Journal of Hazardous Materials 394 (2020) 122537

Contents lists available at ScienceDirect

Journal of Hazardous Materials

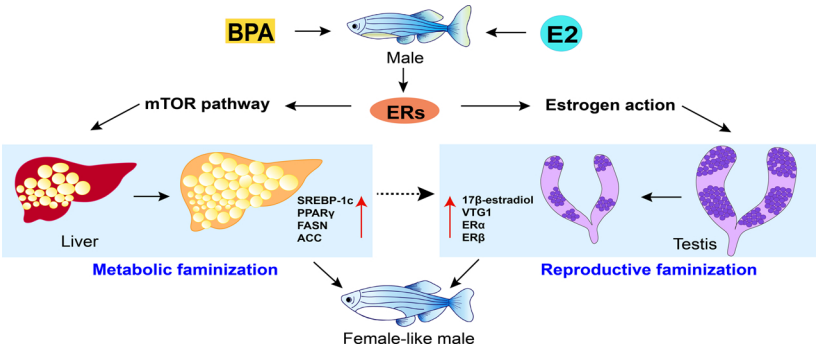
journal homepage: www.elsevier.com/locate/jhazmat

Environmental estrogen exposure converts lipid metabolism in male fish to a female pattern mediated by AMPK and mTOR signaling pathways

Sheng-Xiang Sun[a], Jun-Lin Wu[a], Hong-Bo Lv[a], Hai-Yang Zhang[b], Jing Zhang[c], Samwel Mchele Limbu[a,d], Fang Qiao[a], Li-Qiao Chen[a], Yi Yang[c,e], Mei-Ling Zhang[a], Zhen-Yu Du[a,*]

[a] LANEH, School of Life Sciences, East China Normal University, Shanghai 200241, China
[b] Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences, School of Life Sciences, East China Normal University, Shanghai 200241, China
[c] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200062, China
[d] Department of Aquatic Sciences and Fisheries Technology, University of Dar as Salaam, Dar es Salaam, Tanzania
[e] Key Laboratory of Geographic Information Science (Ministry of Education), School of Geographical Sciences, East China Normal University, Shanghai 200241, China

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: D. Aga

Keywords:
Environmental estrogens
17β-estradiol (E2)
Bisphenol A (BPA)
Lipid metabolism
Feminization

ABSTRACT

Environmental estrogens, including bisphenol A (BPA) and 17β-estradiol (E2), which are widely used in industries and medicine, pose a severe ecological threat to fish due to feminization induction. However, the related metabolic basis for reproductive feminization in male fish has not been well addressed. We first found that female zebrafish exhibited higher lipid accumulation and lipogenesis activity than males. Next, we exposed male and female zebrafish to E2 (200 ng/L) or BPA (100 μg/L) for six weeks, and observed an early-phase reproductive feminization in males, accompanied with reduced spermatids, significant fat deposition and lipogenic gene expressions that mimicked female patterns. Cellular signaling assays revealed that, E2 or BPA modulated lipid metabolism in males mainly through lowering 5′ AMP-activated protein kinase (AMPK) and upregulating the lipogenic mechanistic target of rapamycin (mTOR) pathways. For the first time, we show that environmental estrogens could alter lipid metabolism in male fish to a female pattern (metabolic feminization) prior to gonad feminization in male fish, to allows males to accumulate efficiently lipids to harmonize with the feminized gonads. This study suggests that negative effects of environmental estrogens, as hazardous materials, on vertebrate health are more complicated than originally thought.

* Corresponding author.
E-mail address: zydu@bio.ecnu.edu.cn (Z.-Y. Du).

https://doi.org/10.1016/j.jhazmat.2020.122537
Received 17 January 2020; Received in revised form 28 February 2020; Accepted 12 March 2020
0304-3894/ © 2020 Elsevier B.V. All rights reserved.

1. Introduction

Environmental estrogens, such as bisphenol A (BPA) and 17β-estradiol (E2) which are widely used in manufacturing industries and medicine, are estrogenic compounds that bind estrogen receptors (ERs) and mainly originate from chemical pollutants and vertebrates wastes (Shore and Shemesh, 2016). They are currently widely distributed in aquatic environment. For example, the natural hormone E2 and the synthetic compound BPA are the two typical environmental estrogens (Shrestha et al., 2012; null[]). The concentration of E2 in effluents in Israel ranged from 48 to 141 ng/L (Kramer et al., 1998), and it was as high as 313 ng/L in the Salt River (Arizona, USA) and Huron River waters (Michigan, USA) (Yoon et al., 2003). The concentration of BPA in river water in the Netherlands reached 21 μg/L (Crain et al., 2007), and as high as 370 μg/L in effluents in Japan (Fukazawa et al., 2001).

A number of environmental estrogens have been reported as risk factors for metabolic syndromes in humans (Lee et al., 2014; Grundy et al., 2004). Furthermore, environmental estrogens are also a serious threat to ecology, especially in aquatic environment (Hallgren et al., 2014; Kidd et al., 2007). Fish, as the largest group of vertebrates are severely affected by environmental estrogens. For example, 17α-ethynylestradiol (EE2) induced reproductive failure and feminization in male fish by altering courtship displays, delaying testis maturity, and causing feminized phenotypes leading to oogonia and vitellogenin (VTG) production (Kidd et al., 2007; Rose et al., 2013). These disturbing effects of environmental estrogens have been reported in numerous fish species (Jobling et al., 1998; Viganò et al., 2001; Mikaelian et al., 2010), and have caused ecological damage globally (Hallgren et al., 2014; Kidd et al., 2007).

Although the feminization effects of environmental estrogens on male fish have attracted much scientific attention, the underlying mechanisms have not been well addressed. Of note, a few descriptive studies showed that during gonad development and/or spawning, female fish have a different nutrient utilization capacity compared to males (Bertoldo et al., 2015; Rljnsdorp and Ibelings, 1989). For instance, post-reproductive Arctic charr females consumed almost 80% of body lipids during winter, whereas males consumed 50–55% of body lipids in the same period (Jøsrgensen et al., 1997). This finding suggested that lipid metabolism differs between female and male fish. Apparently, females need more lipids than males for reproduction. However, the systemic differences in lipid metabolism between female and male fish have not been intensively reported.

Previous fish studies reported that dietary or aquatic exposure to some pollutants, particularly BPA and polychlorinated biphenyls (PCBs), not only induced feminization but also elevated lipid accumulation in the liver and adipose tissue of males (Li et al., 2019; Sun et al., 2019; Forner-Piquer et al., 2018). However, the mechanisms of induced lipid accumulation and feminization due to environmental estrogens exposure have not been well demonstrated. Considering that female fish need more lipid for gonad development and egg formation, it is very likely that during the environmental estrogen-induced feminization in male fish, lipid metabolism would likewise change to adapt to the increased physiological requirement for ovary development as in females. Therefore, we hypothesized that, the lipid metabolism pattern differs between male and female fish, and the lipid metabolism pattern in male fish might change to a female pattern to harmonize with reproductive feminization induced by environmental estrogens.

To verify our hypotheses, we used zebrafish (*Danio rerio*) to test the effects of E2 and BPA on lipid metabolism. First, we investigated the possible differences in patterns of fat accumulation and lipid metabolism in female and male zebrafish through feeding them with diets containing different fat contents for six weeks. Afterwards, we determined the effects of E2, BPA and tamoxifen (TAM), which is an antagonist of ERs, on the gonad development and lipid metabolism patterns in male and female fish. Next, we assayed a number of molecular signaling proteins to illustrate the mechanism during the alteration of the lipid metabolism pattern in male fish subjected to environmental estrogens.

2. Materials and methods

This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (approval NO.: F20140101). The experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China.

2.1. Chemicals and diets preparations

The 17-beta-estradiol (E2), 2,2-bis (4-Hydroxyphenol) propane (BPA) and tamoxifen (TAM) were purchased from Adamas-beta (Shanghai, China). Dimethyl sulfoxide (DMSO), Methanol, n-hexane, dichloromethane and acetonitrile of HPLC grade were supplied by ANPEL (Shanghai, China). The internal standards of E2-d_2 and BPA-d_{16} (E2 > 96.8%, BPA > 99%) were purchased from Dr. Ehrenstrofer GmbH (Augsburg, Germany). The E2 and BPA were dissolved in DMSO to make stock solutions at concentrations of 1 g/L and 100 g /L, respectively. The stock solutions were diluted by using dechlorinated water to obtain the designed concentrations. The final DMSO concentration in cultured water was 0.005 ml/L.

