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Recovery of gonadal development in tiger puffer *Takifugu rubripes* after exposure to 17β-estradiol during early life stages*

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Abstract The aim of the present study was to investigate the long-term effects of 17β-estradiol (E2) exposure on gonadal development in the tiger puffer (*Takifugu rubripes*), which has a genetic sex determination system of male homogametic XY-XX. Tiger puffer larvae were exposed to 1, 10 and 100 μg/L E2 from 15 to 100 days post-hatch (dph) and then maintained in clean seawater until 400 dph. Changes in sex ratio, gonadal structure and gonadosomatic index (GSI) were monitored at 100, 160, 270 and 400 dph. Sex-associated single nucleotide polymorphism (SNP) markers were used to analyze the genetic sex of samples, except those at 100 dph. Exposure had a positive effect on the conversion of genetically male gonads into phenotypically female gonads at 100 dph. However, gonads from 60% of genetic XY males in the 1-μg/L E2 group and 100% in the 10-μg/L E2 group developed intersexual gonads at 160 dph; gonads of all genetic XY males in the two treatment groups reverted to testis by 270 dph. While 38%, 57% and 44% of gonads of XY fish in the 100-μg/L E2 group reverted to intersexual gonads at 160, 270 and 400 dph, respectively, none reverted to testis after E2 treatment. In addition, E2 exposure inhibited gonadal growth of both genetic sexes, as indicated by the clear dose-dependent decrease in GSI at 270 and 400 dph. The results showed that exposure to E2 during the early life stages of tiger puffer disrupted gonadal development, but that fish recovered after migration to clean seawater. The study suggests the potential use of tiger puffer as a valuable indicator species to evaluate the effects of environmental estrogens on marine fish, thereby protecting valuable fishery resources.

Keyword: *Takifugu rubripes*; 17β-estradiol; sex reversal; gonadal structure; morphological recovery

1 INTRODUCTION

Environmental estrogens (EEs) have attracted growing scientific and public concern owing to their ability to mimic natural, endogenous estrogens and disrupt the normal function of the endocrine system in humans and animals (Harrison et al., 1997). EEs have been widely detected in fresh, estuarine and coastal water systems at concentrations ranging from a few nanograms to several micrograms per liter, which have been proven in laboratory studies to have adverse reproductive effects on aquatic organisms (Mills and Chichester, 2005; Rocha et al., 2014; Bhandari et al., 2015). Aquatic species inhabiting such polluted waters are frequently at risk of abnormal development caused by estrogenic exposure (Allner et al., 2010; Bahamonde et al., 2013; Zheng et al., 2015).

Because of their labile sex determination and wide distribution, fish are generally accepted as model organisms for investigating the reproductive effects

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of EEs on aquatic organisms. Much of the research in this area has focused on the effects of EEs in freshwater fish, including model species such as zebrafish (*Danio rerio*) (Segner, 2009), medaka (*Oryzias latipes*) (Patyna et al., 1999) and fathead minnow (*Pimephales promelas*) (Kramer et al., 1998). Adverse reproductive effects of EEs in these fish species include delayed gonadal development and maturation (Segner et al., 2003), occurrence of intersexual gonads (Zhao et al., 2014), and skewed sex ratios (Länge et al., 2001). However, few studies have focused on marine fish species, such as sheepshead minnow (*Cyprinodon variegatus* Lacépède) or mummichog (*Fundulus heteroclitus*), in which the reproductive effects of EEs have been shown to differ from freshwater fish (Zillioux et al., 2001; Peters et al., 2010). For example, lifecycle exposure to ethynylestradiol-17α (EE2) at concentrations <10 ng/L was sufficient to skewed the sex ratio and reduce copulation and spawning in fathead minnow, medaka and zebrafish (Länge et al., 2001; Balch et al., 2004; Van den Belt et al., 2004). While sheepshead minnow had reduced reproductive success at EE2 levels of 20–200 ng/L, mummichog experienced altered steroid synthesis, reproductive success and sex differentiation at >10 ng/L EE2 (Zillioux et al., 2001; Peters et al., 2010). These discrepancies may be the result not only of the diverse reproductive strategies employed by different fish species but also of differences in the biological availability of the contaminants due to variations in the physicochemical characteristics of water and/or fish physiology (Peters et al., 2010; Murua, 2014).

Tiger puffer (*Takifugu rubripes*), a gonochoristic fish with an XX-XY sex determination system, is widely distributed in the northwestern Pacific (Kakimoto et al., 1994; Katamachi et al., 2015). Tiger puffer spawn in estuaries during spring, where larvae develop into juveniles and remain until late summer, and then migrate to deep water for overwintering (Katamachi et al., 2015). Intersex tiger puffer individuals have been observed in wild populations (Suzuki, 1997), which may have resulted from estrogen exposure during their early life history. Differentiation and development of the gonads in tiger puffer is not easily affected by environmental factors, such as temperature (Lee et al., 2009b; Hattori et al., 2012); nevertheless, intersex gonads were observed in tiger puffer fed a high dose of 17β-estradiol in their diet (E2; 100 μg/g diet) from 21 to 80 days post-hatch (dph) (Lee et al., 2009a).

A trans-species missense single nucleotide polymorphism (SNP) in the anti-Müllerian hormone type II receptor (Amhr2) has been identified to be associated with sex determination in tiger puffer (Kamiya et al., 2012). By analyzing this SNP marker, it is possible to identify the genetic sex of exposed tiger puffer and ascertain changes in the gonadal development of both genetic genders. The purpose of this study was to evaluate the effects of exogenous E2 on gonadal development in tiger puffer after long-term exposure during the larval and juvenile stages. Larval tiger puffer (15 dph) were intermittently exposed to E2 throughout the juvenile stage until 100 dph and were then transferred to fresh seawater until 400 dph. Changes in the sex ratio, gonadosomatic index (GSI) and gonadal structure were examined.

2 MATERIAL AND METHOD

2.1 Larvae rearing

Newly hatched tiger pufferfish larvae were obtained from a commercial hatchery in Weihai City, China, and reared in seawater aquaria at the Tianyuan Fisheries Company, Yantai City, China. At 15 dph, about 4 000 larvae were randomly assigned to four 500-L aquaria containing ~1 000 larvae each. The larvae were fed with live rotifers from 16 to 25 dph, and *Artemia* nauplius from 26 to 40 dph. After 40 dph, metamorphosed juveniles were weaned onto commercial pellets (Marine Yu Bao, Hayashikane Sangyo Co. Ltd., Japan). Water temperature was maintained at 18–21°C during the rearing period.

