The Chinese rare minnow (Gobiocypris rarus) as an in vivo model for endocrine disruption in freshwater teleosts: a full life-cycle test with diethylstilbestrol

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1. Introduction

In recent years, global concern has been raised about natural steroid hormones and other hormone mimics present in the environment. Many of these compounds have been shown to modulate endocrine response, alter sexual development, or reduce reproductive success in fish at environmental concentrations much lower than those causing acute or chronic toxicity(Gimeno et al., 1996; Jobling et al., 1998; Gronen et al., 1999).. Thus, many organizations have involved in toxicological risk assessment, such as the Organization for Economic Cooperation and Development, and developed test methods to identify the chemicals possessing hormonal activity in aquatic environment. Among the methods, the fish full life-cycle test (FFLC) has been proposed as a confirmatory test because endocrine-disrupting chemicals (EDCs) can profoundly disturb the early development period (especially during sexual differentiation) and the reproductive stage. Moreover, this test is considered useful because a concern exists that exposure of parental fish to EDCs may have adverse effects on the next generation.

Rare minnow, *Gobiocypris rarus,* is a Chinese freshwater cyprinid. It has many attractive features that make it a suitable organism in aquatic toxicity tests. So, in the present study, a full life cycle experiment was performed with rare minnow from a few hours postfertilzation to maturation. The aim of this study was to investigate the responses of rare minnow to diethylstilbestrol (DES) in the development and reproduction. An understanding of the responses of rare minnow to DES will help us to determine whether rare minnow is suitable for fish full life cycle test in assessing the environmental impact of EDCs.

2. Meterials and methods

2.1. Experiment animals

The Chinese rare minnow (*Gobiocypris rarus)* were raised in 24 L flow-through chambers with dechlorinated tap water. Fish were maintained in a light/dark cycle of 14: 10 h at 23 - 26 ºC and fed once a day.

2.2. Exposure design

Exposure was initiated at < 24 h after fertilization. The 300 embryos used for each treatment were randomly separated into three groups. In each group, the embryos were placed in a cylindrical glass cup containing about 1 L test solution and the test solution was renewed every day. After hatching, the larvae were fed with adequate amount of *Artemia* nauplii(< 24 h after hatching) twice a day. The mortality, abnormal behavior and appearance of the larvae were recorded until 30 days posthatch. At 30 days posthatch, fish were transferred to the continuous-flowing system and fed with *Limnodrilus spp*. At 64 days posthatch, fish were sampled to measure body weight, body length and then stored at –80 ºC for Vtg analysis and total thyroxine (TT_4) measurement. At 179 days posthatch, twenty fish were sacrificed. Gonads were removed and fixed with Bouin's solution for histological observation. At 200 days posthatch six mating pairs were selected from control group for examination of fecundity and fertility. No male from 0.05, 0.5, 1 and 5 mg/l treatment groups could be obtained because sex ratio completely skewed to female. So, six females from these groups were transferred to clean dilution water and paired with non-exposed males. Each pair was assigned to a test chamber. The eggs spawned from each female were counted and assessed for viability over 7 consecutive days. At the end of reproductive phase, fish remained were sacrificed and measured body weight and body length.

The eggs spawned from the females during the first 7 days were kept in clean dilution water and subjected to the study of F_1 generation. To evaluate their hatchability and hatching time, the fertilized eggs collected from each group

were placed in a glass cup under the same condition as those for F_0 generation. The mortality, abnormal behavior, and appearance of the F_1 larval were observed and recorded. At 30 days posthatch, the larval were randomly separated into four groups of 50 and transferred into the continuous-flow system described previously for the F_0 generation. At 118 days posthatch, the fish were sampled from each group for Vtg, TT4 and total triiodothyronine (TT3) measurements. At 182 days after hatch, four females and four males were sampled for sex steroids measurement. At the end of the experiment, fish remaining were weighed and gonads were removed for gonadal somatic index (GSI) calculation.

2.3. Statistical analysis

Values are expressed as mean ± SD. One way analysis of variance (ANOVA) followed by Spjotvoll/stoline test was used to determine the effects of DES on fish exposed to DES and its offspring. *P* values below 0.05 were set as significant.

3. Results and discussion

3.1. F_0 generation

The body length, body weight and TT_4 level of F_0 fish reduced significantly at 5 mg/L DES exposure at 64 days posthatch. The WBH Vtg concentrations in rare minnow exposed to DES increased significantly. For females at 200 days posthatch, there was also a significant reduction in body weight and body length in 0.5, 1 and 5 mg/L groups compared to the controls ($P < 0.05$) (Table 1). These agreed with the results of Länge et al (2001) and Belt et al (2003). The reduction of body weight and body length may be due to the effects of xenoestrogens on Vtg synthesis. Vitellogenesis has been demonstrated to compromise growth in fish by diverting energy stores as well as producing a burden of protein for the kidneys. In the present study, the change of TT_4 and the retardation of maturation are also confirmations of growth or development defects.

Fish terminated at 179 days posthatch were evaluated for gonadal development. Sex ratios following the gonadal development are given in the Table 1. Sex ratio in the control was 44:56 (Male: Female). In contrast, all groups exposed to DES showed an elevated ratio of females. No males were observed at 0.5, 1 and 5 mg/L groups. Moreover, in the fish exposed to 0.05 mg/L DES, testes-ova could be detected in an incidence of 2% (Table1). Although the mechanism of the development of test-ova in rare minnow with estrogen is unknown, the elevated Vtg level and completely skewed sex ratios of F_0 fish suggested that the abnormal development in the gonads were induced by the estrogenic activity of DES.