We formulated three purified diets as detailed in Supplementary Table S1. The purified diets were necessary in order to avoid extra sources of environmental estrogens. The three diets contained fat at 1% (low-fat diet, LFD), 7% (normal-fat diet, NFD) and 13% (high-fat diet, HFD).

2.2. Fish source and acclimatization

The AB strain adult zebrafish (0.3 ± 0.08 g each, about five months old) were obtained from the Chinese National Zebrafish Resource Center (Wuhan, China). They were acclimated for one week and fed twice daily with a commercial zebrafish diet (Shengsuo, Yantai, China). During acclimation and formal trial, all fish were cultured in dechlorinated tap water with 12 h light to dark photoperiod. The water temperature, pH, dissolved oxygen and ammonia nitrogen were kept at 26 ± 2 °C, 7.99 ± 0.23, 5.5 ± 1.2 mg/L and below 0.02 mg/L, respectively.

2.3. Dietary fat levels treatment

To delineate the sexual dimorphism on lipid metabolism, 36 visually healthy male or female zebrafish were selected (three replicates, 12 fish for each). The fish in each replicate were cultured in a 10 L glass tank and fed with either LFD, or NFD or HFD at a feeding rate at 4% of their body weight, twice daily for six weeks. All fish in each tank were weighed biweekly and the daily feeding amounts were adjusted accordingly. One third of cultured water by volume in each tank was replaced with fresh-dechlorinated water every day.

2.4. Estrogens exposure treatment

In order to choose a proper exposure dose of E2 and BPA for the mechanistic studies, the male and female zebrafish fed on NFD were divided and exposed to control (fresh-dechlorinated water), E2 (50, 100, 200, 300 and 400 ng/L) and BPA (50, 100, 200, 300, 400 μg/L), respectively. The 60 female or male zebrafish were grouped into one treatment (20 fish per replicate), and were reared into 30 L glass tanks for six weeks. Because E2 of 200 ng/L and BPA of 100 μg/L in female, and E2 of 100–300 ng/L and BPA of 50–100 μg/L in male, which are all environmental concentrations, could induce considerable fat deposition in body (Fig. S2), therefore, E2 of 200 ng/L and BPA of 100 μg/L were chosen for the following formal exposure experiment. In order to verify the effects of TAM, which is a well-known estrogen receptor antagonist,

on fish growth, lipid metabolism and estrogen receptor expression, the zebrafish fed on NFD were divided and exposed to control (fresh-dechlorinated water), TAM-50 (50 μg/L TAM) and TAM-250 (250 μg/L TAM), respectively. The 30 fish were grouped into one treatment (10 fish per replicate), and were reared into 30 L glass tanks for four weeks. The results showed that either 50 μg/L or 250 μg/L TAM could significantly lower expressions of estrogen receptors, but didn't significantly change fish growth, lipid deposition and the related lipogenesis gene expression (Fig. S3). Therefore, in the formal experiment, 50 μg/L of TAM was used. In the formal experiment, 90 male or female zebrafish in three replicates (30 fish per replicate) were reared into 30 L glass tanks. The fish were fed on LFD or HFD and exposed to E2, BPA, E2 + TAM (ET), and BPA + TAM (BT) for six weeks (Fig. 2A). A control group was also included, in which fish were fed on either LFD or HFD only. Half of the culture water by volume in the control tanks were replaced with fresh-dechlorinated water every 24 h, while those in exposure treatments were replaced with fresh-dechlorinated water containing the initial concentrations. The whole culture water was replaced every seven days. The experimental conditions were maintained as those used in the dietary experiment.

In the formal experiment, the real exposure concentrations of E2 and BPA were randomly measured on the 10^{th} and 38^{th} days. The E2 concentrations in the water of control and E2 treatments were 0.56 ± 0.05 ng/L and 171.75 ± 6.37 ng/L, respectively. The BPA concentrations in the water of control and BPA treatments were 0.11 ± 0.01 μg/L and 77.45 ± 3.72 μg/L, respectively (Fig. S4B). The contents of E2 and BPA in LFD and HFD are shown in Fig. S4A.

2.5. Fish sampling

At the end of the dietary and exposure trials, all fish were fasted for 12 h. A total of eight fish from each replicate were sampled, anesthetized by using MS-222 and sacrificed for tissue samples collection. The liver, muscle, viscera (including intestines, spleen and abdominal fat tissue), testis and six whole fish from each tank were collected and stored at -80 °C for biochemical assays and molecular measurements.

2.6. Lipid content and histological assays

A total of 12 fish from each treatment were collected for total fat content analysis. The total fat content was measured by using the classic methanol-chloroform method (Bligh and Dyer, 1959). Another sample of 10 fish from each treatment (two fish as a pooled sample) were sampled and used for determination of hepatic, viscera and muscle triglyceride (TG) assays by using specific commercial kit (Jiancheng Biotechnology Co., China). A part of testis and liver from four fish in each treatment were fixed by using 4% paraformaldehyde for histological analysis based on the literature (Betancor et al., 2015).

2.7. Quantitative real-time PCR assay

Liver, muscle and viscera tissues from 12 fish were prepared for total RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR), as described in our previous study (Limbu et al., 2018). Elongation factor 1 (*ef1α*) and β-actin were used as reference genes, due to their stable expression in different treatments. All the gene primer sequences used for qPCR analysis are listed in Table S2. The relative mRNA expressions of target genes were calculated by using the $2^{-\Delta\Delta Ct}$ method, thereof, $\Delta Ct = Ct_{target} - (Ct_{ef1\alpha} + Ct_{\beta\text{-}actin})/2$. The qPCR efficiency was between 99% and 102%, and the correlation coefficient was above 0.98 for each gene.

2.8. Transcriptomic assay

Nine liver samples from each selected treatment were used for the RNA-seq transcriptomic assay. The mRNA isolation, invalid reads and unigenes assembly were performed as described by Lu et al (Lu et al., 2019). To confirm the accuracy of transcriptomic data, we chose female-HFD and male-HFD treatments in dietary experiment as the representative for validation (Fig. S1).

2.9. Western blot assay

A total of six liver tissues were homogenized on ice by using the mixture of ice-cold PIPA lysis buffer (Beyotime Biotechnology) and 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology), and their cells were lysed for 30 min for protein extraction. The detail methods and antibody information of western blot assay are presented in the Supplementary data.

2.10. Determination of E2 and BPA by UHPLC-MS/MS

A total of 30 fish, 12 L culture water and 300 g diets from each exposure treatment were prepared for E2 and BPA detections by using ultra-high-performance liquid chromatography equipped with tandem mass spectrometry (UHPLC-MS/MS), as described by Ye et al. (2013). The detailed methods for determination of E2 and BPA are presented in the Supporting Information.

2.11. Statistical analyses

All values are presented as mean ± standard error of means (SEM). Statistical differences between female and male fish fed with the same diet were tested by using the independent sample *t*-test ($P < 0.05$). Statistical differences among female or male fish for more than two treatments were compared by using one-way analysis of variance (ANOVA) ($P < 0.05$). All statistical analyses were conducted by using SPSS 23 statistical software (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. The lipid metabolism pattern differs between male and female zebrafish

We first fed both sexes on diets with the same fat content for six weeks in order to understand the lipid metabolism pattern. The total fat and TG contents in the liver and viscera were higher in female fish than in male fish (Fig. 1A–D). Similarly, female fish also had more and larger lipid vacuoles than males (Fig. 1E). During transcriptomic assay, lipid metabolism was the main pathway significantly changed between the female and male fish, especially those fed on HFD (Fig. 1F). Likewise, female fish had higher expression of genes related to lipogenesis, such as fatty acid synthase (*fasn*), stearoyl-CoA desaturase (*scd*), acetyl-CoA carboxylase (*acc*), sterol regulatory element binding transcription factor 1 (*srebp1*), and peroxisome proliferator activated receptor gamma (*pparγ*) (Fig. 1G).