2.2 17β-estradiol exposure

The experiment consisted of one control group and three treatment groups (1, 10 and 100 μg/L E2). In each treatment group, juveniles were exposed to E2 at the set concentration for 2 h once a day from 15 to 100 dph. Stock solutions were prepared by dissolving 0.02, 0.2 and 2 mg E2 in 1 000 mL of absolute ethanol to give a concentration of 0.02, 0.2 and 2 mg/mL E2, respectively, and stored in the dark at -20°C until use. Prior to treatment, the water volume in the aquarium of each treatment group was reduced to 200 L and 10 mL of the respective E2 stock solutions was added to each aquarium to give final concentrations of 1, 10 and 100 μg/L E2. The water volume in the control aquarium was also reduced to 200 L and treated with 10 mL of absolute ethanol only. After the 2-h treatment, the water volume of in each aquarium was increased to 500 L and flowing water (75 L/h)

restored. Mortality was monitored daily in each experimental group during exposure. After the final exposure to E2 at 100 dph, juveniles from the three treatment groups and the control group were transferred to four 5-m^3 aquaria and reared in a flow-through seawater system until 400 dph.

2.3 Sample collection

At 100, 160, 270 and 400 dph, the gonads of 30 juveniles from each group were collected, fixed in Davidson's fluid for ~24 h, washed with 50% ethanol and then stored in 70% ethanol at 4°C until histological processing. Simultaneously, except at 100 dph, the body and gonad weights of each sampled juvenile were recorded to calculate the GSI [GSI=(gonad weight/body weight)×100; muscle tissue was also sampled and stored at -20°C for DNA extraction.

2.4 Identification of phenotypic sex

The middle portion of the gonad was used for histological analysis. Tissues were dehydrated through an alcohol series, clarified in dimethylbenzene, and embedded in paraffin. Cross-sections were cut at 5–7 μm with a microtome (Leica RM2235, Nussloch, Germany), mounted on slides, stained with hematoxylin and eosin, and observed and photographed using a light microscope (Olympus DP72, Tokyo, Japan).

2.5 Identification of genetic sex

Identification of genetic sex was conducted at 160, 270 and 400 dph, but not at 100 dph. Total DNA in the muscle tissue samples was extracted using a Marine Animals DNA kit (TLANamp, Beijing, China) following the manufacturer's instructions. The quality of total DNA was assessed by 1% agarose gel electrophoresis and by determining the absorbance ratio of 260 and 280 nm using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

A trans-species missense SNP (G/C) in Amhr2, which was confirmed to be associated with sex determination in tiger puffer (Kamiya et al., 2012), was used to identify the genetic sex of juveniles. A sense (F) 5′-CAGAGTAGCCGTGAAGGTT-3′ and antisense (R) 5′-CCCAGTGTTGCGGTATGTAA-3′ primer pair for the SNP marker were designed with Primer Premier 5.0 software (Prenier Biosoft, Palo Alto, CA, USA). The reaction conditions of the F/R PCR were 5 min at 95°C, followed by 35 cycles of

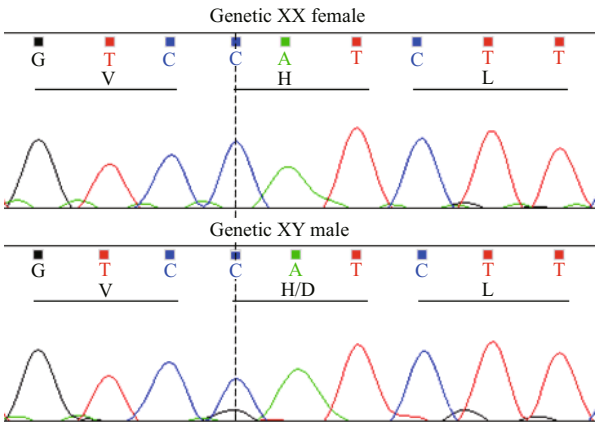

Fig.1 Sample sequence traces of sex-specific single nucleotide polymorphism in Amhr2 from a genetic XX female and a genetic XY male tiger puffer *Takifugu rubripes*

All genetic XX females were homozygous as His/His 384 (C/C; indicated by a vertical bar), while all genetic XY males were heterozygous as His/Asp 384 (C/G).

1 min at 94°C, 40 s at 57°C and 50 s at 72°C, with a final extension at 72°C for 5 min. Then, the products of F/R PCR were sequenced by Sangon Biotech (Shanghai, China). By analyzed the SNP marker in the resulting sequence of Amhr2, the genetic sex of the juveniles was determined with heterozygous bases of C/G for genetic males (XY type) and homozygous bases of C/C for genetic females (XX type) at site His/Asp 384 (Fig.1).

2.6 Statistical analysis

Data, presented as mean±SD, were analyzed using one-way analysis of variance, and Dunnett's tests were conducted for multiple comparisons. Significance was set at $P<0.05$. All statistical analyses were conducted using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

3 RESULT

3.1 Sex ratio

At 100 dph, the survival rates in the control, 1, 10 and 100 μg/L E2 groups were 92.6%, 94.4%, 90.8% and 92.1%, respectively. Changes in the sex ratio of tiger puffer in the control and E2 treatment groups were ascertained according to the phenotype of the gonads (Fig.2). The male/female ratio in the control group was in close to 1:1, and no intersex individuals were observed. At 100 dph, the E2 treatment groups exhibited a high percentage of female, with 90% in the 1-μg/L E2 group and 100% in the 10- and 100-

