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Effects of Bisphenol S Exposure on Endocrine Functions and Reproduction of Zebrafish

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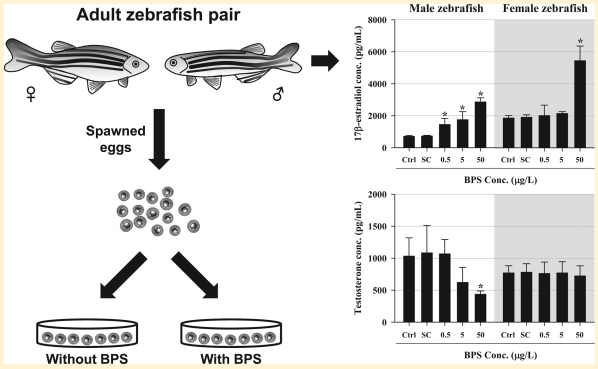
Supporting Information

ABSTRACT: While bisphenol S (BPS) has been frequently detected both in environment and biota, limited information is available on their effects of endocrine system. In the present study, adult zebrafish pairs were exposed to 0.5, 5, and 50 μg/L of BPS for 21 d, and the effects on reproduction, sex steroid hormones, and transcription of the genes belonging to the hypothalamic-pituitary-gonad (HPG) axis were investigated. The adverse effects on performances of F1 generation were further examined with or without subsequent exposure to BPS. Egg production and the gonadosomatic index in female fish were significantly decreased at ≥0.5 μg/L BPS. Plasma concentrations of 17β-estradiol were significantly increased in both male and female fish. In male fish, however, significant decreases of testosterone concentration were observed along with up-regulation of *cyp19a* and down-regulation of *cyp17* and *17βhsd* transcripts. Parental exposure to BPS resulted in delayed and lesser rates of hatching even when they were hatched in clean water. Continuous BPS exposure in the F1 embryos resulted in worse hatchability and increased malformation rates compared to those without BPS exposure. Our observations showed that exposure to low level BPS could affect the feedback regulatory circuits of HPG axis and impair the development of offspring.



INTRODUCTION

Bisphenols have been used in the manufacturing of polycarbonate plastics and epoxy resin. Among these compounds, bisphenol A (BPA) has been frequently used in numerous commercial applications and has been produced over eight billion pounds per year worldwide.[1] BPA can act as a weak estrogen receptor agonist and has been associated with a variety of adverse human health outcomes[2,3] as well as adverse effects on reproduction and development in laboratory animals.[4,5] In 2011, the European Commission applied the precautionary principle on bisphenol A and restricted its use in infant feeding bottles.[6] In response to this restriction, bisphenol S (BPS; 4,4′-sulfonyldiphenol) has been often used as a component of plastic substitutes for the production of baby bottles.[7]

BPS has been detected in both products and biota: e.g., thermal receipt paper, currency bill, and airplane luggage tags,[8] canned foodstuffs,[9] and human urine samples.[1] This compound has been also detected in an environment such as river water up to 3 μg/L.[10] BPS appears to be more resistant to environmental degradation compared to BPA.[11] However, there is only limited information available on their toxicological effects; previous studies have shown that BPS is acutely toxic to *Daphnia magna*,[12] antiandrogenic activity,[13] and estrogenic activity.[7]

Reproductive processes in fish are regulated by coordinated interactions among steroid hormones along the hypothalamic-pituitary-gonad (HPG) axis and by steroidogenesis of gonad tissues.[14] Therefore, environmental contaminants that can affect expression of steroidogenic genes and concentrations of hormones in this axis could affect the function of endocrine system and possibly reproduction of fish.[15] Previous experiments confirmed that BPA induced strong brain-specific overexpression of aromatase (*cyp19b*) mRNA in zebrafish.[16] Significant inhibition of gonadosomatic index (GSI) and egg production and reduced hatchability in the F1 generation were observed at a BPA concentration of ≥640 μg/L.[17] In addition, maternal transfer of BPA has previously been observed in various species, such as fish,[18] mice,[19] and humans.[20] However, the potential effects of BPS on reproduction and transgenerational toxicity have not yet been elucidated.

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To fill this knowledge gap, we investigated the endocrine disrupting effects of BPS on HPG axis and reproduction of adult zebrafish. The effects on the performances of F1 generation were further examined with or without subsequent exposure to BPS at the same concentration.

2. MATERIAL AND METHODS

2.1. Test Chemicals and Instrumental Analysis. BPS (CAS No. 80-09-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). BPS was dissolved in methanol (MeOH), and the final MeOH concentration in the exposure water was 0.1% (v/v).

To measure actual concentrations of the exposure media, water samples were collected from each tank for 3 times during the 21 d exposure period. The samples were analyzed for BPS by following the methods described elsewhere,[1] with some modifications. Briefly, water samples were spiked with the standard and then injected in LC-MS/MS systems before being filtered with nylon syringe filter. An API 4000 electrospray triple quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA, USA), coupled with a high-performance liquid chromatography (HPLC; Agilent 1100 Series, Agilent Technologies, Palo Alto, CA, USA) was used for identification and quantification of BPS (For details, see Supporting Information; Figure S1 and Tables S1 and S2.). The detection limit for BPS was 0.1 μg/L.

2.2. Fish Maintenance, Exposure and Sampling. Male and female adult zebrafish (AB-type, 3−4 mo old) were acclimated in aquaria filled with carbon-filtered water for >2 weeks at 25 ± 1 °C under a photoperiod of 16:8 h light/dark. After the acclimation period, fish were exposed to control, vehicle control (MeOH with a final concentration of 1:1,000 v/v water), 0.5, 5, or 50 μg/L of BPS for 21 d following OECD test guideline 229 with minor modifications.[21] Four male and six female fish were placed in a test aquarium (7.5 L filled in 6 L exposure medium), and two replicate aquaria were allocated per each treatment or control. Exposure medium was prepared in dechlorinated carbon-filtered water and was replaced with freshly prepared medium every 2 d. During the exposure period, eggs were counted and recorded daily. Adult fish were fed twice daily with freshly hatched *Artemia* nauplii. Exposure medium was routinely monitored for pH, temperature, and dissolved oxygen. No mortalities were observed at any treatment during the exposure period. After the exposure, fish were euthanized in 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), and total weight and snout-to-vent length were recorded for each fish. Indices including condition factor (K; 100 × total wt/total length^3), brain-somatic index (BSI; 100 × brain wt/body wt), hepatosomatic index (HSI; 100 × liver wt/body wt), and GSI (100 × gonad wt/body wt) were calculated. For hormone and gene expression analysis, 4 males and females were randomly sampled from each BPS treatment.

On the 16th d of the fish exposure, the fertilized eggs were collected from each aquarium. Then thirty eggs were randomly selected per each aquarium and individually placed in 48-well plate containing 1 mL exposure medium of the same concentration or clean water (control) until 6 d post-fertilization (dpf). Hatchability, time to hatch, and malformation rates were determined from this F1 generation. The developmental status of zebrafish was observed with a microscope (× 5 magnifications, Nikon Eclipse LV100D-U, Nikon Inc., Melville, NY, USA).

2.3. Hormone Measurement. After exposure, blood sample was collected from caudal vein of the fish using a glass capillary tube. Five microliters of blood per each fish was centrifuged at 5,000 × g for 20 min, and the supernatant was stored at −80 °C. Before the hormone measurement, the blood sample was extracted for plasma. Briefly, the supernatant sample was diluted to 400 μL with UltraPure water and extracted twice with 2 mL of diethyl ether at 2,000 × g for 10 min. The solvent used to extract hormones was evaporated under a stream of nitrogen, and the residues were dissolved in 120 μL of ELISA buffer. E2 (Cat No. 582251) and T (Cat No. 582701) were quantified by use of an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer's instructions.

2.4. Real-Time Polymerase Chain Reaction (PCR) Assay. Samples of brain and gonads of each fish were collected after 21 d of exposure and were measured for transcription of 21 genes representing key signaling pathways and functional processes of the HPG axis plus one housekeeping gene (*β-actin*) were measured (For the full name of the genes and the sequences of primers for the genes measured, refer to Supporting Information Tables S3 and S4.). Twenty-one d was determined as an appropriate time for sampling fish for gene transcription analysis, based on a preliminary experiment (For details refer to Supporting Information Figure S2.). Samples of each organ were preserved in RNAlater storage solution (QIAGEN, Korea Ltd., Seoul, Korea) at −80 °C until analysis. Total RNA was isolated from the sample by use of RNeasy mini-kit (QIAGEN), and RNA samples (100 ng RNA of male and female brain, 100 ng RNA of male gonad, and 1 μg RNA of female gonad) were used for reverse transcription using iScript cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). The ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. PCR reaction mixtures (15 μL) contained 1.35 μL (0.9 μM) of forward and reverse primers, 2 μL of cDNA sample, and 7.5 μL of 2 × SYBR Green PCR master mix (Applied Biosystems). The thermal cycle profile was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing together with extension for 1 min at 60 °C. For each sample a dissociation step was performed at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s at the end of the amplification phase to ensure amplification of a single product. Efficiency of each qPCR assay was assessed by construction of standard curves by use of serially diluted cDNA standards. For quantification of PCR results, the threshold cycle (*Ct*) was determined for each reaction. *Ct* values for each gene of interest were normalized to the endogenous control gene, *β-actin*, by using the $^{\Delta\Delta}Ct$ method.[22] Using geNorm analysis, our studies demonstrated that *β-actin* is the most stable gene among the six commonly used housekeeping genes (*β-actin*, *tubulin alpha 1* (*tuba1*), *glyceraldehydes-3-phosphate dehydrogenase* (*gapdh*), *elongation factor 1-alpha* (*elfa*), *18s rRNA* (*18S rRNA*), and *ribosomal protein L8* (*rpl8*)) in both male and female brain and gonad. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values.

