.16 ± 0.03	0.93 ± 0.02	4.91 ± 0.15	1.39 ± 0.08	1.14 ± 0.03	0.24 ± 0.05
	50%	4.97 ± 0.36	1.49 ± 0.40	1.11 ± 0.03	0.86 ± 0.16	4.80 ± 0.20	1.29 ± 0.32	1.09 ± 0.04	0.27 ± 0.09
	100%	5.69 ± 0.15	2.35 ± 0.29	1.22 ± 0.05	0.91 ± 0.08	5.24 ± 0.15	1.77 ± 0.14	1.21 ± 0.02	0.26 ± 0.04

Each value represents the mean ± SD (n = 3).

GSI were observed in either female or male fish exposed to the dilution series of river water sampled from the three representative sections when compared with the control.

VTG levels in juvenile goldfish after 30 days of depuration in clean water are shown in Fig. 3. For all groups treated by experimental solutions, VTG levels in goldfish were reduced after 30 days of depuration. And there was a full clearance of induced VTG in the juvenile fish exposed to 25% or 50% river water from the Sanchahe section and 25% river water from the Daqiao section. VTG were fully cleared in all the treated fish after 75 days of depuration, thereby it was not figured in the present article.

2.3 Sex ratio of juvenile fish after an early life stage exposure to river water

The sex ratio of each test group is shown in Fig. 4. In the control group, the female:male ratios was 49:51. The highest female:male ratios were observed in response to the treatment with 50% or 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section (53:47, 56:44 and 54:46, respectively). But no significant difference in sex ratio in female direction was observed in any treatment group compared with the control.

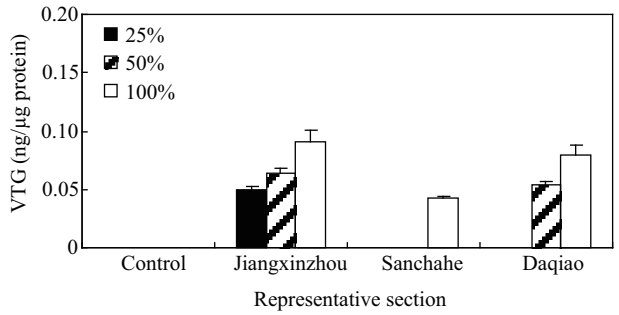

Fig. 3 Whole-body VTG levels in juvenile goldfish after 30 days of depuration in clean water ($n = 3$).

3 Discussion

3.1 Effects of exposure to water samples on larval goldfish development and vitellogenesis

No significant effect on the time to hatch was observed in any treatment group after the early life stage exposure when compared to the control. Zha et al. (2008) reported that time to hatch of Chinese rare minnows (*Gobiocypris rarus*) exposed to 17α-ethinylestradiol (EE_2) was not significantly different from control values. Other studies on known environmental estrogens showed that there were no effects of them on hatching success or survival (Brion et al., 2004; Imai et al., 2005; Panter et al., 2006). It could be concluded that some other chemicals in the river water should be responsible for the reduced survival.

CF can serve as an index of environmental or feeding conditions (Ballón et al., 2008). In the present study, exposure to the high concentrations of river water had an adverse effect on the development of larval goldfish. Environmental estrogens in the river water could be responsible for it. Many studies had reported that exposure to environmental estrogens in early life stage could affect the development of fish. In the juvenile exposure experiment (21–42 days post-fertilization), when the zebrafish (*Danio rerio*) reached maturity, the length and weight of the adult females were significantly reduced at and above 25 and 100 ng/L E_2 respectively ($P < 0.01$) and CF of adult males from the 25 ng/L E_2 group was significantly lower than that of the control males ($P < 0.05$) (Brion et al., 2004). Imai et al. (2005) reported that Java-medaka (*Oryzias javanicus*) had a significantly lower body weight after exposure to > 9.5 ng/L E_2 for 187 days after hatching, while those exposed to 16 ng/L E_2 had a significantly shorter length in comparison to that of the control group. Body length of Japanese medaka fish (*Oryzias latipes*) treated by 200 μg/L BPA were consistently smaller than age related controls ($P < 0.01$) (Ramakrishnan and Wayne, 2008).