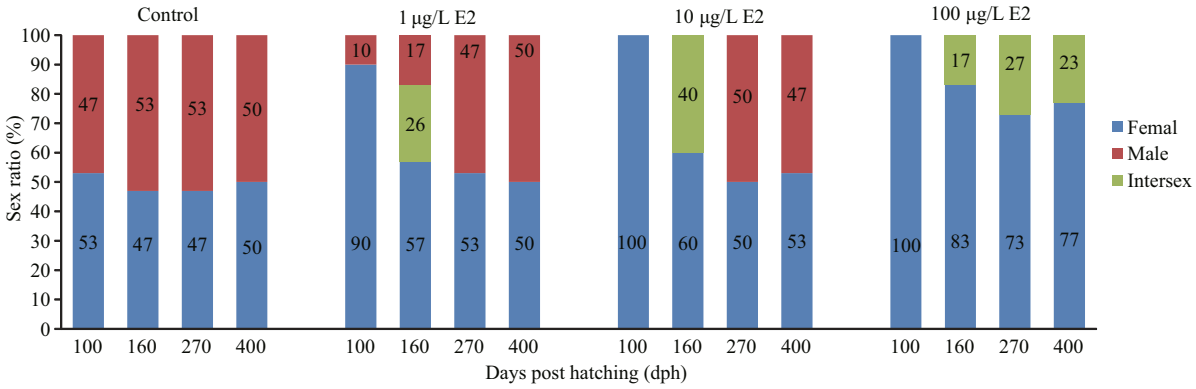

Fig.2 Variations in sex ratio of tiger puffer *Takifugu rubripes* in the control and treatment groups after E2 exposure according to phenotype of the gonads

Dph: days post-hatching.

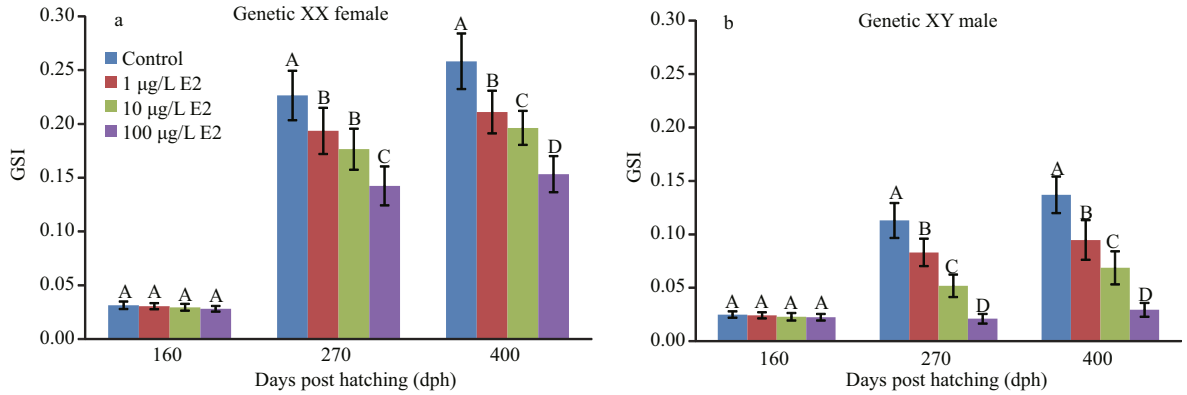

Fig.3 Variations in gonadosomatic index (GSI) of genetic XX female (a) and genetic XY male (b) tiger puffer *Takifugu rubripes* in the control and treatment groups after E2 exposure

Data presented as the mean±SD (n=12). Uppercase letters indicate significant differences between groups within the same time period (P<0.05).

μg/L E2 groups. However, at 160 dph, the proportion of female in the 1- and 10-μg/L E2 groups fell to ~60%, and the proportion in the 100-μg/L E2 group was ~83%. The remaining fish in these three groups were identified as male or intersex. At 270 and 400 dph, no intersex individuals were recorded in the 1- and 10-μg/L E2 groups, and the male/female ratio was close to 1:1. A high percentage of females (>70%) was still observable in the 100-μg/L E2 group, while the remaining fish were all intersex.

In the control group, the phenotype of the gonads corresponded totally with the genetic sex of each fish. All females in the 1- and 10-μg/L E2 groups were of the genetic XX type, and both male and intersex fish were genetic XY. In the 100-μg/L E2 group, 26% (n=8), 20% (n=6) and 30% (n=9) of the females were identified as genetic XY at 160, 270 and 400 dph, respectively, and all intersex fish were genetic XY.

3.2 Gonadosomatic index (GSI)

No significant differences in GSI were observed among genetic XX females (Fig.3a) or genetic XY males (Fig.3b) from the four experimental groups at 160 dph. However, a clear dose-dependent decrease in the GSI was observed for E2-treated genetic XX females and genetic XY males at 270 and 400 dph. Although all genetic XY males in the 100-μg/L E2 group presented as female or intersex, no significant difference was observed in the GSI.

3.3 Gonadal structure

At 100 dph, the ovarian lamellae of the control group contained a large number of perinucleolar oocytes and a small number of previtellogenic oocytes (Fig.4a). Spermatogonia were observed along the circumference of the testes in the control group (Fig.4b). The histological structure of ovaries and testes in the 1-μg/L E2 group was similar to those observed in the control group. Two different types of ovaries were observed in the 10-μg/L E2 group: one type that was similar in histological structure to the control group and a second type that was much thinner