2.5. Data Analysis. Normality of distribution and homogeneity of variance of each sample set were assessed with Kolomogrov-Smirnov test and Levene's test, respectively. If necessary, data were log-transformed to approximate

Table 1. Effects on Somatic Indices of Zebrafish after 21 d Exposure to Bisphenol S[e]

	male					female				
group	*N*	*K*[a]	BSI[b]	HSI[c]	GSI[d]	*N*	*K*[a]	BSI[b]	HSI[c]	GSI[d]
control	8	8.74 ± 1.94	1.16 ± 0.18	1.19 ± 0.30	1.13 ± 0.26	12	9.16 ± 1.15	1.14 ± 0.24	1.32 ± 0.20	14.00 ± 1.62
MeOH	8	9.08 ± 1.48	1.14 ± 0.14	1.24 ± 0.19	1.09 ± 0.24	12	9.28 ± 0.94	1.16 ± 0.18	1.39 ± 0.10	14.01 ± 0.77
BPS 0.5 μg/L	8	8.76 ± 1.33	1.14 ± 0.10	1.19 ± 0.28	0.89 ± 0.14	12	9.31 ± 0.94	1.21 ± 0.17	1.33 ± 0.22	11.41 ± 2.18*
BPS 5 μg/L	8	8.26 ± 1.21	1.17 ± 0.18	1.21 ± 0.12	0.90 ± 0.11	12	9.06 ± 1.53	1.10 ± 0.29	1.41 ± 0.16	9.74 ± 1.05*
BPS 50 μg/L	8	8.32 ± 1.93	1.05 ± 0.26	1.20 ± 0.33	0.83 ± 0.16*	12	9.40 ± 1.48	0.99 ± 0.16	1.49 ± 0.20	8.94 ± 2.00*

[a]K = weight (g)/snout−vent length (cm)^3 × 100. [b]BSI = brain weight ×100/body weight. [c]HSI = liver weight ×100/body weight. [d]GSI = gonad weight ×100/body weight. [e]The values are mean ± standard deviation. Asterisk indicates significant difference between exposure and control group.

normality. Differences between control and exposure groups were evaluated by Dunnett's one-way analysis of variance (ANOVA). To evaluate dose−response relationship in somatic indices, plasma hormones, and expression of mRNA, the linear regression analysis was conducted using IBM SPSS Statistic 19 (IBM Corp., New York, USA). The viability of the F1 generation (hatchability, time to hatch, and malformation rates) between exposure water group and clean water group were compared by independent t test.

To identify which gene factors were most responsible for the changes observed in steroid hormones and consequently reproduction, the following statistics were performed. First, Spearman correlation analysis was performed to determine the bivariate correlation between 21 gene transcriptions in the HPG axis. Principle component analysis (PCA) was used to transform a number of potentially correlated independent variables (gene transcriptions) into a smaller number of uncorrelated variables called "principal components (PCs)". The first two PCs identified were then used in regression analysis to evaluate the relationship between gene transcriptions and hormone concentrations. P-values less than 0.05 were considered significant. Statistical analyses were carried out using SAS (Version 9.2, SAS Institute, Cary, NC, USA) and IBM SPSS Statistic 19 (IBM Corp.).

3. RESULTS

3.1. Chemical Analysis. The mean measured concentrations during the exposure period ranged from 80% to 110% of the nominal concentrations of BPS (Table S5). Since good agreement existed between the nominal and actual exposure concentrations, subsequent analyses of biological effects were based on nominal concentrations for simplicity.

3.2. Survival and Organism Level Changes of Adult Fish. The effects of BPS on K, BSI, HSI, and GSI of adult zebrafish are summarized in Table 1 and Figure S3. There were no tank effects between two replicate tanks in terms of tested somatic indices. The GSI in both male and female fish were significantly decreased at 50 μg/L and ≥0.5 μg/L BPS, respectively, in a dose−response manner (β = −0.513 in males and β = −0.762 in females, $p < 0.05$). In females, BSI and HSI exhibited slight decrease (β = −0.235, $p < 0.05$) and increase (β = 0.262, $p < 0.05$), respectively, but were not statistically significant in ANOVA statistics. K, BSI, and HSI in males were not influenced at the range of concentrations tested in the present study.

3.3. Concentrations of Hormones in F0 Fish. Concentrations of plasma E2 were significantly increased in both male and female fish exposed to ≥0.5 μg/L and 50 μg/L BPS, respectively (Figures 1A and S4). Concentrations of plasma T were significantly decreased in males exposed to 50 μg/L BPS, while no significant difference was observed in females (Figures 1B and S4). The E2 to T ratio (E2/T) was significantly increased at ≥0.5 μg/L and 50 μg/L BPS in male and female fish, respectively (Figures 1C and S4).

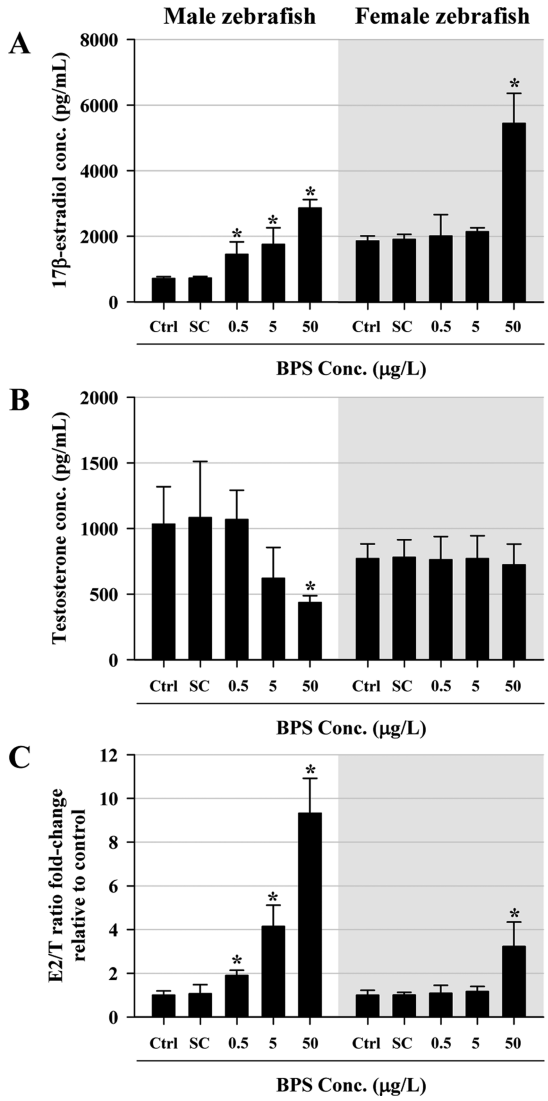

Figure 1. Effects of bisphenol S on (A) 17β-estradiol (E2) concentration, (B) testosterone (T) concentration, and (C) E2/T ratio in plasma. The results are shown as mean ± standard deviation of four fish replicates. Asterisk indicates significant difference from control ($p < 0.05$).

3.4. mRNA Expression of Genes in F0 Fish. The transcriptions of the genes of HPG axis were affected by exposure to BPS (Table 2, Figures S5 and S6). In males, expressions of *gnrh3*, *gnrhr1*, *gnrhr2*, *fshβ*, *lhβ*, and *cyp19b*

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Table 2. Transcriptional Response Profiles of the Genes of Hypothalamic-Pituitary-Gonad Axis in Male and Female Zebrafish after 21 d Exposure to Bisphenol S[a]