Fecundity is widely considered to be a key parameter affecting the sustainability of fish populations. Recent workshops have highlighted the need to address the impacts of endocrine disruptor on the demographics and reproductive health of fish populations. So, the evaluation of the reproductive effects of xenoestrogen has been a key goal in the FFLC study with fish. In recent years, many studies have found that the female

Table 1 Effects of DES on growth of F_0 generation at 200 days posthatch and sex ratio as determined by gonadal histology

Test concentration (µg/L)	Female		Male		Percentage of fish with		
	Body length (cm)	Body weight (g)	Body length (cm)	Body weight (g)	Testis	Ovary	Testis-ova
Control	5.13±0.57	1.73±0.82	4.78±0.34	1.17±0.85	44	56	0
0.05	4.61±0.35	1.23±0.34	4.25±0.24	1.1±0.32	11	87	2
0.5	4.41±0.41*	1.01±0.38*	-	-	0	100	0
1	4.7±0.54*	1.16±0.34*	-	-	0	100	0
5	3.89±0.52*	0.79±0.3*	-	-	0	100	0

reproductive success of fish has been impaired after exposed to EDCs. In the present study, a reduced egg production has been also observed in the 0.5 and 5 mg/L DES treated females after breeding with non-exposed males. The number of eggs per batch in each treatment has been also found to decrease although only 5 mg/L DES treatment is significantly different from control (Fig 1). This indicates that DES exposure impair the female reproductive success of rare minnow. In this study, the egg number produced by females exposed to DES at 1 mg/L has also been found to decrease, but not significant. This is probably due to the large variation in egg number produced by different individual pairs of rare minnow. However, the number of abnormal fry and the percentage of survival fry of F_1 generation after 30 days hatching have been found to significantly decrease when compared with the control group. Since successful development and hatching of embryos are dependent upon the quality of gametes as well as nutritional content of eggs, it also indicates the reproductive impairment by DES treatment.

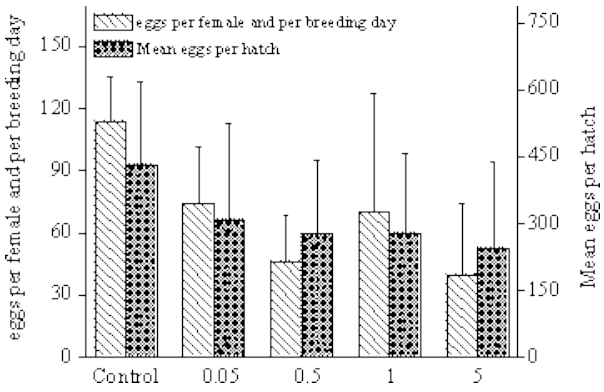

Fig. 1. Reproduction by rare minnow females exposed to DES from 200 to 336 days post-hatch (n = 6).

3.2. F_1 generation

Survival of control fish was 91.59 ± 5.80 % at 30 days posthatch. For the fish exposed to 50 ng/L DES, survival was 83.27 ± 17.19 % and was not statistically significant different from the control. However, for the fish exposed to 0.5 mg/L, 1 mg/L and 5 mg/L DES, survivals were significantly reduced. Survival was 51.59 ± 14.73 % at 0.5 mg/L, 42.44 ± 21.86 % at 1 mg/L and 64 ± 16.06 % at 5 mg/L.

At 246 days posthatch, significant reduction was observed in the GSI of adult male, but not in female. No significant change on sex ratio was found in all DES treated groups compared to the controls. In the whole body homogenates of male offspring of fish exposed to DES, the analysis of T concentration revealed a significant decrease compared to control values. E_2 levels also showed decrease (Fig.2). In the whole body homogenates of female progeny of DES-exposed fish, E_2 levels were, although not significantly, elevated compared to control fish. Testosterone levels remained unchanged (Fig.2). These results indicate that exposure to xenoestrogen causes a broad variety of transgenerational effects and that rare minnow is suitable for FFLC test in assessing the environmental impact of EDCs.

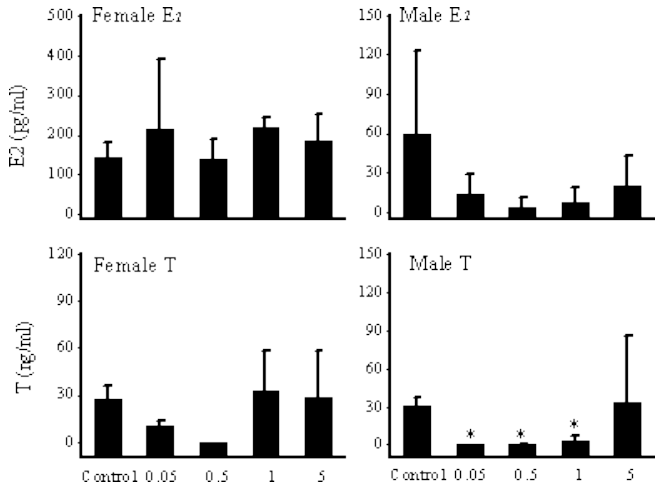

Fig. 2. Effects of long-term DES exposure on sex steroid levels of F1 generation at 182 days posthatch.

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