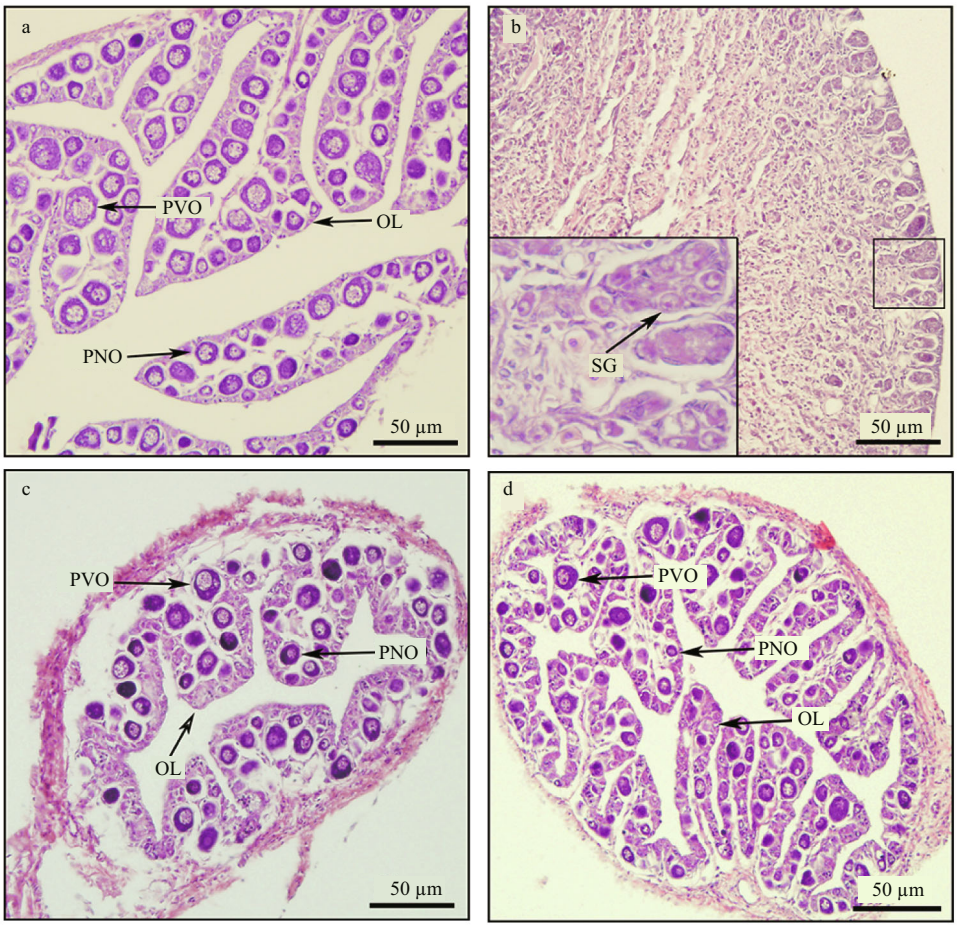

Fig.4 Photomicrographs of gonads of tiger puffer *Takifugu rubripes* from the control and E2 treatment groups at 100 dph

a. ovary from the control group; b. testis from the control group; c. abnormal ovary from the 10-μg/L E2-treated group; d. ovary from the 100-μg/L E2-treated group. OL: ovarian lamellae; PNO: perinucleolar oocyte; PVO: previtellogenic oocyte; SG: spermatogonium.

and contained fewer oocytes (Fig.4c). Ovaries from fish in the 100-μg/L E2 treated group were smaller than those from the control or the other two E2-treated groups (Fig.4d).

No structural difference in XX ovaries was observed between control and the three treatment groups at 160 dph. Ovarian lamellae in these ovaries were well developed and full of perinucleolar and previtellogenic oocytes (Fig.5a). Spermatogonia appeared in the inner tissue of XY testes of the control group (Fig.5b), and the histological structure of XY testes (40%) in the 1-μg/L E2 group was similar to those of XY testes in the control group. The XY intersexual gonads (60% of XY gonads in the 1-μg/L E2 group, 100% in the 10-μg/L E2 group and 40% in the 100-μg/L E2 group) consisted of testicular-like tissue but with very few perinucleolar and previtellogenic oocytes (Fig.5c). XY ovaries (60%) in the 100-μg/L E2 group were thinner and shorter than XX ovaries in the control group, with a small number of perinucleolar and previtellogenic oocytes in the initial ovarian lamellae (Fig.5d).

No significant differences were observed between XX ovaries at 160 and 270 dph within each treatment group. Groups of spermatocytes were observed in XY testes of the control group, but not in XY testes of the 1- or 10-μg/L E2 groups at 270 dph (Fig.6a, b). In the 100-μg/L E2 group, XY intersexual gonads and XY ovaries maintained their structure at this time period (Fig.6c, d).

At 400 dph, oocytes were still present at the previtellogenic stage in XX ovaries of both the control and the three treatment groups (Fig.7a). No structural difference was observed between XY testes in the 1- and 10-μg/L E2 groups and XY testes of the control group at this time, i.e. 400 dph (Fig.7b). In the 100-μg/L E2 group, spermatocytes were observed in XY intersexual gonads (Fig.7c), and a greater number of oocytes was present in XY ovaries at 400 dph compared with XY ovaries at 270 dph (Fig.7d).

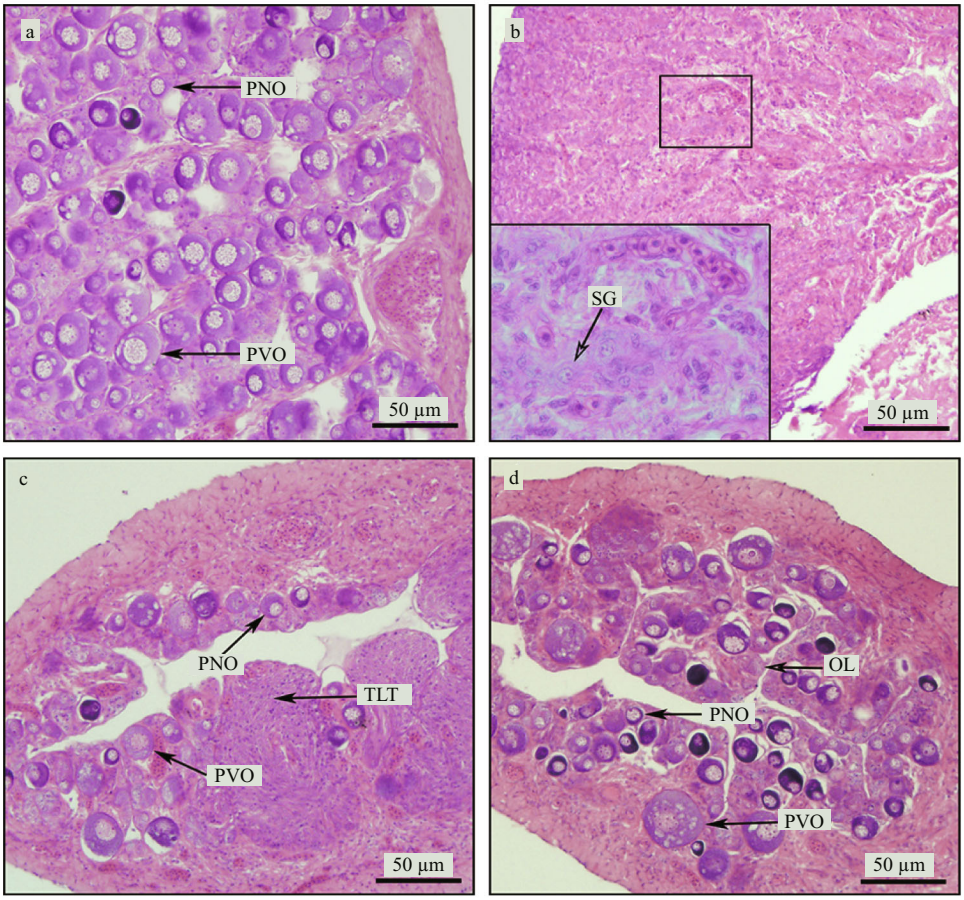

Fig.5 Photomicrographs of gonads of tiger puffer *Takifugu rubripes* from the control and E2 treatment groups at 160 dph