Our results on sexual dimorphism in lipid metabolism provide new insights because such investigations are mostly performed in mammals, but seldom in fish (Watkins et al., 1972; Soler-Argilaga and Heimberg, 1976). In our study, the lipid metabolism pattern exhibited sexual dimorphism, with females displaying higher fat deposition and lipid synthesis compared with males (Fig. 1). The differences in lipid metabolism between sexes in fish and mammals indicate a universal phenomenon in animals. Since female fish consume more body lipid than male fish in the reproductive period (Jøsrgensen et al., 1997), they reserve large amounts of lipid to meet the energy demands during reproductive development. Furthermore, the major difference in lipid metabolism between sexes was found mainly in the liver, for which female fish had higher degrees of increase in TG compared to males (Fig. 1B). The liver of fish not only regulates lipid metabolism, but also influences reproductive development (Canbay et al., 2007; Kley and Krüskemper, 1976). Accordingly, mature female fish in the reproductive period produce large amounts of VTG in the liver, which are

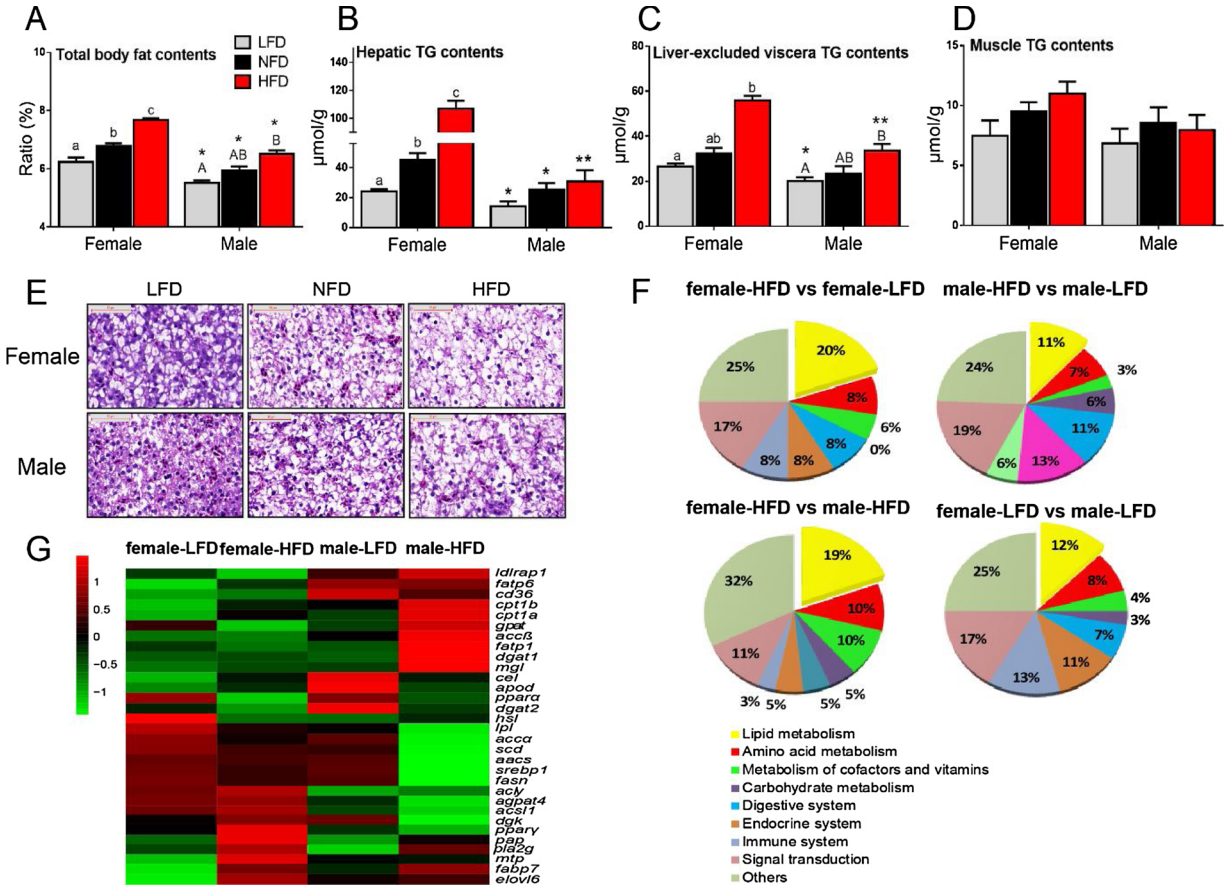


Fig. 1. Differences of lipid metabolism between female and male zebrafish. Total body fat contents **(A)**, tissue TG contents **(B-D)** and lipid vacuoles in the liver histological pictures **(E)** are shown in the female and male zebrafish. The female and male zebrafish were fed on low fat diet (LFD), normal fat diet (NFD) or high fat diet (HFD), respectively. Data are expressed as means ± SEM (n = 5). Statistical differences between female and male fish fed with the same diet are marked with the asterisk "*" ($P < 0.05$). Statistical differences among dietary treatments in female or male fish are marked with different lowercases or capitals, respectively ($P < 0.05$). The scale bar in the photomicrographs represents 50 μm. After performing a transcriptomic assay in fish liver, the comparisons of functional classification (**F**) and the heatmap of gene expressions involved in lipid metabolism (**G**) are presented among the four treatments, including female-LFD (female fed with LFD), female-HFD (female fed with HFD), male-LFD (male fed with LFD) and male-HFD (male fed with HFD). Red and green colors in the heatmaps represent upregulation and downregulation of the gene expressions, respectively. The full names of gene abbreviation at the right side of heatmap are described in Table S3. In short, these data show that the female zebrafish exhibited higher lipid accumulation and lipogenesis activity than the males.

transferred to the ovaries to provide sufficient nutrients for eggs (Romano et al., 2004). Hence, the observed differences in lipid metabolism in the fish liver between females and males might be caused by variations in reproductive demands.

3.2. Zebrafish accumulates E2 and BPA after exposure

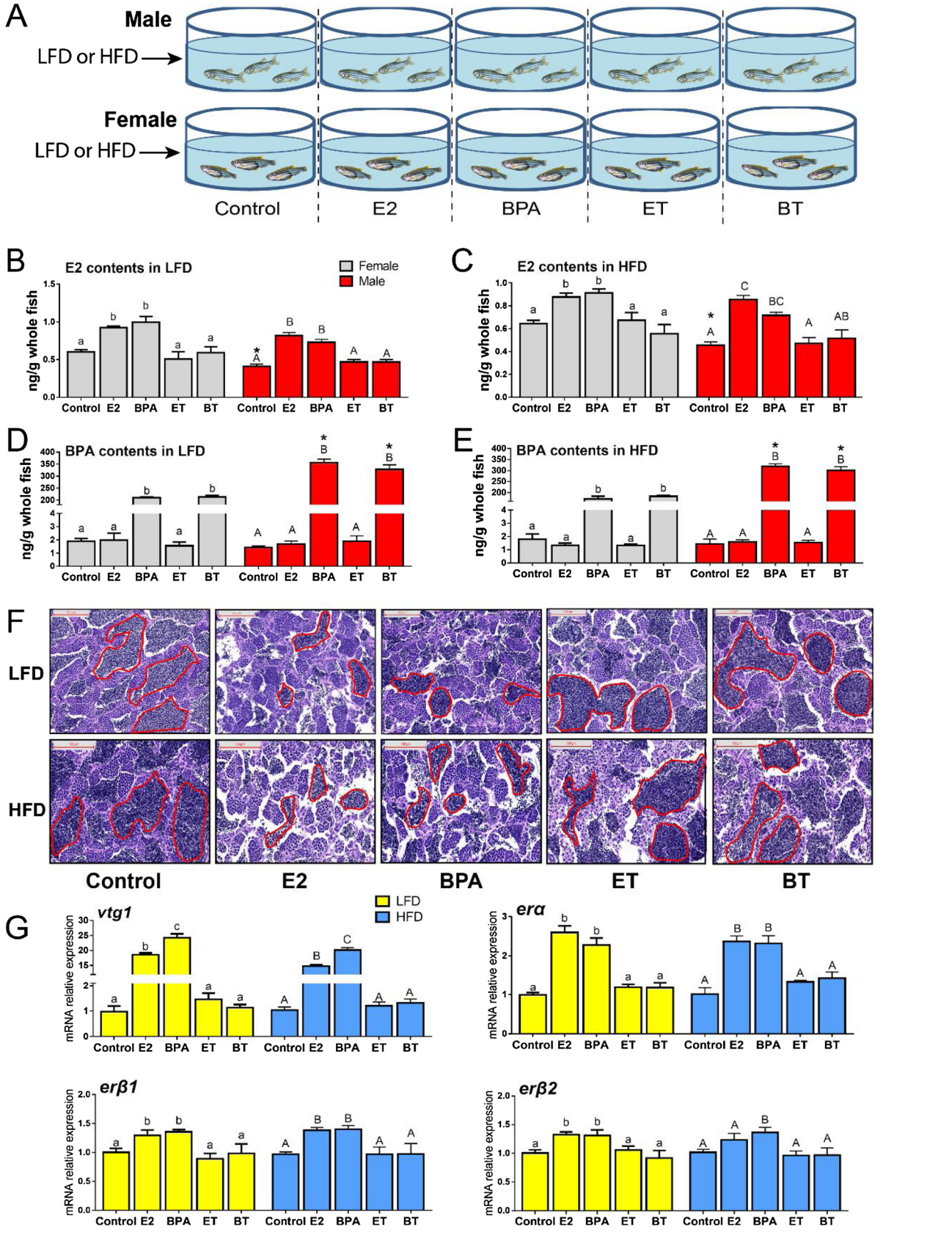
To furtherly verify the effects of E2 and BPA exposure in zebrafish, we exposed males and females fed on LFD and HFD to E2 (200 ng/L) and BPA (100 μg/L) for six weeks. The E2 content in females and males was significantly higher in the E2 treatments than in the controls. However, co-exposure of E2 and TAM significantly reduced the accumulation of E2 in males and females (Fig. 2B, C). Interestingly, exposure to BPA significantly increased E2 content in males and females, and the co-exposure to BPA and TAM significantly reduced the E2 content in both sexes. An increase in E2 levels was also reported in goldfish (*Carassius auratus*) after injecting 50 or 100 mg/kg of BPA (Li et al., 2008). These results suggest that BPA increase endogenous synthesis of E2 in fish. Generally, E2 levels are higher in female fish than in males throughout the life period (Pottinger and Carrick, 2010). In the present study, the E2 concentration in male fish exposed to E2 and BPA was comparable to females (Fig. 2B, C), suggesting E2 and BPA induce effects in males based on E2 levels.