sex	tissue	gene	control	MeOH	BPS 0.5 μg/L	BPS 5 μg/L	BPS 50 μg/L
male	brain	*gnrh2*	1.00 ± 0.28	1.03 ± 0.24	0.92 ± 0.19	1.23 ± 0.32	1.32 ± 0.22
		gnrh3	1.00 ± 0.30	0.93 ± 0.12	1.07 ± 0.27	1.53 ± 0.07	2.22 ± 0.81*
		gnrhr1	1.00 ± 0.36	1.17 ± 0.51	1.46 ± 0.46	1.36 ± 0.26	1.80 ± 0.18*
		gnrhr2	1.00 ± 0.14	0.91 ± 0.31	0.91 ± 0.36	1.72 ± 0.34	2.19 ± 0.60*
		gnrhr4	1.00 ± 0.23	0.99 ± 0.21	0.86 ± 0.34	0.94 ± 0.36	0.97 ± 0.42
		fshβ	1.00 ± 0.08	1.01 ± 0.35	1.35 ± 0.38	1.24 ± 0.51	2.09 ± 0.69*
		lhβ	1.00 ± 0.10	1.14 ± 0.25	1.56 ± 0.45	2.01 ± 0.71	2.32 ± 0.42*
		cyp19b	1.00 ± 0.16	0.96 ± 0.13	1.04 ± 0.42	2.23 ± 0.52	2.65 ± 0.24*
		erα	1.00 ± 0.36	1.08 ± 0.31	0.83 ± 0.14	0.66 ± 0.15	0.53 ± 0.22
		er2β	1.00 ± 0.07	0.98 ± 0.29	0.90 ± 0.60	0.82 ± 0.20	0.73 ± 0.33
		ar	1.00 ± 0.13	0.88 ± 0.18	0.98 ± 0.30	1.09 ± 0.35	0.95 ± 0.41
	gonad	*fshr*	1.00 ± 0.13	1.07 ± 0.43	1.01 ± 0.45	1.39 ± 0.73	2.65 ± 0.41*
		lhr	1.00 ± 0.09	1.04 ± 0.32	2.59 ± 0.90	2.89 ± 0.99	3.18 ± 1.00*
		hmgra	1.00 ± 0.46	1.21 ± 0.28	1.24 ± 0.43	2.19 ± 0.35	2.29 ± 0.29*
		hmgrb	1.00 ± 0.15	1.20 ± 0.45	1.33 ± 0.17	1.75 ± 0.50	1.96 ± 0.78*
		star	1.00 ± 0.20	0.91 ± 0.26	0.81 ± 0.22	0.67 ± 0.35	0.53 ± 0.19
		cyp11a	1.00 ± 0.31	1.14 ± 0.45	1.17 ± 0.07	1.60 ± 0.38	2.37 ± 0.92*
		3βhsd	1.00 ± 0.30	1.16 ± 0.22	1.60 ± 0.54	1.65 ± 0.62	2.07 ± 0.77*
		cyp17	1.00 ± 0.05	1.05 ± 0.21	1.37 ± 0.25	1.14 ± 0.48	0.47 ± 0.10*
		17βhsd	1.00 ± 0.16	1.10 ± 0.23	0.54 ± 0.18	0.51 ± 0.18	0.39 ± 0.13*
		cyp19a	1.00 ± 0.35	1.00 ± 0.19	1.54 ± 0.79	1.65 ± 0.39	2.81 ± 0.69*
female	brain	*gnrh2*	1.00 ± 0.16	1.10 ± 0.52	1.14 ± 0.49	0.93 ± 0.51	0.53 ± 0.22
		gnrh3	1.00 ± 0.29	1.05 ± 0.25	1.29 ± 0.16	0.80 ± 0.35	0.49 ± 0.18*
		gnrhr1	1.00 ± 0.25	0.98 ± 0.31	1.10 ± 0.54	1.30 ± 0.43	0.85 ± 0.68
		gnrhr2	1.00 ± 0.36	1.09 ± 0.40	1.29 ± 0.17	1.06 ± 0.05	0.62 ± 0.21
		gnrhr4	1.00 ± 0.40	1.10 ± 0.44	1.22 ± 0.33	1.28 ± 0.31	0.58 ± 0.19
		fshβ	1.00 ± 0.10	1.26 ± 0.19	0.96 ± 0.33	0.68 ± 0.26	0.46 ± 0.22*
		lhβ	1.00 ± 0.25	1.10 ± 0.16	0.96 ± 0.30	0.86 ± 0.27	0.73 ± 0.36
		cyp19b	1.00 ± 0.13	1.28 ± 0.31	0.90 ± 0.18	0.85 ± 0.44	0.61 ± 0.28
		erα	1.00 ± 0.38	1.05 ± 0.79	0.99 ± 0.41	0.76 ± 0.20	0.44 ± 0.17
		er2β	1.00 ± 0.47	1.00 ± 0.70	1.19 ± 0.50	1.25 ± 0.23	0.98 ± 0.52
		ar	1.00 ± 0.14	1.13 ± 0.62	1.15 ± 0.21	1.27 ± 0.28	0.99 ± 0.52
	gonad	*fshr*	1.00 ± 0.26	1.15 ± 0.28	1.24 ± 0.52	1.16 ± 0.51	1.40 ± 0.39
		lhr	1.00 ± 0.26	1.01 ± 0.36	1.10 ± 0.27	1.19 ± 0.78	1.34 ± 0.49
		hmgra	1.00 ± 0.14	1.02 ± 0.30	0.46 ± 0.36	0.38 ± 0.12	0.28 ± 0.21*
		hmgrb	1.00 ± 0.31	1.30 ± 0.37	1.43 ± 0.30	0.53 ± 0.21	0.44 ± 0.22*
		star	1.00 ± 0.26	1.06 ± 0.21	1.29 ± 0.46	1.09 ± 0.30	0.65 ± 0.16
		cyp11a	1.00 ± 0.12	1.08 ± 0.24	1.22 ± 0.16	0.72 ± 0.43	0.86 ± 0.35
		3βhsd	1.00 ± 0.27	1.18 ± 0.16	1.17 ± 0..04	1.06 ± 0.33	1.20 ± 0.30
		cyp17	1.00 ± 0.40	1.09 ± 0.51	1.14 ± 0.26	0.93 ± 0.16	0.61 ± 0.11
		17βhsd	1.00 ± 0.11	1.13 ± 0.27	1.17 ± 0.25	0.83 ± 0.43	0.99 ± 0.37
		cyp19a	1.00 ± 0.22	1.11 ± 0.14	1.44 ± 0.52	0.96 ± 0.45	1.07 ± 0.09

[a]mRNA expression is expressed as the fold change compared to that of the control. The results are shown as mean ± standard deviation of four biological replicate samples. Asterisk indicates significant difference from control ($p < 0.05$).

mRNA in brain and *fshr*, *lhr*, *hmgra*, *hmgrb*, *cyp11a*, *3βhsd*, and *cyp19a* mRNA in testis were significantly up-regulated by exposure to BPS. Expressions of *cyp19b* and *cyp19a* mRNA in male fish exposed to 50 μg/L BPS were greater by 2.65-fold ($\beta = 0.837$, $p < 0.05$) and 2.81-fold ($\beta = 0.750$, $p < 0.05$), respectively, compared to the expressions in control male fish. Expressions of *cyp17* and *17βhsd* mRNAs in male zebrafish exposed to 50 and ≥0.5 μg/L BPS were significantly lesser by 0.47-fold and 0.39-fold, respectively, compared to control male fish. In females, significant down-regulation of *gnrh3* ($\beta = -0.515$, $p < 0.05$) and *fshβ* ($\beta = -0.688$, $p < 0.05$) mRNAs in brain were observed in fish exposed to 50 μg/L BPS. Expressions of *hmgra* ($\beta = -0.771$, $p < 0.05$) and *hmgrb* ($\beta = -0.568$, $p < 0.05$) mRNA in ovary from female fish exposed to BPS were significantly down-regulated in a dose−response manner.

The relationship between gene transcriptions and sex steroid hormone concentrations in male and female fish was evaluated. Since several genes were highly correlated with each other among the 21 genes investigated ($r > 0.5$, $p < 0.01$, Supporting Information Table S6), PCA was used to reduce the number of independent variables to fewer factors (PCs). PCA of gene transcription profiles in male fish showed that PC1 and PC2 account for 47% and 15% of the total variances, while in female fish PC1 and PC2 account for 29% and 14%, respectively (Figure 2, Supporting Information Table S7). The first component (PC1) was highly influenced by variables such as *cyp19a*, *cyp19b*, *gnrh3*, *gnrhr2*, *lhβ*, *lhr*, *hmgra*, *hmgrb*, and

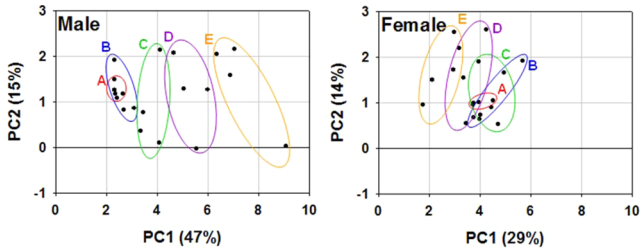

Figure 2. Plot of the first two factors of the principal component (PC) analysis of gene transcription along the hypothalamic-pituitary-gonad axis. Each dot represents PC scores along PCs 1 and 2 for each fish. The clusters grouped as red (A), blue (B), green (C), purple (D), and orange ovals (E) represent the fish from control, solvent control, bisphenol S (BPS) 0.5 μg/L, BPS 5 μg/L, and BPS 50 μg/L, respectively.

cyp11a and was significantly correlated with the concentrations of E2 ($\beta = 0.286$, $p < 0.0001$) and T ($\beta = -0.221$, $p = 0.007$) in male fish (Supporting Information Table S7). In females, PC1 ($\beta = -0.291$, $p < 0.0001$) and PC2 ($\beta = 0.225$, $p = 0.0145$) were significantly correlated with the concentration of E2 but not that of T. The PCA found that three concentrations of BPS clustered along PC1 separately from each other in male and female zebrafish.

3.5. Reproduction Performance and F1 Generation Effects. The average number of eggs spawned was significantly less upon the exposure to ≥0.5 μg/L BPS (Figure 3A). Parental exposure to 50 μg/L and ≥5 μg/L of BPS resulted in delayed and lesser rates of hatching of eggs, respectively, even when the eggs were transferred to clean water (Figure 3B and 3C). Continuous BPS exposure during the hatching period resulted in reduced hatchability and increased malformation rates compared to those which did not receive BPS exposure during the hatching period (Figure 3B and 3D). Phenotypic observation revealed that subsequent exposure to BPS in F1 generation caused cardiac edema, shortened tails, and severe spinal kyphosis (Figure 3E). The embryos that did not hatch within 6 dpf were dead.