a. XX ovary from the control group; b. XY testis from the control group; c. XY intersexual gonad from the 1-μg/L E2-treated group; d. XY ovary from the 100-μg/L E2-treated group. OL: ovarian lamellae; PNO: perinucleolar oocyte; PVO: previtellogenic oocyte; SG: spermatogonium; TLT: testicular-like tissue.

4 DISCUSSION

The early life stages of fish are particularly sensitive to environmental estrogens. A number of studies have indicated that estrogen exposure during this critical period could permanently disrupt normal gonadal development, and even delay gonadal maturity (Piferrer, 2001; Brion et al., 2004; Baumann et al., 2013). E2 has been reported to disrupt the endocrine system in juvenile and female *Pomatoschistus microps* (Dias et al., 2014). In this study, tiger puffer were exposed to E2 from 15 to 100 dph at higher than environmentally applicable values, and the long-term effects on gonadal development were examined. The study demonstrated that E2 had a positive effect on the conversion of genetically male gonads into phenotypically female gonads (XY type). All XY ovaries induced by treatment with 1 and 10 μg/L E2 reconverted to testes after a specific depuration period. However, ~50% of XY ovaries induced by 100 μg/L E2 developed into intersex gonads, with all other XY ovaries retaining their morphology.

In gonochoristic fish, changes in gonadal development induced by estrogens are mainly dependent on exposure time and concentration, as well as fish species. In general, developmental sensitivity to estrogenic estrogen occurs during a period prior to, or concomitant with, histological differentiation of the primordial gonad in fish (Devlin and Nagahama, 2002). The concentrations of E2 used in the current study were capable of inducing irreversible feminization in many other fish species during their periods of gonadal differentiation. For example, 1 μg/L E2 could induce permanent sex reversal in the South American cichlid fish (*Cichlasoma dimerus*) (Meijide et al., 2016) and chum salmon (*Oncorhynchus masou*) (Nakamura, 1984). In zebrafish and medaka, 0.1 and 0.12 μg/L E2

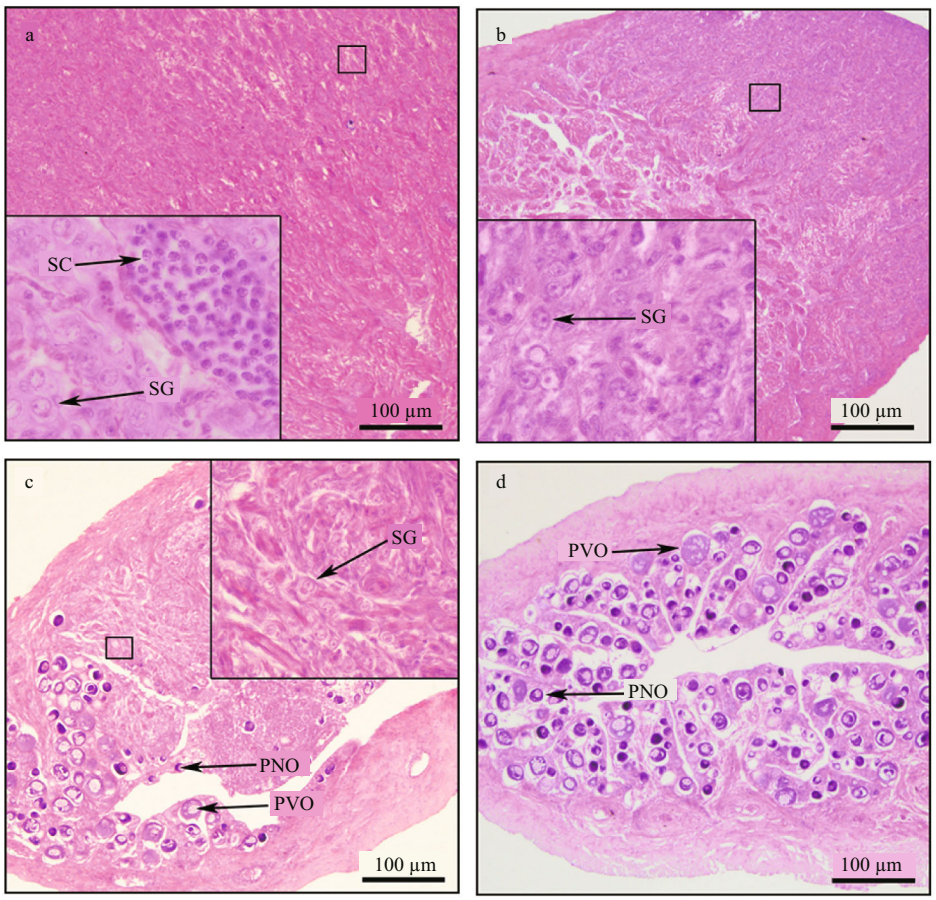

Fig.6 Photomicrographs of gonads of tiger puffer *Takifugu rubripes* from control and E2 treatment groups at 270 dph

a. XY testis from the control group; b. XY testis from the 1-μg/L E2-treated group; c. XY intersexual gonad from the 100-μg/L E2-treated group; d. XY ovary from the 100-μg/L E2-treated group. OL: ovarian lamellae; PNO: perinucleolar oocyte; PVO: previtellogenic oocyte; SC: spermatocyte; SG: spermatogonium.

were, respectively, sufficient to transform genetic males to phenotypic females (Brion et al., 2004; Nimrod and Benson, 1998). A previous study in our laboratory demonstrated that the time window for the differentiation of ovary and testis in tiger puffer is 40–82 and 63–103 dph, respectively (Hu et al., 2015). Interestingly, irreversible feminization was not achieved in the current study by exposing tiger puffer to E2 at concentrations of 1 and 10 μg/L. Our results suggested that tiger puffer may be resistant to the induction of sex reversal with E2 after migration to clean seawater.