VTG, a precursor protein for egg yolk, has proved to be a sensitive biomarker for estrogen exposure in aquatic environments. Normal physiological expression of VTG

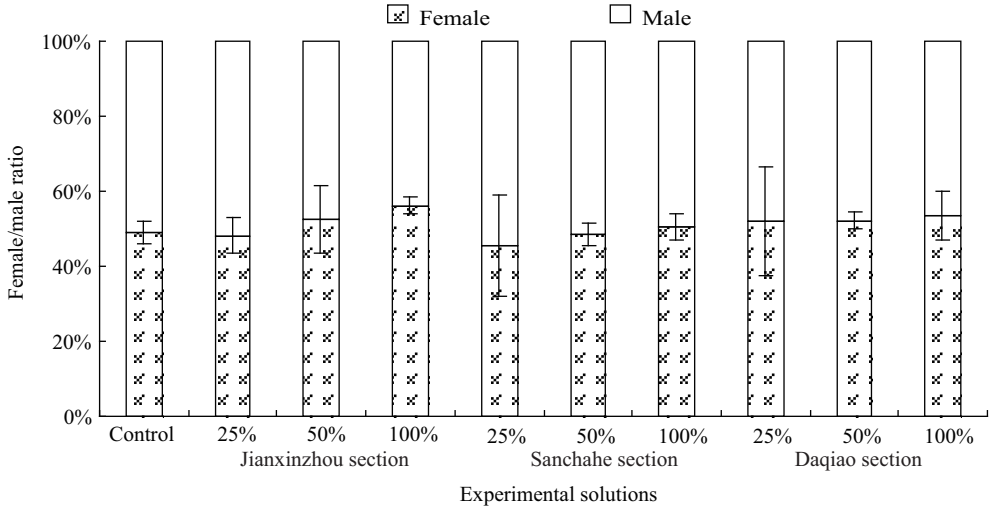

Fig. 4 Female to male ratio of juvenile fish after an early life stage exposure to water samples collected from Jiangxinzhou section, Sanchahe section and Daqiao section. Error bar presents mean ± SD ($n = 15$).

is female-specific and not apparent in male and juvenile fish. But males or juveniles can be induced to synthesize VTG by exposure to estrogens or estrogen-mimics that can activate the estrogen receptor (ER) (Tilton et al., 2002). Therefore, VTG in male or juvenile fish is an excellent indicator of exposure to bioavailable ER-agonists or compounds which may indirectly stimulate estrogenic activity.

The observed significant levels of VTG indicated that fish in the studied area had been exposed to environmental estrogens. VTG expressions in larvae after exposure to estrogens had been reported in many previous studies. Whole body VTG increased in fathead minnow (*Pimephales promelas*) exposed to EE_2 for 142 dph (Länge et al., 2001). Medaka exposed to two estrogenic alkylphenols for 60 dph had concentration-dependent increases in hepatic VTG in both sexes (Seki et al., 2003). VTG were substantially increased in embryo, larval, juvenile and adult life stage zebrafish after 7 days of exposure to low concentrations of E_2 (Jin et al., 2009). Except the six estrogenic chemicals (E_1, E_2, E_3, OP, NP and BPA) detected in the Yangtze River (Nanjing section), some other environmental estrogens may exist in the Yangtze River. Organochlorine pesticides including hexachlorobenzene (HCB) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (p,p'-DDE) were detected in surface sediments from the lower reaches of the Yangtze River (Shi et al., 2010). p,p'-DDE had been shown estrogenic and could induce VTG expression in male fish. Male juvenile (20 dph) Japanese medaka was exposed to 1, 5, 20, and 100 μg/L p,p'-DDE for two months. It was found that gene expressions of VTG in the liver of the fish were significantly up-regulated by p,p'-DDE exposure (Zhang and Hu, 2008). In addtion, phthalic acid esters (PAEs) were also detected in the upper reaches of the Yangtze River (Tian et al., 2004). Chen et al. (2008) reported that exposure of PAEs induced VTG expression in male fish and resulted in feminization.