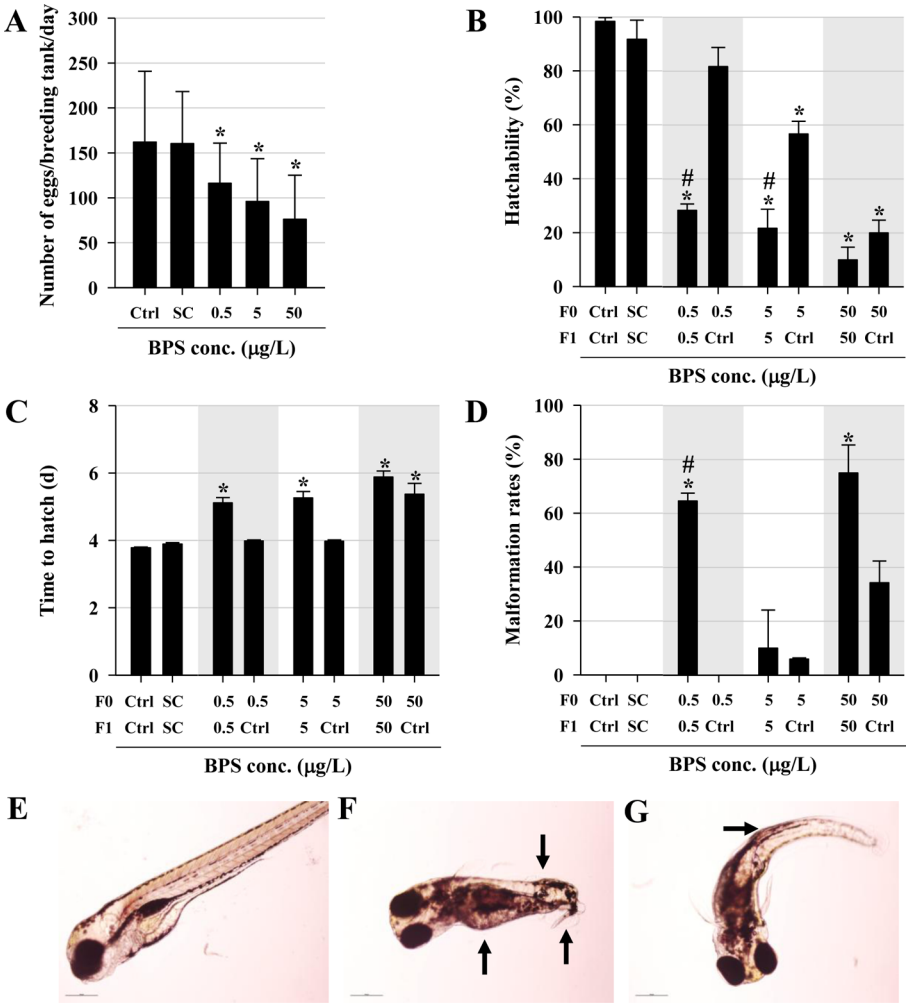

Figure 3. Reproductive performances of F0 fish and the toxic effects in offspring after maternal exposure to bisphenol S (BPS). (A) Number of eggs/breeding tank/day, (B) hatchability (%), (C) time to hatch (day), (D) malformation rate (%), and phenotypic changes in F1 larvae at 120 h post fertilization (E: control larva fish, F: larva fish continuously exposed to 50 μg/L BPS with kyphosis, cardiac edema, and shortened tails, G: larva fish continuously exposed to 0.5 μg/L BPS with kyphosis). The results are shown as mean ± standard deviation of four fish replicates. Asterisk (*) indicates significant difference from control and # indicates significant difference between continuous exposure group and without exposure group in F1 generation ($p < 0.05$). Arrows indicate morphological malformations observed in larvae. SC: solvent control (MeOH).

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4. DISCUSSION

The results of our study demonstrate that BPS causes reproductive dysfunction and alters plasma sex hormone levels as well as gene transcription in the HPG axis in adult zebrafish. Up-regulation of *gnrh3*, *gnrhr1*, and *gnrhr2* genes in male zebrafish suggests that BPS could modulate concentrations of GnRHs in fish, which could subsequently affect production of gonadotropin hormones. In vertebrates, gonadotropin-releasing hormone (GnRH) has a crucial role on the control of reproduction through HPG axis[23] and regulates the synthesis and release of gonadotropin hormone. Zebrafish have two types of GnRH (GnRH2 and GnRH3) and four different GnRH receptors (GnRHR).[24] The up-regulation of *fshβ*, *lhβ*, *fshr*, and *lhr* genes in male observed in the present study corresponds well with the fact that BPS can indirectly affect gonadotropin hormones.

Measurement of sex steroid hormones has been suggested to be one of the most integrative, functional end points for reproduction and has been conducted previously in zebrafish.[14] In the present study, significantly greater production of E2 and lesser production of T were observed after exposure to BPS in male zebrafish, and this was accompanied by up-regulation of the aromatase (*cyp19a* and *cyp19b*) genes and down-regulation of *cyp17* and *17βhsd* genes. Significant decrease of T has also been previously reported in fish exposed to BPA, but the effective concentrations were similar or up to 20-fold greater than those of our study, e.g., decrease of T was observed in mature male common carp exposed to 1000 μg/L BPA for 14 d,[25] in juvenile turbot (*Psetta maxima*) exposed to 59 μg/L BPA for 21 d,[26] and in larvae brown trout (*Salmo trutta*) exposed to 50 μg/L BPA for 63 d.[27] These results suggest that the endocrine disruption potential of BPS is no less than that of BPA. In addition, a significantly greater E2/T ratio was observed in both male and female zebrafish after exposure to BPS, indicating that balance of sex hormones was disrupted by BPS. Altered sex hormone balance could further result in adverse effects on gametogenesis, sexual development, or reproduction in fish. The changes in sex hormones, e.g., increase of E2 and decrease of T concentrations, are well supported by significant up-regulation of *cyp19a* and *cyp19b* genes that were observed in the present study. Aromatase enzyme (cyp19) catalyzes the final step in conversion of androgen to estrogen, and the mRNA level of *cyp19* is well correlated with activity of aromatase enzyme.[28]

Interestingly, the adverse effects of BPS on hormone levels as well as gene transcriptions were sex dependent, with males being more sensitive than females. In females, significantly greater concentrations of E2 were observed at 50 μg/L BPS, while no significant differences were observed in plasma T concentration and gonadal transcription of *cyp19a* gene. One explanation for this observation is that BPS has the potential to increase E2 in female fish by other mechanisms, e.g., inhibition of E2 metabolism, rather than enhancement of aromatase activity. Sulfotransferase (SULT) enzymes may inactivate E2 by sulfonation,[29] and it has been shown that BPA might increase estrogenicity as a result of inhibition of estrogen sulfotransferase activity.[30]

The change in sex steroid hormone levels may cause subsequent reproductive dysfunction by interfering with the regulatory mechanisms of the HPG axis.[19] Our observation that the altered plasma levels of E2 and T were accompanied by significantly reduced egg production in the zebrafish exposed to ≥0.5 μg/L BPS supports this hypothesis. BPA was also reported to inhibit egg production of fathead minnow but at a much greater concentration of 1280 μg/L.[17] In the present study, significant decrease on the relative weight of reproductive organ was observed in environmentally relevant concentrations, supporting that BPS has the potential to inhibit the normal growth of gonad. A lesser GSI value accompanied by an inhibition of egg production has been reported in fish exposed to estrogenic compounds.[31]

The results of PCA show the relationship between gene transcriptions and hormone concentrations. The uncorrelated variables of gene transcriptions were generated by PCA, and the cluster of the genes constituting the first two PCs were different between male and female fish. In male fish, major contributors to the first PC (*cyp19a*, *cyp19b*, *gnrh3*, *gnrhr2*, *lhβ*, *lhr*, *hmgra*, *hmgrb*, and *cyp11a*) were positively correlated with the concentrations of E2 but negatively correlated with those of T. In females, major contributors to PC1 (*cyp19b*, *gnrh2*, *fshβ*, *lhβ*, and *erα*) and PC2 (*gnrhr1*, *er2β*, *ar*, *fshr*, *lhr*, and *17βhsd*) were negatively and positively correlated with the concentrations of E2, respectively. These results also agree with the hypothesis of negative E2 feedback actions in the hypothalamus and pituitary of females to maintain hormonal homeostasis.

In the present study, parental exposure to environmentally relevant concentrations of BPS resulted in delayed and damaged hatching and increased malformation rates of the offspring generation. These results are in good agreement with those reported for BPA: BPA has been reported for its adverse effects on offspring development of rainbow trout through maternal transfer.[18] BPA has been also reported for severe sublethal effects on the developmental stage of zebrafish embryos, e.g., no blood flow, cardiac edema, delayed hatching, and tail deformations,[32] which have been also noted among the F1 generation fish of the present study. Although the present study design did not allow detailed understanding of the transfer processes, it clearly indicates that parental exposure to low concentrations of BPS could affect the performance of F1 generation. Parental exposure to BPS can be more important because there might be no excretion mechanism for BPS in the eggs as suggested for BPA.[33]

BPA and related compounds are well-known as weak estrogen agonists.[13,34] However, the results of our study indicate that exposure to BPS is associated with changes of sex hormone regulation in HPG axis, and these changes were unlikely due to the effects of BPS as an ER agonist. It has been reported that steroidogenesis is a major target for endocrine disrupting chemicals including BPA.[35] The contribution of BPA to the hormonal changes in HPG axis rather than ER-mediated mechanism have frequently been reported.[19,36] Although the action of BPS on steroidogenesis and feedback regulatory circuits in the HPG axis were observed in this study, the precise mechanisms of BPS warrants further investigation. The present study is the first report to show adverse effects of BPS on reproduction of zebrafish and its progeny generation at environmental relevant levels of exposure.

■ ASSOCIATED CONTENT

Supporting Information

Additional methods and results as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Effects of bisphenol S exposure on endocrine functions and reproduction of zebrafish

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Number of Tables: 7

Contents of Supporting Information

Supporting Materials

Analysis of bisphenol S (BPS) and quality assurance

Water samples were extracted and analyzed for BPS by following the methods described elsewhere, with some modifications (Liao et al., 2012). Briefly, an aliquot of 500 μL water samples was spiked with 10 μL of internal standards (1 μg/mL of bisphenol A). Calibration standards were prepared with ultrapure water samples previously spiked with standard mixture (ng/mL) and internal standards (Figure S1).

For quantification of BPS, an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA) was used in electrospray ionization (ESI) and multiple reaction monitoring (MRM) modes. MRM transitions and collision energy employed in the analysis are described in Table S1. Quantification was based on the peak areas relative to the corresponding internal standards. The accuracy and reliability were estimated by replicate analysis of water samples at low (0.5 ng/mL) and high-concentration (5.0 ng/mL). The accuracy was calculated as % recovery. The overall precision of the analytical procedure was measured as the coefficient of variations (CVs) determined from 7 replicate analysis of standards within the range of low- to high-concentration. The accuracy and precision of analysis for BPS are shown in Tables S2-S3.