Morphological recovery after insufficient exposure time has been reported in medaka (Paul-Prasanth et al., 2011). Diethylstilbestrol exposure failed to induce somatic cell reversal in medaka which was believed to play a critical role in the developmental fate of gonads (Shinomiya et al., 2002): after exposure, gonadal development in recoverable males was influenced by the unchanged somatic cells inside the gonads. A previous study in tiger puffer also suggested that E2 treatment induced ovarian development by modifying the gonadal somatic cells in genetic males (Lee et al., 2009a).

Intersex is identified as the simultaneous presence of female and male gonadal tissues in an individual of a gonochoristic species. The presence of intersex has always been considered direct evidence of feminization in wild fish exposed to estrogenic estrogens, or a result of insufficient estrogen exposure in experimental laboratory fish (Jobling et al., 2002; Bahamonde et al., 2013). For example, partial intersex could be induced in rainbow trout (*Oncorhynchus mykiss*) by a low concentration of EE2 (0.1 μg/L) from 60 to 136 days post-fertilization, while 100% feminization was accomplished by 10 μg/L EE2 (Depiereux et al., 2014). Similar result have also been reported in medaka (Zhao et al., 2014): 0.04 μg/L E2

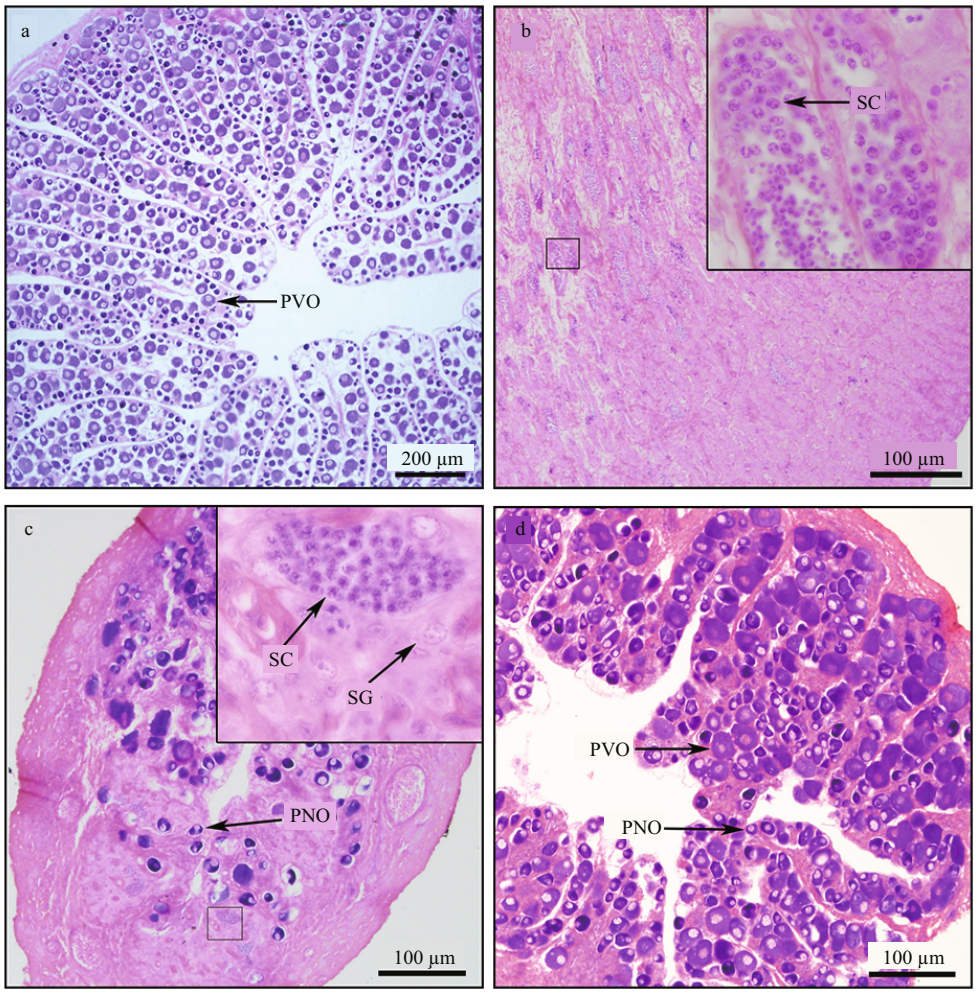

Fig.7 Photomicrographs of gonads of tiger puffer *Takifugu rubripes* from control and E2 treatment groups at 400 dph

a. XX ovary from the control group; b. XY testis from the control group; c. XY intersexual gonad from the 100-μg/L E2-treated group; d. XY ovary from the 100-μg/L E2-treated group. PNO: perinucleolar oocyte; PVO: previtellogenic oocyte; SC: spermatocyte; SG: spermatogonium.

induced intersex in male medaka from 0 and 30 dph only, while 0.08 μg/L E2 caused sex reversal in most male medaka. In our study, intersex gonads induced by 100 μg/L E2 treatment could retain their morphology after transformation from ovaries, demonstrating that this high concentration was insufficient to induce complete feminization in tiger puffer. These findings support our hypothesis that tiger puffer may be resistant to the induction of sex reversal with E2 after migration to clean seawater.