Similar to E2 deposition, BPA residue was significantly elevated in male and female fish exposed to BPA, but the co-exposure of BPA and TAM did not affect the BPA residue in the fish (Fig. 2D, E). Natural estrogens have a short half-life and are readily eliminated compared to synthetic ones (Yin et al., 2003; Ferreiraleach and Hill, 2001). Therefore, when the ERs were occupied by TAM in the co-exposure treatments, the dissociative E2 as a natural estrogen may be rapidly degraded. However, the dissociative BPA as a typical synthetic estrogen may exhibit a slower degradation compared with E2. The different residues between BPA and E2 are possibly attributed to stability and degradation properties.

3.3. Exposure to E2 and BPA feminizes male zebrafish

Previous studies indicated that feminized male fish produce oocytes or oviducts in the testis, and then develop into intersex (Jobling et al., 1998; Ortiz-Zarragoitia et al., 2014). In the present study, neither oocytes nor oviducts were observed in the testis of male zebrafish exposed to E2 and BPA (Fig. 2F). However, we found fewer spermatids in testis of males exposed to E2 and BPA than in the control and TAM co-exposure treatments (Fig. 2F), similar to delayed spermatid maturation in zebrafish exposed to E2 from 100 to 270 ng/L (Scholz and Klüver, 2009) and in Japanese medaka (*Oryzias latipes*) exposed to BPA ranging from 50 to 335 μg/L (Scholz and Klüver, 2009; Yokota et al., 2000). Moreover, carp males exposed to 10 and 100 μg/L E2 for 20 days

produced oviducts in testis (Gimeno et al., 1998), while Japanese medaka males exposed to 1820 μg/L BPA for 60 days produced oocytes (Yokota et al., 2000). These studies suggest that the extent of feminization induced by environmental estrogens depends on the exposure concentrations and their duration, and fish species. In the present study, the inhibition of sperm maturation in the male fish is



(caption on next page)

Fig. 2. Reproductive feminization of the male zebrafish exposed to E2 and BPA. Experimental design of E2 and BPA exposure are shown (**A**). The male or female zebrafish fed with LFD or HFD were exposed to E2, BPA, or TAM, respectively, for six weeks. Exposure treatments are abbreviated as Control, E2, BPA, ET (co-exposure of E2 and TAM), and BT (co-exposure of BPA and TAM). Concentrations of E2 (**B-C**) and BPA (**D-E**) in the whole fish are presented. Data are expressed as means ± SEM (n = 3). Statistical differences between female and male fish fed with the same diet are marked with the asterisk "*" ($P < 0.05$). Statistical differences among exposure treatments in female or male fish are marked with different lowercases or capitals, respectively ($P < 0.05$). Testis histological biopsy of male zebrafish represent the damage of E2 and BPA on sperm development (**F**). The dense dark-purple dots enclosed by red lines on the testis biopsies represent the spermatids, and the scale bar represents 100 μm. Gene expressions involved in gonad feminization in male liver are presented (**G**). Statistical differences among exposure treatments in the male fish fed on LFD or HFD are marked with the different lowercases or capitals, respectively ($P < 0.05$). In short, these data present that E2 and BPA induce the early-phase gonad feminization in the male zebrafish.

regarded as early-phase feminization induced by exposure to E2 and BPA. Moreover, the hepatic mRNA expressions of *erα*, *erβ1*, *erβ2*, and *vtg1*, which are all molecular markers for predicting feminization in fish (Romano and Limatola, 2000; Kime et al., 1999), were upregulated in male fish exposed to the E2 and BPA compared to the male controls and the TAM co-exposure treatments (Fig. 2G). Of note, the individual exposure of TAM could significantly inhibit expressions of estrogen receptors in zebrafish (Fig. S3), and the E2 concentrations in the ET and BT groups were also significantly reduced (Fig. 2B, C). Therefore, the alleviated feminization in the male fish in the TAM co-exposure groups could be caused by the TAM-induced inhibition of estrogen actions. Taken together, exposure to E2 and BPA at environmental concentrations for six weeks increased their residues in male zebrafish, and induced early-phase feminization. However, the inhibition of estrogen actions would block this feminization in male fish.

3.4. Exposure to E2 and BPA in fish fed on HFD induces sex-dependent lipid deposition

In the fish fed on HFD, the whole body fat and tissue TGs contents were higher in male zebrafish exposed to E2 and BPA than the controls and TAM co-exposure treatments, especially in liver (Fig. 3A–D). However, the female zebrafish exposed to E2 and BPA elevated only TG content in the liver, but not in other tissues (Fig. 3A–D). Similarly, in the fish fed on LFD, E2 and BPA did not affect significantly lipid accumulation in female fish (Fig. S5), but also increased significantly hepatic TG content in male fish (Fig. S5B). The male fish exposed to E2 or BPA increased the number and size of lipid vacuoles (Fig. 3E), similar to female fish. Notably, in both sexes, the co-exposure of TAM decreased the E2- and BPA-induced fat accumulation to levels comparable with the controls, particularly in the fish fed on HFD. In liver, the fish exposed to E2 and BPA upregulated lipogenic genes expression including *srebp-1c* and *fasn* in females and males, whereas TAM co-exposure did not affect the expression compared to the controls (Figs. 3F and S6). Similar expression patterns in lipid synthesis occurred in viscera and muscle tissues, but the extent of changes was weaker than in the liver (Figs. S7A, S7C, S8A and S8C). However, the mRNA expression of genes involved in lipid catabolism (peroxisome proliferator activated receptor alpha, *ppara*; carnitine palmitoyltransferase 1a, *cpt1a*) and lipid transport (long-chain fatty acid transport protein 1, *fatp1*; cluster determinant, *cd36*) were not significantly affected in fish exposed to E2, BPA or TAM (Fig. 3G, H).

The above data suggest that, males are more sensitive to environmental estrogens exposure compared to females. Notably, males increased lipid accumulation, especially in the liver, resulting in similar lipid metabolism characteristics as in females. Lipogenesis, but not lipid catabolism, mainly contributed to the E2- or BPA-induced fat accumulation. In addition, the results suggest that the effects of environmental estrogens on lipid accumulation in male fish are also dependent on the animal's nutritional background. As shown in the above results, environmental estrogens could induce lipid deposition in the males fed with either LFD or HFD, and this environmental estrogens-induced fat deposition was strengthened by HFD feeding. Considering HFD has been widely used in the practical aquaculture to save dietary protein and increase feed utilization in farmed fish (Boujard et al., 2004; Hillestad et al., 1998), the combination of environmental estrogens and HFD feeding could reflect a more real situation when people evaluate the effects of environmental estrogens on fish production and human health.

Higher lipid accumulation and expressions of lipid metabolism-related genes were also found in zebrafish and bluefin tuna males exposed to PCBs and organochlorine pesticides (OCPs) compared to females (Li et al., 2019; Maisano et al., 2016). Thus, environmental estrogen-induced sex-dependent metabolic alterations in fish are likely to be a common phenotype in response to different organic pollutants. Interestingly, we found that compared to the individual exposure of E2 and BPA, the TAM co-exposure treatments significantly decreased lipid deposition and increased lipogenesis gene expressions in fish. Moreover, our pre-experiment also indicated that the individual exposure of TAM only downregulated gene expressions of *erα* and *erβ1*, but didn't significantly affect lipid deposition and lipogenesis gene expressions (Fig. S3). This indicates that the TAM co-exposure-induced fat reduction was mainly caused by the inhibited estrogen actions. It has been reported that TAM, as an antagonist for ERs, could prevent estrogen actions of BPA and E2 (Kumar et al., 2016), and lipid metabolism was regulated by estrogen through ERs in mammals (Sara et al., 2011; Lin et al., 2013). Therefore, the E2- and BPA-induced lipid deposition in fish could also result from estrogen-like actions through the ERs.