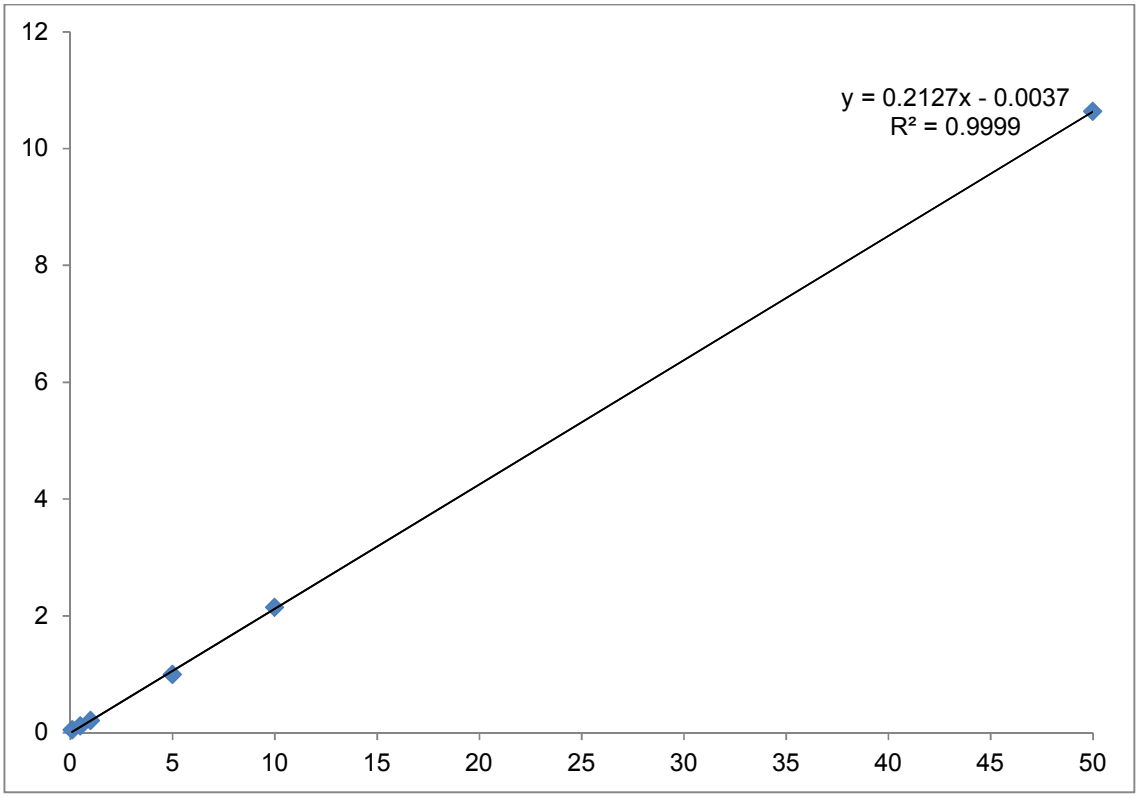

Figure S1. Linearity of the calibration curves for bisphenol S in water samples.

Table S1. Parameters for analysis and limit of detection levels for bisphenol S

Compound	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy (V)	Limit of detection (ng/mL)
Bisphenol S	249	108	-30	0.1

Table S2. Recovery and precision of bisphenol S from water samples

Compound	Spiking level (ng/mL)	Recovery (%)		Precision CV(%)
		Mean ± SD	Range	
Bisphenol S	0.5	86.4 ± 7.00	79.3 ~ 95.2	8.1
	5.0	97.3 ± 7.07	89.6 ~ 109.6	7.3
	total	91.9 ± 7.03	79.3 ~ 109.6	7.7

Real-time PCR assay

Table S3. Gene list of HPG axes of zebrafish

Abbreviation	Gene name	Category
gnrh	Gonadotropin-releasing hormone	Hormone
gnrhr	Gonadotropin-releasing hormone receptor	Peptide receptor
fshβ	Follicle stimulating hormone β	Hormone
lhβ	Luteinizing hormone β	Hormone
cyp19b	Cytochrome P450 19B	Steroidogenesis
er	Estrogen receptor	Steroid receptor
ar	Androgen receptor	Steroid receptor
fshr	Follicle stimulating hormone receptor	Peptide receptor
lhr	Luteinizing hormone receptor	Peptide receptor
hmgr	Hydroxymethylglutaryl CoA reductase	Steroidogenesis
star	Steroidogenic acute regulatory protein	Steroidogenesis
cyp11a	Cytochrome P450 side-chain cleavage	Steroidogenesis
3βhsd	3β-hydroxysteroid dehydrogenase	Steroidogenesis
cyp17	Cytochrome P450 17	Steroidogenesis
17βhsd	17β-hydroxysteroid dehydrogenase	Steroidogenesis
cyp19a	Cytochrome P450 19A	Steroidogenesis

Table S4. Sequences of primers for the genes measured

Gene name	Accession No.	Description	Sequence (5'-3')
β-actin	NM_131031	Forward	TGCTGTTTTCCCCTCCATTG
		Reverse	TCCCATGCCAACCATCACT
gnrh2	AY657018	Forward	CTGAGACCGCAGGGAAGAAA
		Reverse	TCACGAATGAGGGCATCCA
gnrh3	NM_182887	Forward	TTGCCAGCACTGGTCATACG
		Reverse	TCCATTTCACCAACGCTTCTT
gnrhr1	NM_001144980	Forward	ACCCGAATCCTCGTGGAAA
		Reverse	TCCACCCTTGCCCTTACCA
gnrhr2	NM_001144979	Forward	CAACCTGGCCGTGCTTTACT
		Reverse	GGACGTGGGAGCGTTTTCT
gnrhr4	NM_001098193	Forward	CACCAACAACAAGCGCAAGT
		Reverse	GGCAACGGTGAGGTTCATG
fshβ	NM_205624	Forward	GCTGTCGACTCACCAACATCTC
		Reverse	GTGACGCAGCTCCCACATT
lhβ	NM_205622	Forward	GGCTGCTCAGAGCTTGGTTT
		Reverse	TCCACCGATACCGTCTCATTTA
cyp19b	AF183908	Forward	GTCGTTACTTCCAGCCATTCG
		Reverse	GCAATGTGCTTCCCAACACA
erα	NM_152959	Forward	CAGACTGCGCAAGTGTTATGAAG
		Reverse	CGCCCTCCGCGATCTT
er2β	NM_174862	Forward	TTCACCCCTGACCTCAAGCT
		Reverse	TCCATGATGCCTTCAACACAA
ar	NM_001083123	Forward	TCTGGGTTGGAGGTCCTACAA
		Reverse	GGTCTGGAGCGAAGTACAGCAT
fshr	NM_001001812	Forward	CGTAATCCCGCTTTTGTTCCT
		Reverse	CCATGCGCTTGGCGATA
lhr	AY424302	Forward	GGCCATCGCCGGAAA
		Reverse	GGTTAATTTGCAGCGGCTAGTG
hmgra	BC155135	Forward	GAATCCACGGCCTCTTCGT
		Reverse	GGGTTACGGTAGCCACAATGA
hmgrb	NM_001014292	Forward	TGGCCGGACCGCTTCTA
		Reverse	GTTGTTGCCATAGGAACATGGA
star	NM_131663	Reverse	GGTCTGAGGAAGAATGCAATGAT
		Reverse	CCAGGTCCGGAGAGCTTGT
cyp11a	NM_152953	Forward	GGCAGAGCACCGCAAAA
		Reverse	CCATCGTCCAGGGATCTTATTG
3βhsd	AY279108	Forward	AGGCACGCAGGAGCACTACT
		Reverse	CCAATCGTCTTTCAGCTGGTAA
cyp17	AY281362	Forward	TCTTTGACCCAGGACGCTTT
		Reverse	CCGACGGGCAGCACAA
17βhsd	AY306005	Forward	TGCATCTCGCATCAAATCCA
		Reverse	GTCCAAGTTCCGCATAGTAGCA
cyp19a	AF226620	Forward	GCTGACGGATGCTCAAGGA
		Reverse	CCACGATGCACCGCAGTA

A preliminary experiment was conducted to determine an appropriate duration of exposure (or sampling time point) that would lead to significant changes of the target genes. For this purpose, five male fish were placed in a test aquarium and four replicate aquaria were allocated per each group. Fish were exposed to control, vehicle control (MeOH with a final concentration of 1:1,000 v/v water) and 50 μg/L BPS for 21 d. During this exposure period three fish per treatment or control were collected at day 2, 7, 14, and 21, and were measured for the transcriptions of two genes.

Expressions of *cyp19b* mRNA in brain and *cyp19a* mRNA in gonad showed time-dependent transcriptional response (see the figure below). Exposure for 2 d, however, did not cause significant change in transcription of any tested genes. While the transcription of *cyp19b* mRNA in brain and *cyp19a* mRNA in testis were slightly up-regulated at days 7 and 14, no statistical significance was observed. As shown below, however, after 21 d of exposure, the transcription of both mRNAs were significantly increased, therefore 21 d was determined as the biological sampling time for gene expression analysis.

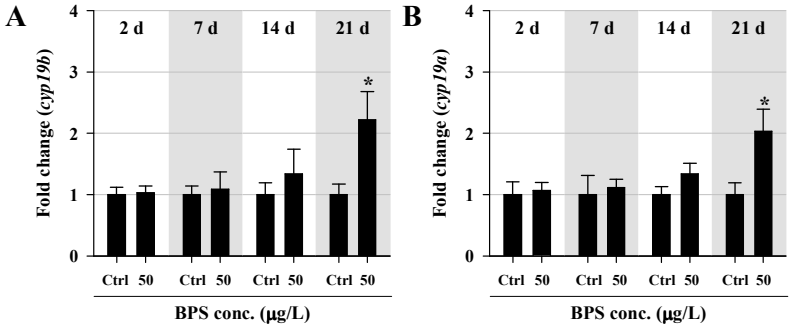

Figure S2. Changes in transcription of *cyp19b* mRNA in brain and *cyp19a* mRNA in gonad following 2, 7, 14, and 21 d of exposure to BPS. Based on this preliminary experiment, 21 d was determined as an appropriate time point of biological sample collection for gene transcription analysis.