The GSI is an indicator of gonadal growth and maturation in fish and, thus, a useful marker for assessing the effects of EEs. A large number of studies have shown that EEs inhibit the growth of testicular tissue and delay spermatogenesis, with consequent decreased GSI values in males (Jobling et al., 1996; Meijide et al., 2016). The GSI may recover when morphology recovery occurs after estrogenic exposure, as shown in medaka (Paul-Prasanth et al., 2011). In the present study, the reversion of XY ovaries to testes occurred in the 1- and 10-μg/L E2-treated males after 270 dph, while gonadal growth in the XY males failed to recover and the GSI exhibited a dose-dependent reduction. Conversely, studies in bluegill sunfish (*Lepomis macrochirus*) (Wang et al., 2008), medaka (Lei et al., 2014), and sea bass (*Dicentrarchus labrax*) (Gorshkov et al., 2004) demonstrated that the GSI of sex-reversed females had increased compared with control males when the testes were replaced by ovaries. In our study, GSI values were lowest in XY females induced by 100 μg/L E2 treatment, as observed in the XY intersex in the same group. Our results suggest that, in tiger puffer, E2 exposure had a dose-dependent and unrecoverable inhibiting effect on the gonadal growth of genetic males, regardless of gonad condition.

Decreased GSI values, caused by estrogen exposure, have also been observed in the females of many species, including zebrafish (Van den Belt et al., 2004), medaka (Lei et al., 2014) and rare minnow (Zhang et al., 2008). The inhibitory effect of estrogenic estrogens may be a result of their action on germ-cell proliferation in ovaries, as shown in medaka (Paul-Prasanth et al., 2011). Scholz and Gutzeit (2000) have also demonstrated that EE2 exposure in medaka had long-term effects on ovarian growth and that the GSI values of female did not recover after exposure. In our study, even after a prolonged period of recovery, decreased GSI values in genetic females could not be reversed at 270 or 400 dph. The results suggested that, in tiger puffer, exogenous E2 at concentrations >1 μg/L could permanently inhibit ovarian growth of genetic females.

5 CONCLUSION

The results of our study indicate that long-term exposure to E2, at concentrations higher than environmentally applicable values, could impair sexual development of tiger puffer during the early life stages (15–100 dph). However, tiger puffer has the ability to recover from the feminizing effect, but not from the inhibiting effect, after a specific depuration period. The data suggest the potential use of tiger puffer as a valuable indicator species to evaluate the effects of environmental estrogens on marine fish, especially migratory species, and the protection of valuable fishery resources. Further studies are needed to assess possible delays in the reproductive capacity of E2-treated tiger puffer, as reported in medaka and zebrafish (Seki et al., 2002; Xu et al., 2008), and to understand the mechanisms underlying the reversibility of feminization. Changes at the level of mRNA expression of relevant markers also require further study with respect to their recovery from E2 exposure.

References

Allner B, von der Gönna S, Griebeler E M, Nikutowski N, Weltin A, Stahlschmidt-Allner P. 2010. Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment. *Environmental Science and Pollution Research*, **17**(2): 505-518.

Bahamonde P A, Munkittrick K R, Martyniuk C J. 2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? *General and Comparative Endocrinology*, **192**: 25-35.

Balch G C, Mackenzie C A, Metcalfe C D. 2004. Alterations to gonadal development and reproductive success in Japanese medaka (*Oryzias latipes*) exposed to 17α-ethinylestradiol. *Environmental Toxicology and Chemistry*, **23**(3): 782-791.

Baumann L, Holbech H, Keiter S, Kinnberg K L, Knörr S, Nagel T, Braunbeck T. 2013. The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test. *Aquatic Toxicology*, **128-129**: 34-42.

Bhandari R K, Deem S L, Holliday D K, Jandegian C M, Kassotis C D, Nagel S C, Tillitt D E, vom Saal F S, Rosenfeld C S. 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. *General and Comparative Endocrinology*, **214**: 195-219.

Brion F, Tyler C R, Palazzi X, Laillet B, Porcher J M, Garric J, Flammarion P. 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquatic Toxicology*, **68**(3): 193-217.

Depiereux S, Liagre M, Danis L, De Meulder B, Depiereux E, Segner H, Kestemont P. 2014. Intersex occurrence in rainbow trout (*Oncorhynchus mykiss*) male fry chronically exposed to ethynylestradiol. *PLoS One*, **9**(7): e98531.

Devlin R H, Nagahama Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, **208**(3-4): 191-364.

Dias L C A, Soares A M V M, Ferreira A L G, Santos C S, Monteiro M S. 2014. Biomarkers of endocrine disruption in juveniles and females of the estuarine fish *Pomatoschistus microps*. *Marine Pollution Bulletin*, **84**(1-2): 314-321.

Gorshkov S, Gorshkova G, Colorni B, Gordin H. 2004. Effects of natural estradioI-17β and synthetic 17α-ethynylestradiol on direct feminization of European Sea Bass *Dicentrarchus labrax*. *Journal of the World Aquaculture Society*, **35**(2): 167-177.

Harrison P T C, Holmes P, Humfrey C D N. 1997. Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? *Sci. Total Environ*., **205**(2-3): 97-106.

Hattori N, Miyashita S, Sawada Y. 2012. Effective masculinization method of tiger puffer by temperature control under culture condition. *Nippon Suisan Gakkaishi*, **78**(1): 87.

Hu P, Liu X F, Liu B, Wen H S, Yang Z, Lei J L. 2015. Histological observation on the gonadal differentiation of tiger puffer (*Takifugu rubripes*). *Periodical of Ocean University of China*, **45**(10): 25-30. (in Chinese with English abstract)

Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty G C, Sumpter J P, Tyler C R. 2002. Altered sexual maturation and gamete production in Wild Roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents.

Biology of Reproduction, **66**(2): 272-281.

Jobling S, Sumpter J P, Sheahan D, Osborne J A, Matthiessen P. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environmental Toxicology and Chemistry*, **15**(2): 194-202.

Kakimoto Y, Aida S, Arai K, Suzuki R. 1994. Induction of gynogenetic diploids in ocellated puffer *Takifugu rubripes* by cold and heat treatments. *J. Fac. Appl. Biol. Sci. Hiroshima Univ*., **33**(2): 103-112.

Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N, Fujita M, Suetake H, Suzuki S, Hosoya S, Tohari S, Brenner S, Miyadai T, Venkatesh B, Suzuki Y, Kikuchi K. 2012. A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu). *PLoS Genetics*, **8**(7): e1002798.

Katamachi D, Ikeda M, Uno K. 2015. Identification of spawning sites of the tiger puffer *Takifugu rubripes* in Nanao Bay, Japan, using DNA analysis. *Fisheries Science*, **81**(3): 485-494.