In the present study, the hepatic lipid contents in the male zebrafish exposed to E2 and BPA were significantly increased, and reached almost the same level as the hepatic lipid content in the control females (Fig. 3B, E). In fact, this estrogens-induced accumulation of lipid in liver has been widely reported in female fish during the ovarian development. For example, during the ovarian development in the female Arctic charr, the liver lipid content increased from 3.5% to 14.7%, whereas it decreased again to 3.1% in the post-spawning females (Jørsgensen et al., 1997). Similarly, several other studies in female fish also found the remarkable fat deposition in liver during the ovarian development (Medford and Mackay, 1978; Diana and Mackay, 1979), and found the deposited fat in liver was mobilized for gonad maturation (Krivobok and Tokareva, 1972; Reshetnikov et al., 1970). The VTGs, which are the large lipid transfer proteins (LLTP), are synthesized in liver during the female sexual maturation and have been verified to transport liver fat to ovary for egg production and providing energy (Wang et al., 2005). In numerous environmental toxicological studies, the high expression of VTG in male fish liver has been identified as an important marker of feminization (Sumpter and Jobling, 1995). Therefore, these literatures indicate that the high accumulation of fat in liver is a specific physiological response, but not pathologic outcome, during the ovarian development in female fish. Accordingly, we found in the present study that the environmental estrogens exposure in the male fish not only induced high hepatic fat accumulation and gonad feminization, but also significantly increased the gene expressions of *vtg1* with 14–20 folds (Fig. 2G). Therefore, it is reasonable to conclude that the increased lipid deposition in the male fish exposed to environmental estrogens could be a metabolic change to adapt to gonad feminization.

3.5. Exposure to E2 and BPA converts lipid metabolism in male fish to a female pattern by changing gene transcription and signaling pathways

To systemically detect the lipid metabolism pattern in male fish

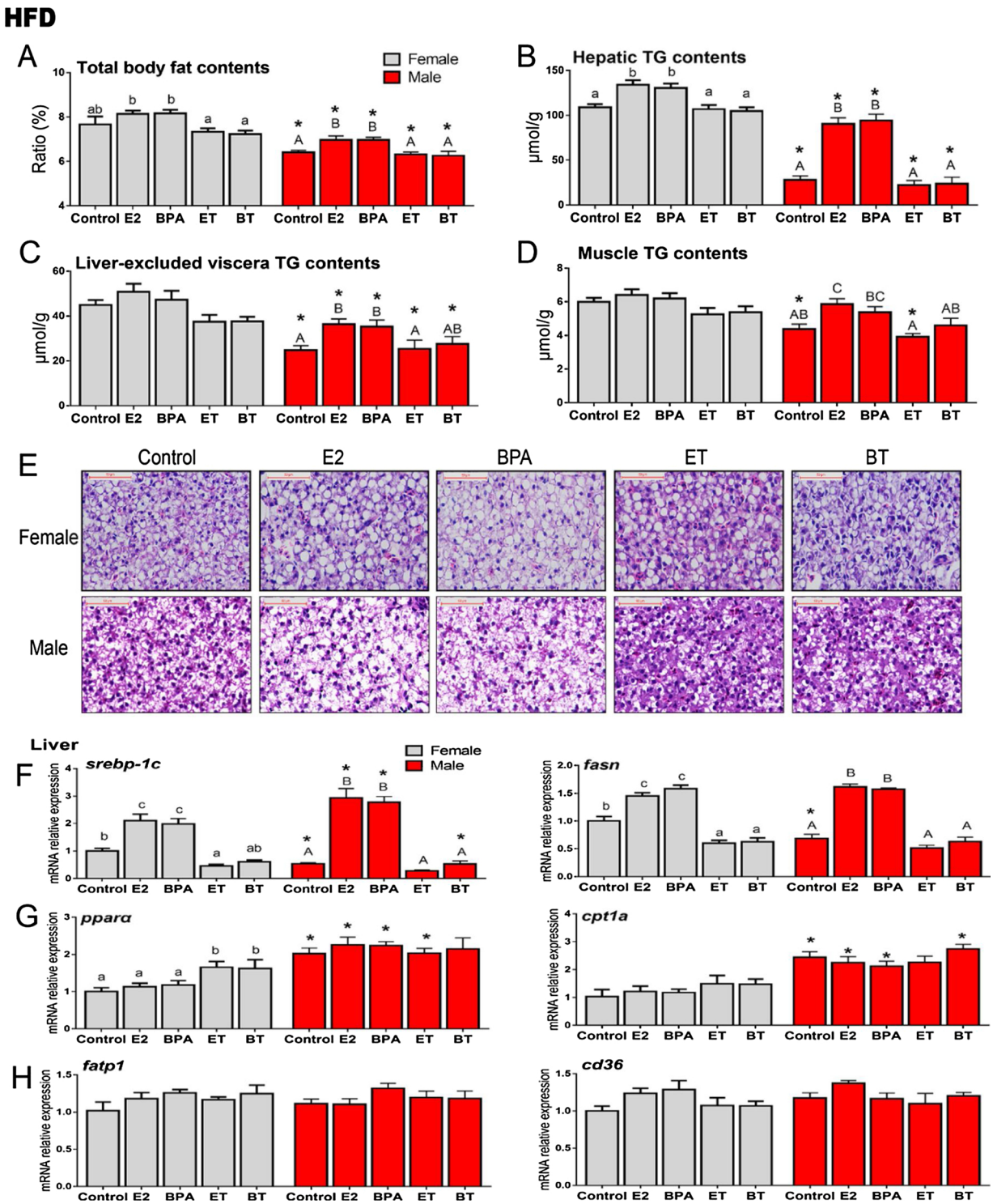


Fig. 3. Alteration of lipid metabolism in the HFD-fed male zebrafish exposed to E2 and BPA. Total body fat contents (**A**), tissue TG contents (**B-D**) and lipid vacuoles in liver histological biopsy (**E**) in the HFD-fed male or female zebrafish exposed to E2, BPA or TAM are presented. Scale bar of photomicrographs represents 50 μm. Gene expressions involved in lipid synthesis (**F**), lipid catabolism (**G**) and lipid transport (**H**) in fish liver are also presented. Data are expressed as means ± SEM (n = 5). Statistical differences between female and male fish fed with the same diet are marked with the asterisk "*" ($P < 0.05$). Statistical differences among exposure treatments in female or male fish are marked with different lowercases or capitals, respectively ($P < 0.05$). In short, these data show that the exposure to E2 and BPA in male fish increased fat deposition and lipogenic gene expressions that mimicked those shown in females.

exposed to E2 and BPA, we performed a hepatic transcriptomic analysis in the fish fed on HFD (control females and males) and males fed on HFD and exposed to E2 and BPA. The genes related to lipid metabolism separated clearly between the control females and males (Fig. 4A). The males exposed to E2/BPA showed similar genes expression to those in the control females (Fig. 4A). Control females had higher expressions of a number of lipogenic genes, including *fasn*, *srebp-1*, *acca*, *scd*, elongation of very-long-chain fatty acids protein 6 (*elovl6*), ATP-citrate synthase (*acly*) and *pparγ* compared to control males (Fig. 4B, C). Moreover, the lipogenesis-related genes, such as *fasn*, *srebp-1*, *acca*, *scd*,