Supporting Results

Chemical analysis

Table S5. Nominal and measured concentrations of bisphenol S (BPS) at the beginning of and after the 48 h exposure

Nominal concentration (μg/L)	Measured concentration (μg/L)	
	beginning of exposure	after 48 h exposure
Control	<LOD	<LOD
MeOH control	<LOD	<LOD
Bisphenol S 0.5	0.46 ± 0.13	0.46 ± 0.20
Bisphenol S 5	4.49 ± 1.25	4.59 ± 1.34
Bisphenol S 50	43.98 ± 15.86	42.10 ± 12.81

Limit of detection is 0.10 μg/L. Values are mean ± standard deviation of the 6 samples (two replicate samples collected from three different days).

Organism level changes of adult fish

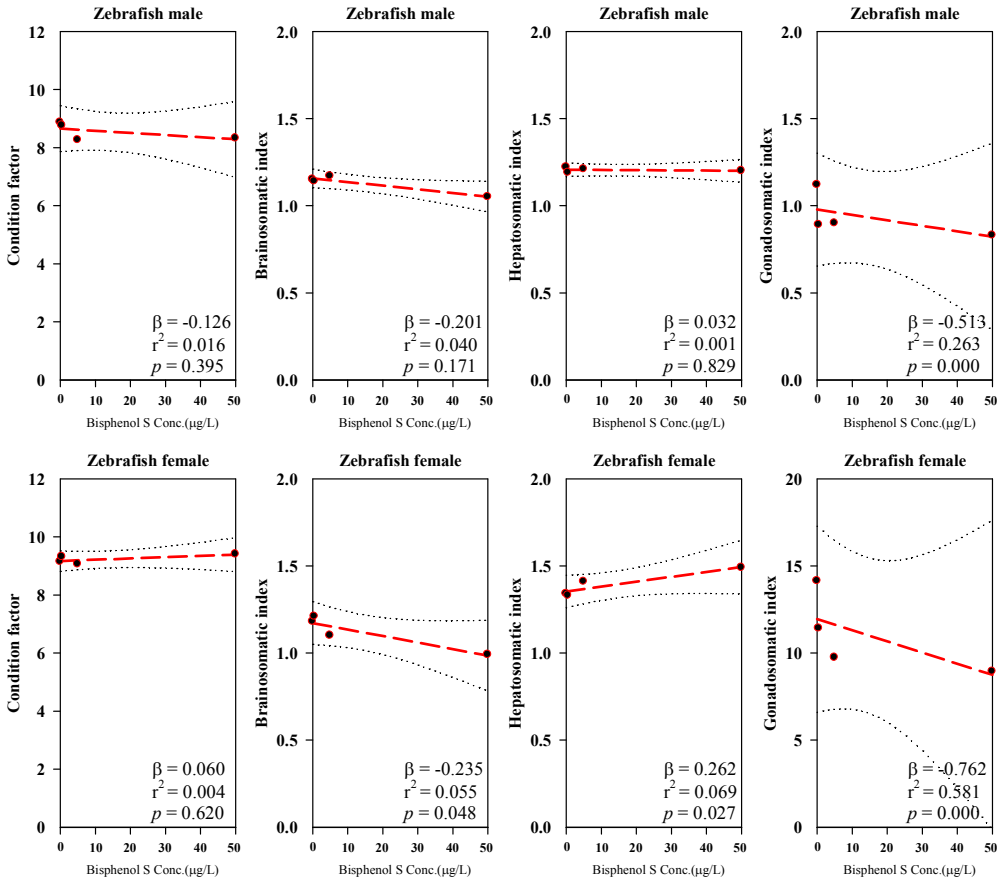

Figure S3. Effects on somatic indices of zebrafish after 21 d exposure to bisphenol S. Bold dotted line shows least-squares linear regression line, and dotted lines indicate 95% confidence band.

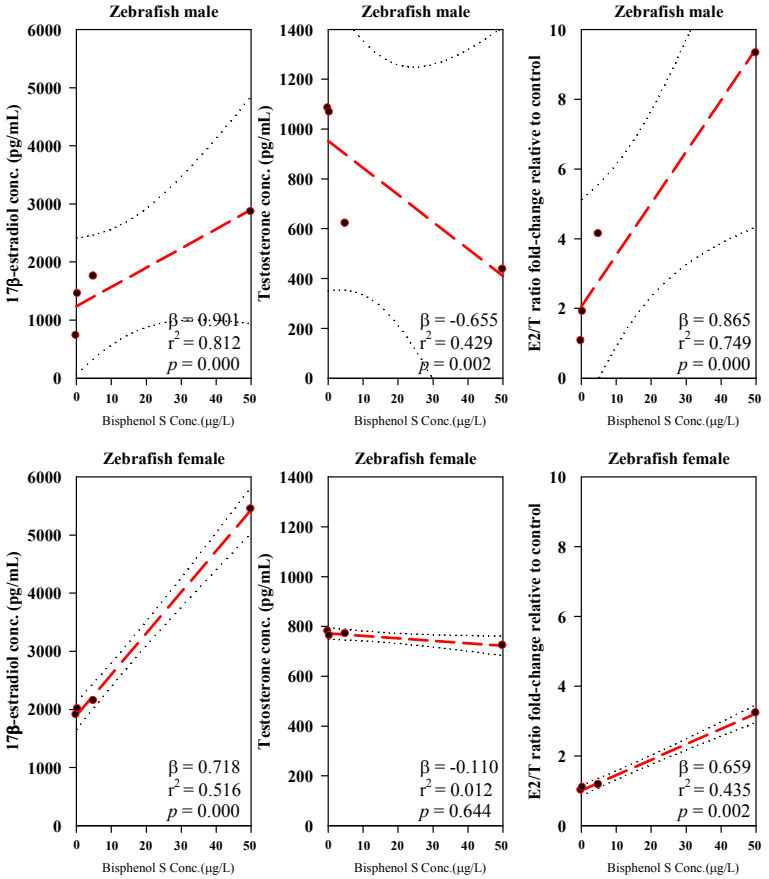

Figure S4. Effects of bisphenol S on 17β-estradiol (E2) and testosterone (T) concentrations and E2/T ratio. Bold dotted line shows least-squares linear regression line, and dotted lines indicate 95% confidence band.

Effects of BPS on gene and hormone levels in F0 fish

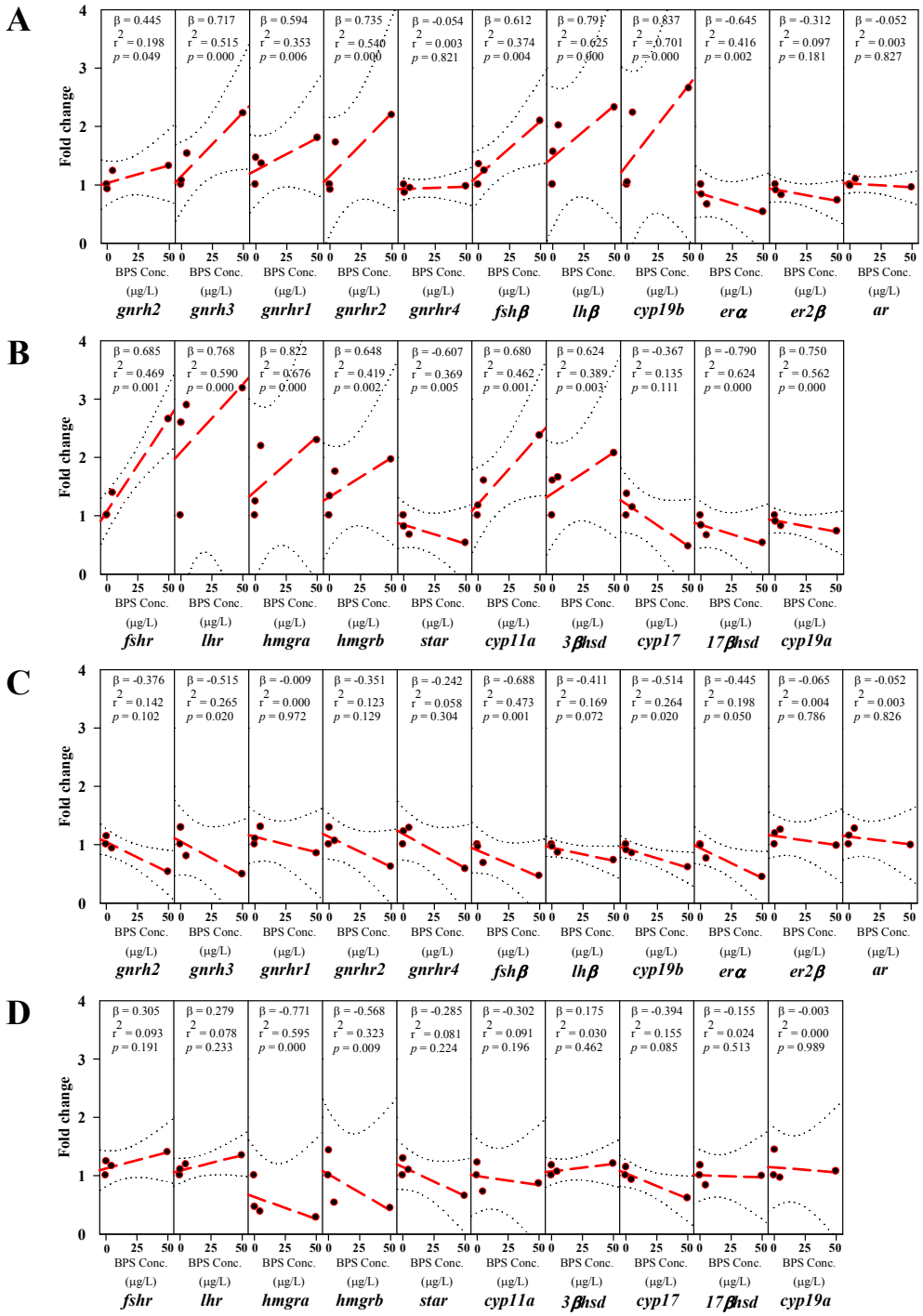

Figure S5. Effects of bisphenol S on expression of the mRNAs of hypothalamic-pituitary-gonad axis in male and female zebrafish after 21 d exposure. Observations in male brain (A), male gonad (B), female brain (C), and female gonad (D). Bold red dotted line shows least-squares linear regression line, and black thin dotted lines indicate 95% confidence band.