Kramer V J, Miles-Richardson S, Pierens S L, Giesy J P. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. *Aquatic Toxicology*, **40**(4): 335-360.

Länge R, Hutchinson T H, Croudace C P, Siegmund F, Schweinfurth H, Hampe P, Panter G H, Sumpter J P. 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, **20**(6): 1 216-1 227.

Lee K H, Yamaguchi A, Rashid H, Kadomura K, Yasumoto S, Matsuyama M. 2009a. Estradiol-17β treatment induces intersexual gonadal development in the pufferfish, *Takifugu rubripes*. *Zoological Science*, **26**(9): 639-645.

Lee K H, Yamaguchi A, Rashid H, Kadomura K, Yasumoto S, Matsuyama M. 2009b. Germ cell degeneration in high-temperature treated pufferfish, *Takifugu rubripes*. *Sexual Development*, **3**(4): 225-232.

Lei B L, Kang J, Yu Y X, Zha J M, Li W, Wang Z J, Wang Y P, Wen Y. 2014. Long-term exposure investigating the estrogenic potency of estriol in Japanese medaka (*Oryzias latipes*). *Comparative Biochemistry and Physiology Part C*: *Toxicology & Pharmacology*, **160**: 86-92.

Meijide F J, Rey Vázquez G, Piazza Y G, Babay P A, Itria R F, Lo Nostro F L. 2016. Effects of waterborne exposure to 17β-estradiol and 4-tert-octylphenol on early life stages of the South American cichlid fish *Cichlasoma dimerus*. *Ecotoxicology and Environmental Safety*, **124**: 82-90.

Mills L J, Chichester C. 2005. Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment*, **343**(1-3): 1-34.

Murua H. 2014. Fish reproduction assortment: a wonderful diversity. *Environmental Biology of Fishes*, **97**(3): 329-333.

Nakamura M. 1984. Effects of estradiol-17β on gonadal sex differentiation in two species of salmonids, the masu salmon, *Oncorhynchus Masou*, and the chum salmon, *O. Keta*. *Aquaculture*, **43**(1-3): 83-90.

Nimrod A C, Benson W H. 1998. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquatic Toxicology*, **44**(1-2): 141-156.

Patyna P J, Davi R A, Parkerton T F, Brown R P, Cooper K R. 1999. A proposed multigeneration protocol for Japanese medaka (*Oryzias latipes*) to evaluate effects of endocrine disruptors. *The Science of the Total Environment*, **233**(1-3): 211-220.

Paul-Prasanth B, Shibata Y, Horiguchi R, Nagahama Y. 2011. Exposure to diethylstilbestrol during embryonic and larval stages of medaka fish (*Oryzias latipes*) leads to sex reversal in genetic males and reduced gonad weight in Genetic Females. *Endocrinology*, **152**(2): 707-717.

Peters R E M, Courtenay S C, Hewitt L M, MacLatchy D L. 2010. Effects of 17α-ethynylestradiol on early-life development, sex differentiation and vitellogenin induction in mummichog (*Fundulus heteroclitus*). *Marine Environmental Research*, **69**(3): 178-186.

Piferrer F. 2001. Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture*, **197**(1-4): 229-281.

Rocha M J, Cruzeiro C, Reis M, Pardal M Â, Rocha E. 2014. Spatial and seasonal distribution of 17 endocrine disruptor compounds in an urban estuary (Mondego River, Portugal): evaluation of the estrogenic load of the area. *Environmental Monitoring and Assessment*, **186**(6): 3 337-3 350.

Scholz S, Gutzeit H O. 2000. 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquat. Toxicol*., **50**(4): 363-373.

Segner H, Caroll K, Fenske M, Janssen C R, Maack G, Pascoe D, Schäfers C, Vandenbergh G F, Watts M, Wenzel A. 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicology and Environmental Safety*, **54**(3): 302-314.

Segner H. 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comparative Biochemistry and Physiology Part C*: *Toxicology & Pharmacology*, **149**(2): 187-195.

Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias Latipes*). *Environmental Toxicology and Chemistry*, **21**(8): 1 692-1 698.

Shinomiya A, Shibata N, Sakaizumi M, Hamaguchi S. 2002. Sex reversal of genetic females (XX) induced by the transplantation of XY somatic cells in the medaka, *Oryzias latipes*. *Int. J. Dev. Biol*., **46**(5): 711-717.

Suzuki N. 1997. Histological study on intersexual gonads observed in wild tiger puffer, *takifugu rubripes*. *Bull. Nansei Natl. Fish. Res. Inst*., **30**: 101-113.

Van den Belt K, Berckmans P, Vangenechten C, Verheyen R, Witters H. 2004. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethynylestradiol and nonylphenol. *Aquatic Toxicology*, **66**(2): 183-195.

Wang H P, Gao Z X, Beres B, Ottobre J, Wallat G, Tiu L, Rapp D, O'Bryant P, Yao H. 2008. Effects of estradiol-17β on survival, growth performance, sex reversal and gonadal structure of bluegill sunfish *Lepomis macrochirus*. *Aquaculture*, **285**(1-4): 216-223.

Xu H, Yang J, Wang Y X, Jiang Q, Chen H, Song H Y. 2008. Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). *Aquatic Toxicology*, **88**(1): 1-8.

Zhang X Y, Zha J M, Wang Z J. 2008. Influences of 4-nonylphenol on doublesex- and mab-3-related transcription factor 1 gene expression and vitellogenin mRNA induction of adult rare minnow (*Gobiocypris rarus*). *Environmental Toxicology and Chemistry*, **27**(1): 196-205.

Zhao Y B, Wang C, Xia S, Jiang J Q, Hu R, Yuan G X, Hu J Y. 2014. Biosensor medaka for monitoring intersex caused by estrogenic chemicals. *Environmental Science & Technology*, **48**(4): 2 413-2 420.

Zheng B H, Liu R Z, Liu Y, Jin F, An L H. 2015. Phenolic endocrine-disrupting chemicals and intersex in wild crucian carp from Hun River, China. *Chemosphere*, **120**: 743-749.

Zillioux E J, Johnson I C, Kiparissis Y, Metcalfe C D, Wheat J V, Ward S G, Liu H. 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17α-ethynylestradiol. *Environmental Toxicology and Chemistry*, **20**(9): 1 968-1 978.