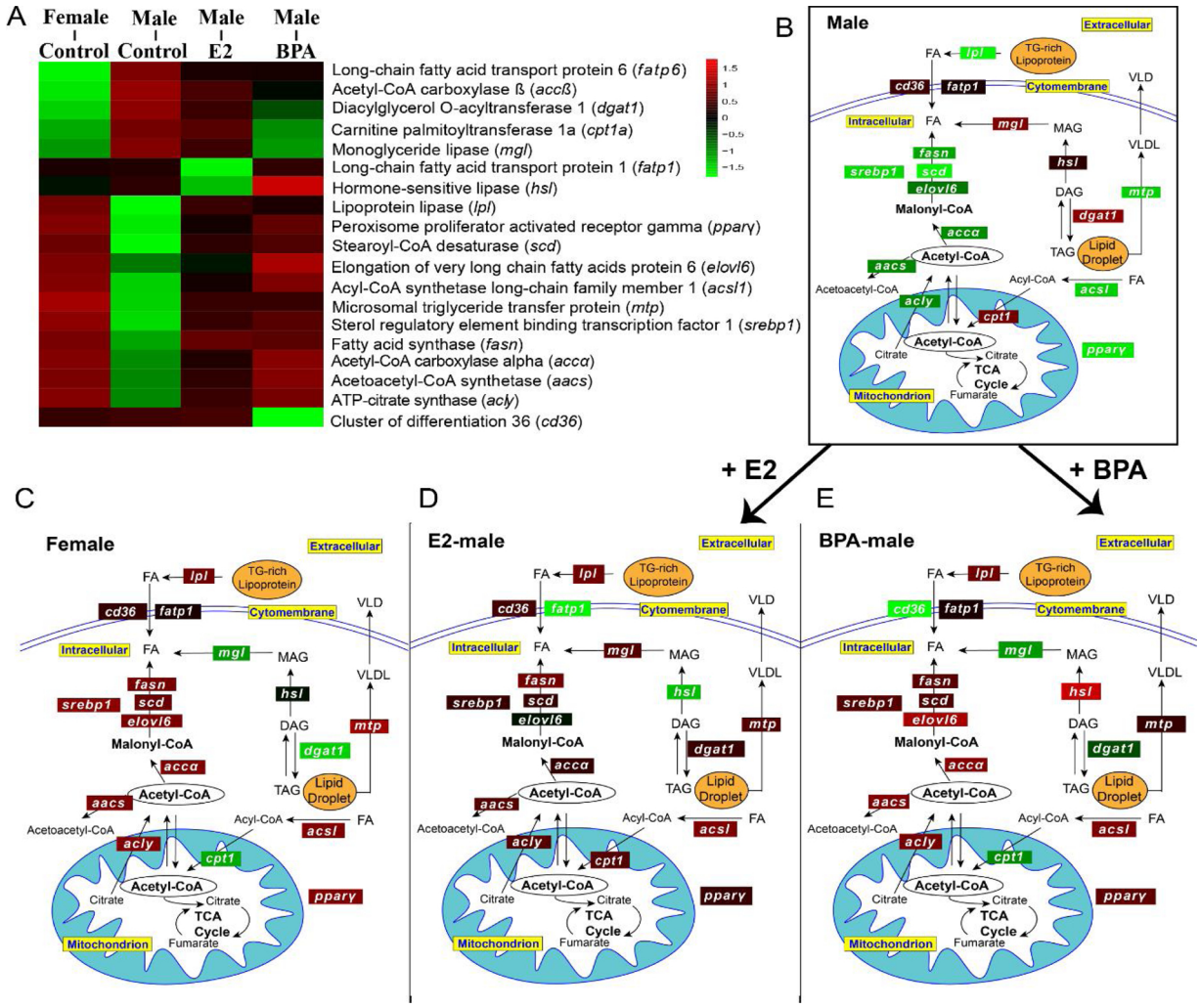


Fig. 4. The female-like patterns of lipid metabolism in the HFD-fed male zebrafish exposed to E2 or BPA. The transcriptomic heatmap of the genes involved in liver lipid metabolism shows that the gene expression pattern in the HFD-fed males exposed to E2 or BPA is similar as that in the female control (A). Systemic pathways of liver lipid metabolism in the male control (B), female control (C), male exposed to E2 (E2-male) (D) or BPA (BPA-male) (E) are presented. Red and green colors in the heatmap and pathways represent the upregulation and downregulation of gene expressions, respectively. The genes of *fasn*, *scd*, *srebp1*, *elovl6* and *accα* participate in the synthesis of fatty acid (FA) from acetyl-CoA. The lipid catabolism from the TG-rich lipoprotein and lipid droplets to FA is modulated by genes *lpl*; *hsl* and *mgl*, respectively. The genes *cd36* and *fatp1* are responsible for the FA transport from extracellular matrix to hepatocytes. The gene *mtp* is involved in the transport of very low density lipoprotein (VLDL) from hepatocytes to extracellular matrix. In short, these data show that E2 and BPA convert lipid metabolic pattern in males to a female pattern at transcript level.

acly and *elovl6*, were mostly activated in males exposed to E2 or BPA (Fig. 4B–E). These data clearly indicate that exposure to environmental estrogens converted the lipid metabolism in male fish to the female pattern.

To investigate the molecular basis of lipid metabolism transformation in male fish exposed to E2 or BPA, we further identified the key signaling pathways involved in estrogen and lipid metabolism in fish fed on HFD. Previous mammalian studies have shown that, E2 modulates lipid metabolism mainly through the mechanistic target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) pathways (Ciruelos, 2014; Palmisano et al., 2017). Signal transducer and activator of transcription 3 (STAT3), which is a direct target for E2, also plays a role in regulating lipid metabolism (Gao et al., 2006; Ueki et al., 2004). In the present study, the female zebrafish had higher lipid synthesis than the control males, through activating the mTOR pathway by upregulating the SREBP-1c and pS6 expressions (Fig. 5A). Nevertheless, pAMPK expression was lower in females compared to control males (Fig. 5A), similar to results obtained in humans (Guadalupe-Grau et al., 2016) and oysters (Guevelou et al., 2013).

The male fish exposed to E2 and BPA decreased the STAT3 gene and protein expressions, which inhibits *srebp-1c* (Ueki et al., 2004), compared to control males (Fig. 5A, B). Notably, male fish exposed to E2 and BPA activated the mTOR-lipogenic pathway manifested by the higher expressions of pS6 and SREBP-1c compared to control males (Fig. 5A). Moreover, the protein expressions of pS6 and SREBP-1c in males exposed to E2 and BPA were similar to those in the control females (Fig. 5A). Correspondingly, the lipogenic genes modulated by SREBP-1c, including *acc* and *fasn* were upregulated in the male fish exposed to E2 and BPA (Fig. 5B). Consequently, E2 and BPA altered the lipid metabolism from the male to the female pattern, mainly through activating the mTOR lipogenic pathway. Of note, although the mTOR pathway was stimulated in males exposed to E2 and BPA, phosphorylated phosphatidylinositol 3-kinase (PI3K) and pAKT, which are the upstream molecules of mTOR, were not upregulated compared to the control males (Fig. 5A). At the same time, the expression of pAMPK, an inhibitor of mTOR phosphorylation decreased dramatically in the male fish exposed to E2 and BPA (Fig. 5A). Taken together, the decline in pAMPK, pPI3K and pAKT expressions suggest that the activated mTOR pathway in males is mainly attributed to AMPK pathway inhibition, and not upregulation of pPI3K and pAKT. We summarized the signaling