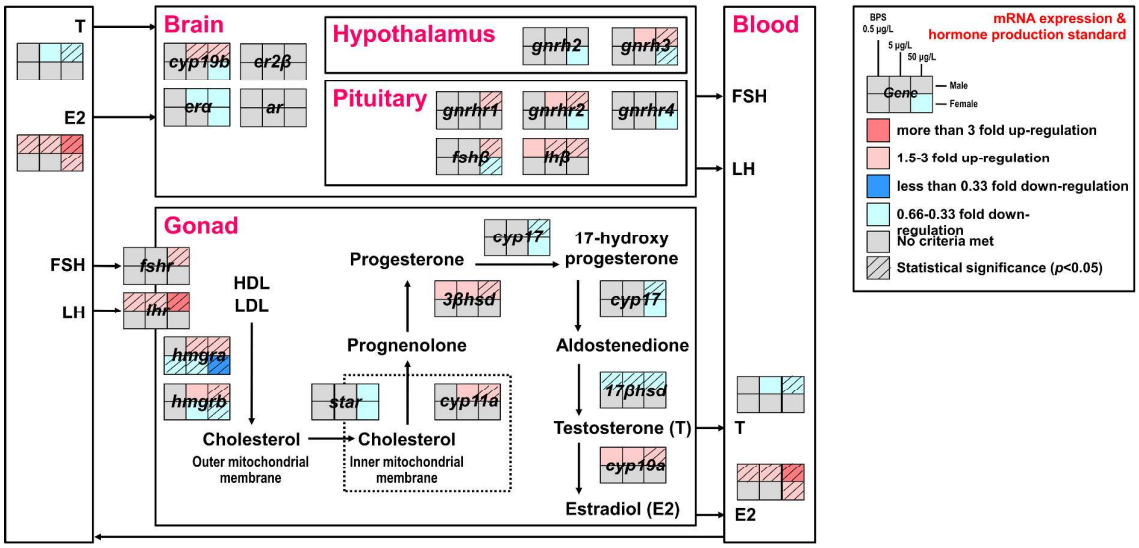

Figure S6. Effects of bisphenol S (BPS) on gene transcription and sex steroid hormones in the hypothalamic-pituitary-gonad (HPG) axes in male and female zebrafish. Gene transcription concentrations in male (upper) and female (lower) zebrafish treated by 0.5, 5, and 50 μg/L BPS are shown as striped color sets on the selected endocrine pathways along the HPG axis. The colors describe different fold thresholds. Gene acronyms are defined in Table S2.

Correlation analysis and principal component analysis

Table S6. Spearman correlation coefficients (r) between mRNA expressions of the genes along the HPG axis in male and female zebrafish after BPS exposure

Male	**Brain**											**Gonad**									
	gnrh2	*gnrh3*	*gnrhr1*	*gnrhr2*	*gnrhr4*	*fshβ*	*lhβ*	*cyp19b*	*erα*	*er2β*	*ar*	*fshr*	*lhr*	*hmgra*	*hmgrb*	*star*	*cyp11a*	*3βhsd*	*cyp17*	*17βhsd*	*cyp19a*
Brain																					
gnrh2	1.000	**0.527**	0.129	**0.549**	0.133	0.415	0.265	0.232	-0.299	-0.033	0.244	**0.461**	0.270	0.431	0.437	-0.115	0.366	0.148	-0.362	-0.305	**0.593**
		(0.016)	(0.586)	**(0.012)**	(0.575)	(0.068)	(0.257)	(0.324)	(0.199)	(0.887)	(0.298)	**(0.040)**	(0.249)	(0.057)	(0.053)	(0.626)	(0.112)	(0.532)	(0.116)	(0.189)	**(0.005)**
gnrh3		1.000	0.374	**0.677**	-0.179	0.268	**0.517**	**0.547**	**-0.518**	-0.341	-0.075	**0.537**	**0.498**	**0.624**	**0.525**	-0.288	**0.505**	**0.450**	-0.378	**-0.738**	**0.562**
			(0.103)	**(0.001)**	(0.449)	(0.252)	**(0.019)**	**(0.012)**	**(0.019)**	(0.140)	(0.751)	**(0.014)**	**(0.025)**	**(0.003)**	**(0.017)**	(0.217)	**(0.023)**	**(0.046)**	(0.100)	**(0.000)**	**(0.009)**
gnrhr1			1.000	0.297	0.066	0.395	**0.627**	**0.565**	-0.289	-0.091	0.103	0.207	**0.473**	**0.460**	0.183	**-0.445**	0.206	0.259	-0.249	**-0.495**	**0.531**
				(0.202)	(0.779)	(0.084)	**(0.003)**	**(0.009)**	(0.215)	(0.700)	(0.665)	(0.380)	**(0.034)**	**(0.041)**	(0.438)	**(0.049)**	(0.381)	(0.270)	(0.289)	**(0.026)**	**(0.015)**
gnrhr2				1.000	0.038	**0.600**	**0.657**	**0.727**	**-0.487**	-0.198	0.121	**0.665**	**0.592**	**0.632**	**0.637**	**-0.443**	**0.617**	0.425	**-0.537**	**-0.555**	**0.706**
					(0.872)	**(0.005)**	**(0.001)**	**(0.000)**	**(0.029)**	(0.401)	(0.608)	**(0.001)**	**(0.005)**	**(0.002)**	**(0.002)**	**(0.049)**	**(0.003)**	(0.061)	**(0.014)**	**(0.011)**	**(0.000)**
gnrhr4					1.000	0.156	0.228	0.063	0.301	**0.739**	**0.608**	0.128	-0.226	-0.071	**-0.486**	-0.251	-0.006	-0.366	-0.283	0.298	-0.116
						(0.510)	(0.332)	(0.788)	(0.195)	**(0.000)**	**(0.004)**	(0.588)	(0.337)	(0.764)	**(0.029)**	(0.284)	(0.979)	(0.111)	(0.226)	(0.201)	(0.624)
fshβ						1.000	**0.691**	0.386	-0.286	-0.052	0.106	**0.446**	0.424	**0.494**	0.403	-0.409	0.244	0.203	**-0.446**	**-0.456**	**0.454**
							(0.000)	(0.092)	(0.220)	(0.825)	(0.656)	**(0.048)**	(0.062)	**(0.026)**	(0.078)	(0.072)	(0.298)	(0.389)	**(0.048)**	**(0.042)**	**(0.044)**
lhβ							1.000	**0.734**	-0.329	-0.190	0.178	0.395	**0.677**	**0.694**	0.373	-0.437	0.346	**0.526**	-0.268	**-0.708**	**0.519**
								(0.000)	(0.155)	(0.421)	(0.450)	(0.084)	**(0.001)**	**(0.000)**	(0.104)	(0.053)	(0.134)	**(0.017)**	(0.252)	**(0.000)**	**(0.018)**
cyp19b								1.000	**-0.553**	-0.183	0.147	0.375	**0.544**	**0.711**	0.372	**-0.470**	**0.496**	**0.445**	**-0.507**	**-0.566**	**0.494**
									(0.011)	(0.438)	(0.534)	(0.102)	**(0.013)**	**(0.000)**	(0.106)	**(0.036)**	**(0.026)**	**(0.048)**	**(0.022)**	**(0.009)**	**(0.026)**
erα									1.000	0.442	0.071	**-0.553**	-0.382	**-0.571**	**-0.564**	0.293	**-0.660**	-0.406	0.298	0.422	**-0.452**
										(0.050)	(0.763)	**(0.011)**	(0.096)	**(0.008)**	**(0.009)**	(0.209)	**(0.001)**	(0.075)	(0.201)	(0.063)	**(0.045)**
er2β										1.000	**0.468**	-0.102	-0.442	-0.294	**-0.570**	-0.009	-0.348	**-0.649**	-0.155	**0.529**	-0.282
											(0.037)	(0.667)	(0.051)	(0.207)	**(0.008)**	(0.679)	(0.132)	**(0.002)**	(0.512)	**(0.016)**	(0.227)
ar											1.000	0.103	-0.094	-0.124	-0.360	-0.082	-0.228	-0.250	0.026	0.070	0.074
												(0.663)	(0.690)	(0.602)	(0.117)	(0.730)	(0.331)	(0.287)	(0.910)	(0.769)	(0.753)
Gonad																					
fshr												1.000	0.138	0.347	0.400	-0.312	**0.550**	0.185	-0.415	-0.362	**0.470**
													(0.560)	(0.133)	(0.079)	(0.179)	**(0.011)**	(0.434)	(0.068)	(0.115)	**(0.036)**
lhr													1.000	**0.645**	**0.758**	-0.286	**0.488**	**0.808**	-0.013	**-0.727**	**0.765**
														(0.002)	**(0.000)**	(0.220)	**(0.029)**	**(<0.001)**	(0.954)	**(0.000)**	**(<0.001)**
hmgra														1.000	**0.646**	**-0.514**	**0.631**	**0.617**	-0.440	**-0.639**	**0.563**
															(0.002)	**(0.020)**	**(0.002)**	**(0.003)**	(0.051)	**(0.002)**	**(0.009)**
hmgrb															1.000	-0.254	**0.647**	**0.700**	-0.095	**-0.649**	**0.696**
																(0.279)	**(0.002)**	**(0.000)**	(0.688)	**(0.001)**	**(0.000)**
star																1.000	**-0.546**	-0.186	**0.617**	0.291	-0.387
																	(0.012)	(0.432)	**(0.003)**	(0.211)	(0.091)
cyp11a																	1.000	0.502	**-0.526**	-0.345	**0.564**
																		(0.024)	**(0.017)**	(0.135)	**(0.009)**
3βhsd																		1.000	0.019	**-0.689**	**0.602**
																			(0.934)	**(0.000)**	**(0.004)**
cyp17																			1.000	0.068	-0.286
																				(0.775)	(0.220)
17βhsd																				1.000	**-0.541**
																					(0.013)
cyp19a																					1.000