3.2 Convalescence of juvenile goldfish after depuration in clean water

The development of fish was recovering with increasing period of depuration and the effect of aquatic estrogens on CF was reversible after depuration in clean water. It was consistent with previous study. Rare minnow were exposed to 10 and 100 μg/L diethylstilbestrol (DES) in early life stage for 26 days. The results showed that significant length and weight decreases were observed in all treated fish. After a five-month period culture in clean water, significant difference in length and weight were observed in fish treated by 100 μg/L DES but no significant difference in length or weight was observed in fish exposed to 10 μg/L DES when compared to the control (Zhong et al., 2005). In the present study, the reversible effect of aquatic estrogens on CF may be because levels of environmental estrogens in the river water were relative low. Length, weight, CF values and GSI of female were greater than those of male, which was probably as a consequence of the physiological difference.

The effect of aquatic estrogens on VTG induction was reversible too. This would suggest that induction of VTG is unlikely to have any long term detrimental health effects on fish. Previous studies have shown that induction of VTG is reversible after depuration in clean water. In the work of Rodgers-Gray et al. (2001), VTG induced in roach (*Rutilus rutilus*) after exposure to estrogenic effluents during early life was partially (but not completely) cleared from the circulation after 100 days of depuration. In the zebrafish exposed to E_2 during their early life stages, VTG was fully cleared after 118–139 days of depuration (Brion et al., 2004).

3.3 Effect of exposure to water samples on sex differentiation

The sex ratios were observed higher in response to the treatment with 50% or 100% river water from the Jiangxinzhou section and 100% river water from the Daqiao section. The three test groups were observed the highest VTG induced and the lowest CF values after the early life stage exposure. All of these indicated that estrogenic chemicals in the river water may be responsible for the sex ratio towards the female sex in the three test groups. Previous studies had reported that early life stage exposure to estrogenic chemicals could result in phenotypic female fish. Japanese medaka were exposed to NP (0.5, 0.8 and 1.9 μg/L), methoxychlor (MXC, 0.2, 0.6 and 2.3 μg/L) and E_2 (0.01, 0.12 and 1.66 μg/L) throughout the first month following hatch. The results showed that all three concentrations of E_2 were sufficient to produce exclusively female populations while no alteration in sex ratios was observed following treatment with NP or MXC (Nimrod and Benson, 1998). Zebrafish were exposed to EE_2 (1, 2, 5, 10 and 25 ng/L) and methyltestosterone (MT, 26, 50, 100, 260, 500 and 1000 ng/L) from 20–60 dph. The results showed that significantly higher proportions of females were detected in all groups exposed to EE_2, with complete sex reversal taking place after exposure to 2 ng/L EE_2. Complete sex reversal was detected in all MT concentrations used (Örn et al., 2003). But in this article the result showed that no significant difference in sex ratio in female direction was observed in any test group when compared to the control. The effects of environmental estrogens in the Yangtze River (Nanjing section) on fish sex ratio following an early life stage exposure should be studied further.

4 Conclusions

This study was undertaken to assess the effects of exposure to river water sampled from three representative sections in the Yangtze River (Nanjing section) during embryo-larvae stage on development, VTG induction, gonadal development and sex ratio in goldfish. This work demonstrated that early life stage of goldfish were sensitive to aquatic estrogens and exposure of river water in early life stage had adverse effects on goldfish survival, development and reproductive health. It highlights that effects of environmental estrogens in the Yangtze River (Nanjing section) on vulnerable developmental stages of fish have to

be considered.

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