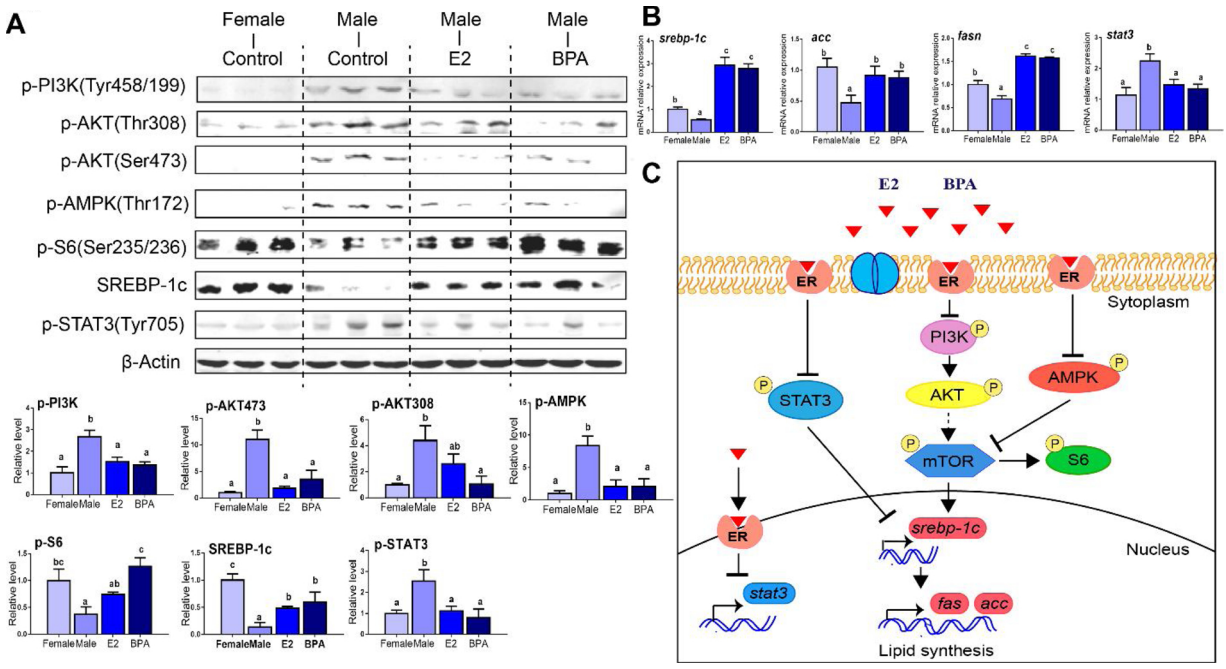


Fig. 5. Verification of cross-pathways connecting lipid metabolism and estrogen actions in the feminization process of the male fish exposed to E2 and BPA. Western blotting (upper image) and quantification of protein (figures below) were used to detect STAT3, mTOR and AMPK pathways in fish liver (n = 3) (A). Relative mRNA expressions of the genes associated with lipid metabolism and in the downstream of STAT3, mTOR and AMPK pathways in fish liver are also presented in (B). The data in (A) and (B) are summarized to show the signaling regulation for metabolic feminization induced by E2 and BPA in male fish (C). Data are expressed as means ± SEM (n = 3). Statistical differences among the four treatments are marked with the different lowercase letters ($P < 0.05$). In short, these data present that the exposure to E2 and BPA in male fish caused lipid accumulation-related metabolic feminization, through lowering AMPK and upregulating the lipogenic mTOR signaling pathways.

pathways connecting the environmental estrogens, ERs, and lipid metabolism in Fig. 5C. All the above data verify that E2 and BPA convert lipid metabolism pattern in males to female pattern via transcriptive and signaling protein levels. The mTOR and AMPK signaling pathways do not only mediate energy homeostasis but also regulate reproductive development and gonad-cell production (Bertoldo et al., 2015; Yaba et al., 2016). Higher AMPK and lower mTOR expressions have been related to nutritional modulation of male gonad and spermatozoa production (Bertoldo et al., 2015; Nguyen, 2017), and benefit male reproduction (Jesus et al., 2017). The present study is the first to show that environmental estrogens convert lipid metabolism from a male pattern to a female pattern, attributed to the AMPK inhibition and mTOR activation signaling pathways.

4. Conclusions

The present results show that patterns of lipid metabolism can differ between female and male fish, whereby the former have a higher capacity for fat deposition than the latter. Exposure to E2 or BPA in male zebrafish not only causes feminization of the male gonads, but also converts the lipid metabolism from a male pattern to a female pattern to accumulate more lipid, especially in liver, through lowering AMPK and upregulating the lipogenic mTOR signaling pathways. This is the first study to show that environmental estrogens could induce metabolic feminization prior to gonad feminization in male fish. Since metabolism is a basic life characteristic related to all physiological processes, future studies on the effects of environmental estrogens should focus on more physiological functions through nutrient metabolism aspects. This approach may provide a more comprehensive understanding of the environmental estrogen effects on living organisms and aquatic ecosystems.

CRediT authorship contribution statement

Sheng-Xiang Sun: Conceptualization, Validation, Formal analysis, Investigation, Visualization, Writing - original draft. **Jun-Lin Wu:** Formal analysis, Investigation. **Hong-Bo Lv:** Formal analysis, Investigation. **Hai-Yang Zhang:** Formal analysis, Investigation. **Jing Zhang:** Data curation. **Samwel Mchele Limbu:** Writing - review & editing. **Fang Qiao:** Writing - review & editing. **Li-Qiao Chen:** Writing - review & editing. **Yi Yang:** Writing - review & editing. **Mei-Ling Zhang:** Writing - review & editing. **Zhen-Yu Du:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

All authors declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

This research was funded by National Key Research and Development Program of China (2018YFD0900400) and National Natural Science Fund (31830102).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.122537.

References

Bertoldo, M.J., Faure, M., Dupont, J., Froment, P., 2015. AMPK: A master energy regulator for gonadal function. Front. Neurosci. 9, 235.