Female	Brain											Gonad									
	gnrh2	*gnrh3*	*gnrhr1*	*gnrhr2*	*gnrhr4*	*fshβ*	*lhβ*	*cyp19b*	*erα*	*er2β*	*ar*	*fshr*	*lhr*	*hmgra*	*hmgrb*	*star*	*cyp11a*	*3βhsd*	*cyp17*	*17βhsd*	*cyp19a*
Brain																					
gnrh2	1.000	0.308	0.087	**0.532**	**0.558**	**0.577**	**0.548**	**0.592**	**0.695**	0.356	0.088	-0.024	-0.202	0.397	0.321	0.193	0.218	-0.196	0.343	0.342	-0.275
		(0.185)	(0.714)	**(0.015)**	**(0.010)**	**(0.007)**	**(0.012)**	**(0.005)**	**(0.000)**	(0.123)	(0.711)	(0.392)	(0.392)	(0.082)	(0.166)	(0.413)	(0.355)	(0.406)	(0.137)	(0.139)	(0.240)
gnrh3		1.000	0.060	**0.624**	**0.544**	0.358	0.028	0.180	0.248	-0.051	-0.075	-0.416	-0.136	0.419	**0.596**	**0.579**	0.112	-0.010	0.339	0.009	0.227
			(0.800)	**(0.003)**	**(0.013)**	(0.121)	(0.904)	(0.446)	(0.291)	(0.828)	(0.752)	(0.067)	(0.565)	(0.065)	**(0.005)**	**(0.007)**	(0.638)	(0.964)	(0.143)	(0.969)	(0.335)
gnrhr1			1.000	0.027	0.034	-0.088	-0.126	0.189	0.223	0.230	0.318	-0.036	-0.037	0.171	-0.225	**0.536**	-0.098	-0.270	0.017	0.262	0.260
				(0.909)	(0.884)	(0.711)	(0.595)	(0.422)	(0.342)	(0.328)	(0.170)	(0.879)	(0.874)	(0.469)	(0.338)	**(0.014)**	(0.678)	(0.248)	(0.942)	(0.263)	(0.267)
gnrhr2				1.000	**0.766**	0.412	0.236	0.174	**0.605**	0.194	0.228	-0.336	-0.010	0.412	**0.545**	0.342	0.169	0.181	0.323	0.354	0.231
					(<0.001)	(0.070)	(0.316)	(0.461)	**(0.004)**	(0.412)	(0.333)	(0.146)	(0.964)	(0.070)	**(0.012)**	(0.139)	(0.473)	(0.444)	(0.164)	(0.125)	(0.325)
gnrhr4					1.000	0.324	0.303	0.163	**0.635**	0.403	0.295	-0.181	0.108	0.281	0.260	0.351	0.055	0.000	0.331	0.046	0.087
						(0.162)	(0.193)	(0.491)	**(0.002)**	(0.078)	(0.206)	(0.442)	(0.649)	(0.229)	(0.268)	(0.128)	(0.815)	(0.997)	(0.153)	(0.845)	(0.714)
fshβ						1.000	**0.741**	**0.769**	**0.473**	-0.000	-0.157	0.137	-0.176	**0.594**	**0.698**	0.207	**0.498**	0.044	0.227	0.365	-0.050
							(0.000)	**(<0.000)**	**(0.035)**	(0.997)	(0.507)	(0.562)	(0.457)	**(0.005)**	**(0.000)**	(0.380)	**(0.025)**	(0.853)	(0.335)	(0.112)	(0.832)
lhβ							1.000	**0.626**	**0.488**	0.410	-0.020	0.315	-0.182	0.347	0.426	-0.072	**0.650**	0.044	0.194	**0.464**	-0.131
								(0.003)	**(0.029)**	(0.072)	(0.932)	(0.175)	(0.442)	(0.133)	(0.060)	(0.760)	**(0.001)**	(0.852)	(0.412)	**(0.039)**	(0.580)
cyp19b								1.000	**0.445**	0.216	0.002	0.090	-0.037	**0.519**	0.267	0.339	**0.567**	0.143	0.201	**0.458**	-0.114
									(0.048)	(0.358)	(0.991)	(0.705)	(0.874)	**(0.018)**	(0.253)	(0.142)	**(0.009)**	(0.545)	(0.393)	**(0.004)**	(0.631)
erα									1.000	**0.546**	0.341	-0.088	0.106	0.441	0.274	0.326	0.311	-0.044	0.477	**0.447**	0.013
										(0.0127)	(0.140)	(0.712)	(0.656)	(0.051)	(0.241)	(0.159)	(0.181)	(0.853)	(0.033)	**(0.047)**	(0.954)
er2β										1.000	**0.533**	0.112	-0.027	-0.162	-0.227	0.151	0.383	0.189	-0.018	0.437	-0.267
											(0.015)	(0.635)	(0.907)	(0.493)	(0.333)	(0.524)	(0.094)	(0.424)	(0.937)	(0.053)	(0.255)
ar											1.000	-0.239	-0.021	-0.012	-0.114	0.354	0.177	0.271	0.362	0.409	0.110
												(0.309)	(0.927)	(0.959)	(0.631)	(0.124)	(0.454)	(0.246)	(0.115)	(0.072)	(0.642)
Gonad																					
fshr												1.000	0.281	-0.400	-0.111	-0.304	0.242	-0.034	-0.311	-0.012	0.045
													(0.229)	(0.080)	(0.640)	(0.191)	(0.302)	(0.884)	(0.181)	(0.957)	(0.850)
lhr													1.000	-0.082	-0.311	-0.008	-0.009	0.252	0.023	-0.151	**0.490**
														(0.728)	(0.181)	(0.972)	(0.969)	(0.283)	(0.922)	(0.524)	**(0.028)**
hmgra														1.000	0.436	0.194	0.048	-0.173	0.363	0.114	0.214
															(0.054)	(0.410)	(0.837)	(0.464)	(0.115)	(0.630)	(0.364)
hmgrb															1.000	0.276	0.411	0.052	**0.470**	0.313	0.177
																(0.238)	(0.071)	(0.825)	**(0.036)**	(0.178)	(0.454)
star																1.000	0.167	0.166	**0.455**	0.285	0.216
																	(0.480)	(0.481)	**(0.043)**	(0.222)	(0.360)
cyp11a																	1.000	0.365	0.247	**0.675**	0.094
																		(0.113)	(0.291)	**(0.001)**	(0.691)
3βhsd																		1.000	0.041	0.195	0.205
																			(0.862)	(0.407)	(0.384)
cyp17																			1.000	0.189	0.303
																				(0.424)	(0.193)
17βhsd																				1.000	-0.018
																					(0.937)
cyp19a																					1.000

The values in parentheses are *p* values.

Table S7. Result of principal component analysis

	Males		Females	
	PC1	PC2	PC1	PC2
Eigenvalues	9.83	3.13	6.09	2.89
% Variance	46.8	14.9	29.0	13.8
Accumulative (%)	46.8	61.7	29.0	42.8
Factor loadings				
gnrh2	0.15	0.18	**0.30**	-0.02
gnrh3	**0.26**	-0.04	0.21	-0.33
gnrhr1	0.18	0.22	0.07	**0.30**
gnrhr2	**0.26**	0.09	0.24	-0.18
gnrhr4	-0.04	**0.48**	0.24	-0.14
cyp19b	**0.27**	0.11	**0.30**	0.11
fshβ	0.23	0.08	**0.30**	-0.13
lhβ	**0.25**	0.19	**0.29**	0.02
erα	-0.24	0.15	**0.32**	0.10
er2β	-0.12	**0.41**	0.21	**0.32**
ar	0.01	**0.37**	0.17	**0.26**
fshr	0.23	0.14	-0.03	**0.29**
lhr	**0.26**	-0.10	-0.05	**0.25**
hmgra	**0.26**	0.05	0.19	-0.29
hmgrb	**0.25**	-0.27	0.23	-0.29
star	-0.16	-0.26	0.18	0.13
cyp11a	**0.26**	-0.07	0.27	0.15
3βhsd	0.23	-0.24	0.04	0.24
cyp17	-0.15	-0.25	0.23	-0.08
17βhsd	-0.23	0.06	0.22	**0.33**
cyp19a	**0.28**	-0.02	0.05	-0.05

Values >0.24 are shown in bold.

References

Liao, C.; Liu, F.; Alomirah, H.; Loi, V.D.; Mohd, M.A.; Moon, H.B.; Nakata, H.; Kannan, K. Bisphenol S in urine from the United States and seven Asian countries: occurrence and human exposures. *Environ. Sci. Technol.* **2012**, *46*, 6860-6866.