Betancor, M.B., Sprague, M., Sayanova, O., Usher, S., Campbell, P.J., Napier, J.A., Caballero, M.J., Tocher, D.R., 2015. Evaluation of a high-EPA oil from transgenic *Camelina sativa* in feeds for Atlantic salmon (*Salmo salar L.*): effects on tissue fatty acid composition, histology and gene expression. Aquaculture 444, 1–12.
Bligh, E.L.G., Dyer, W.J.A., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Boujard, T., Gélineau, A., Covès, D., Corraze, G., Dutto, G., Gasset, E., Kaushik, S., 2004. Regulation of feed intake, growth, nutrient and energy utilisation in European sea bass (*Dicentrarchus labrax*) fed high fat diets. Aquaculture 231, 0–545.
Canbay, A., Bechmann, L., Gerken, G., 2007. Lipid metabolism in the liver. Z. Gastroenterol. 45, 35.
Ciruelos, E.G., 2014. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat. Rev. 40, 862–871.
Crain, D.A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., Leblanc, G.A., Guillette Jr., , 2007. An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod. Toxicol. 24, 225–239.
Diana, J.S., Mackay, W., 1979. Timing and magnitude of energy deposition and loss in the body, liver, and gonads of northern pike (*Esox lucius*). J. Fish. Board Canada 36, 481–487.
Ferreiraleach, A.M., Hill, E.M., 2001. Bioconcentration and distribution of 4-tert-octylphenol residues in tissues of the rainbow trout (*Oncorhynchus mykiss*). Mar. Environ. Res. 51, 75–89.
Forner-Piquer, I., Mylonas, C.C., Calduch-Giner, J., Maradonna, F., Gioacchini, G., Allarà, M., Piscitelli, F., Marzo, V.D., Pérez-Sánchez, J., Carnevali, O., 2018. Endocrine disruptors in the diet of male *Sparus aurata*: modulation of the endocannabinoid system at the hepatic and central level by di-isononyl phthalate and bisphenol A. Environ. Int. 119, 54–65.
Fukazawa, H., Hoshino, K., Shiozawa, T., Matsushita, H., Terao, Y., 2001. Identification and quantification of chlorinated bisphenol A in wastewater from wastepaper recycling plants. Chemosphere 44, 973–979.
Gao, H., Bryzgalova, G., Hedman, E., Khan, A., Efendic, S., Gustafsson, J.A., Dahlman-Wright, K., 2006. Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: A possible mechanism is through direct regulation of signal transducer and activator of transcription 3. Mol. Endocrinol. 20, 1287.
Gimeno, S., Komen, H., Gerritsen, A.G., Bowmer, T., 1998. Feminisation of young males of the common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during sexual differentiation. Aquat. Toxicol. 43, 77–92.
Grundy, S.M., Brewer Jr, H.B., Cleeman, J.I., Smith Jr, S.C., Lenfant, C., 2004. Definition of metabolic syndrome: Reprot of the national heart, lung, and blood institute/American heart association conference on scientific issues related to definition. Circulation 109, 433–438.
Guadalupe-Grau, A., Rodriguez-Garcia, L., Torres-Peralta, R., Morales-Alamo, D., Ponce-Gonzalez, J.G., Perez-Suarez, I., Santana, A., Calbet, J.A., 2016. Greater basal skeletal muscle AMPKalpha phosphorylation in men than in women: Associations with anaerobic performance. Eur. J. Sport Sci. 16, 455–464.
Guevelou, E., Huvet, A., Galindo-Sanchez, C.E., Milan, M., Quillien, V., Daniel, J.Y., Quere, C., Boudry, P., Corporeau, C., 2013. Sex-specific regulation of AMP-activated protein kinase (AMPK) in the Pacific oyster *Crassostrea gigas*. Biol. Reprod. 89, 100.
Hallgren, P., Nicolle, A., Hansson, L.A., Brönmark, C., Nikoleris, L., Hyder, M., Persson, A., 2014. Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs. Environ. Toxicol. Chem. 33, 930–936.
Hillestad, M., Johnsen, F., Austreng, E., Asgard, T., 1998. Long-term effects of dietary fat level and feeding rate on growth, feed utilization and carcass quality of Atlantic salmon. Aquacult. Nutr. 4, 89–97.
Jesus, T.T., Oliveira, P.F., Sousa, M., Cheng, C.Y., Alves, M.G., 2017. Mammalian target of rapamycin (mTOR): A central regulator of male fertility? Crit. Rev. Biochem. Mol. Biol. 52, 235–253.
Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.
Jørsrgensen, E.H., Johansen, S.J.S., Jobling, M., 1997. Seasonal patterns of lipid deposition and lipid depletion in anadromous Arctic charr. J. Fish Biol. 51, 312–326.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. 104, 8897–8901.
Kime, D.E., Nash, J.P., Scott, A.P., 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177, 345–352.
Kley, H.K., Krüskemper, H.L., 1976. Liver and steroid hormones: the liver as a regulative organ in hormone metabolism. Med. Klin. 71, 799.
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. Aquat. Toxicol. 40, 335–360.
Krivobok, M., Tokareva, G., 1972. Dynamics of the body weight and of some organs of the Baltic cod under maturation of gonads. Trudy VNIRO 85, 46–55.
Kumar, A., Bean, L.A., Rani, A., Jackson, T., Foster, T.C., 2016. Contribution of estrogen receptor subtypes, ERα, ERβ, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. Hippocampus 25, 1556–1566.
Lee, D.H., Porta, M., Jacobs Jr., D.R., Vandenberg, L.N., 2014. Chlorinated persistent organic pollutants, obesity, and type 2 diabetes. Endocr. Rev. 35, 557–601.
Le Magueresse-Battistoni, B., Labaronne, E., Vidal, H., Naville, D., 2017. Endocrine disrupting chemicals in mixture and obesity, diabetes and related metabolic disorders. World J. Biol. Chem. 8, 108–119.
Li, H.Y., Liu, P.W., Wei, H., 2008. Effects of bisphenol-A on expression of estrogen receptor and level of 17 β-eatradiol in vivo of female *Carassius auratus*. J. Shanghai Fish. Univ. 17, 641–646.
Li, D.L., Huang, Y.J., Gao, S., Chen, L.Q., Zhang, M.L., Du, Z.Y., 2019. Sex-specific alterations of lipid metabolism in zebrafish exposed to polychlorinated biphenyls. Chemosphere 221, 768–777.
Limbu, S.M., Zhou, L., Sun, S.X., Zhang, M.L., Du, Z.Y., 2018. Chronic exposure to low environmental concentrations and legal aquaculture doses of antibiotics cause systemic adverse effects in Nile tilapia and provoke differential human health risk. Environ. Int. 115, 205–219.
Lin, Z., Brown, W.C., Qing, C., Andrée, K., Pierre, C., Mcguinness, O.P., Stafford, J.M., 2013. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. Diabetes 62, 424–434.
Lu, D.L., Ma, Q., Wang, J., Li, L.Y., Han, S.L., Limbu, S.M., Li, D.L., Chen, L.Q., Zhang, M.L., Du, Z.Y., 2019. Fasting enhances cold resistance in fish through stimulating lipid catabolism and autophagy. J. Physiol. 597, 1585–1603.
Maisano, M., Cappello, T., Oliva, S., Natalotto, A., Giannetto, A., Parrino, V., Battaglia, P., Romeo, T., Salvo, A., Spanã^2, N., 2016. PCB and OCP accumulation and evidence of hepatic alteration in the Atlantic bluefin tuna, *T. thynnus*, from the Mediterranean Sea. Mar. Environ. Res. 121, 40–48.
Medford, B.A., Mackay, W.C., 1978. Protein and lipid content of gonads, liver, and muscle of northern pike (*Esox lucius*) in relation to gonad growth. J. Fish. Board Canada 35, 213–219.
Mikaelian, I., De, L.Y., Harshbarger, J.C., Lee, L.L., Martineau, D., 2010. Health of lake whitefish (*Coregonus clupeaformis*) with elevated tissue levels of environmental contaminants. Environ. Toxicol. Chem. 21, 532–541.
Nguyen, T.M., 2017. Impact of 5′-amp-activated protein kinase on male gonad and spermatozoa functions. Front. Cell Dev. Biol. 5, 25.
Ortiz-Zarragoitia, M., Bizarro, C., Rojo-Bartolome, I., de Cerio, O.D., Cajaraville, M.P., Cancio, I., 2014. Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments. Mar. Drugs 12, 4756–4782.
Palmisano, B.T., Lin, Z., Stafford, J.M., 2017. Role of estrogens in the regulation of liver lipid metabolism. Sex Gend. Factors Affecting Metab. Homeost. Diabetes Obes. 227–256.
Pottinger, T.G., Carrick, T.R., 2010. Contrasting seasonal modulation of the stress response in male and female rainbow trout. J. Fish Biol. 56, 667–675.
Reshetnikov, Y.S., Paranyushkina, L., Kiyashko, V., 1970. Seasonal changes of blood serum protein composition and fat content in whitefishes. J. Ichthyol. 10, 804–815.
Rljnsdorp, A.D., Ibelings, B., 1989. Sexual dimorphism in the energetics of reproduction and growth of North Sea plaice, *Pleuronectes platessa* L. J. Fish Biol. 35, 401–415.
Romano, M., Limatola, E., 2000. Oocyte plasma membrane proteins and the appearance of vitellogenin binding protein during oocyte growth in the lizard *Podarcis sicula*. Gen. Comp. Endocrinol. 118, 383–392.
Romano, M., Rosanova, P., Anteo, C., Limatola, E., 2004. Vertebrate yolk proteins: a review. Mol. Reprod. Dev. 69, 109–116.
Rose, E., Paczolt, K.A., Jones, A.G., 2013. The effects of synthetic estrogen exposure on premating and postmating episodes of selection in sex-role-reversed Gulf pipefish. Evol. Appl. 6, 1160–1170.
Sara, D.T., Gianpaolo, R., Clara, M., Alessia, S., Pierre, C., Andrée, K., Cristian, I., Paolo, M., Paolo, C., Adriana, M., 2011. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. Cell Metab. 13, 205–214.
Scholz, S., Klüver, N., 2009. Effects of endocrine disrupters on sexual, gonadal development in fish. Sex. Dev. 3, 136–151.
Shore, L.S., Shemesh, M., 2016. Estrogen as an environmental pollutant. Bull. Environ. Contam. Toxicol. 97, 447–448.
Shrestha, S.L., Casey, F.X.M., Hakk, H., Smith, D.J., Padmanabhan, G., 2012. Fate and transformation of an estrogen conjugate and its metabolites in agricultural soils. Environ. Sci. Technol. 46, 11047–11053.
Soler-Argilaga, C., Heimberg, M., 1976. Comparison of metabolism of free fatty acid by isolated perfused livers from male and female rats. J. Lipid Res. 17, 605–615.
Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, 173–178.
Sun, S.X., Zhang, Y.N., Lu, D.L., Wang, W.L., Limbu, S.M., Chen, L.Q., Zhang, M.L., Du, Z.Y., 2019. Concentration-dependent effects of 17β-estradiol and bisphenol A on lipid deposition, inflammation and antioxidant response in male zebrafish (*Danio rerio*). Chemosphere 237, 124422.
Ueki, K., Kondo, T., Tseng, Y.H., Kahn, C.R., 2004. Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. Proc. Natl. Acad. Sci. U. S. A. 101, 10422–10427.
Viganò, L., Arillo, A., Bottero, S., Massari, A., Mandich, A., 2001. First observation of intersex cyprinids in the Po River (Italy). Sci. Total Environ. 269, 189–194.
Wang, H., Tan, J.T., Emelyanov, A., Korzh, V., Gong, Z., 2005. Hepatic and extrahepatic expression of vitellogenin genes in the zebrafish, *Danio rerio*. Gene 356, 91–100.
Watkins, M.L., Fizette, N., Heimberg, M., 1972. Sexual influences on hepatic secretion of triglyceride. Biochim. Biophys. Acta 280, 82–85.
Yaba, A., Bozkurt, E.R., Demir, N., 2016. mTOR expression in human testicular seminoma. Andrologia 48, 702–707.
Ye, A.L., Yang, Y., Zhang, J., Liu, M., Hou, L.J., Zhou, J.L., 2013. Simultaneous determination of steroidal and phenolic endocrine disrupting chemicals in fish by ultra-high-performance liquid chromatography–mass spectrometry/mass spectrometry. J. Chromatogr. A 1278, 126–132.
Yin, G.G., Kookana, R.S., Ru, Y.J., 2003. Occurrence and fate of hormone steroids in the environment. Environ. Int. 28, 545–551.
Yokota, H., Tsuruda, Y., Maeda, M., Oshima, Y., Tadokoro, H., Nakazono, A., Honjo, T., Kobayashi, K., 2000. Effect of bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 19, 1925–1930.
Yoon, Y., Westerhoff, P., Snyder, S.A., Esparza, M., 2003. HPLC-fluorescence detection and adsorption of bisphenol A, 17β-estradiol, and 17α-ethynyl estradiol on powdered activated carbon. Water Res. 37, 3530–3537.