1 **Differential effects of bisphenol A toxicity on oyster**
2 **(*Crassostrea angulata*) gonads as revealed by label-free**
3 **quantitative proteomics**

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ABSTRACT

Bisphenol A (BPA) is widely used in industry, and is released in large amounts into the environment. BPA is a teratogen and an estrogen receptor agonist and negatively affects reproduction, particularly in aquatic animals, which is of concern for the aquaculture industry. Although there is a large body of literature on the mechanisms that underlie BPA disruption and the effects of different toxicities on invertebrate reproduction, many of the mechanisms involved in invertebrate responses to BPA remain unknown. In this study, we investigated the effects of BPA on the reproduction of female and male oysters (*Crassostrea angulata*), and measured BPA bioaccumulation, the gonad-somatic index (GSI), and gonadal protein profiles in oysters exposed to BPA. Compared to controls, approximately 160-times more BPA accumulated in the gonads of male and female oysters after exposure to 2 mg.L⁻¹ BPA for 16 days. Gonadal development was negatively affected in males, but was accelerated in females when exposed to BPA, based on GSI analysis and a visual inspection of histological sections of the gonads. BPA exposure induced the differential expression of many important proteins such as vitellogenin, periostin, phosphoglucomutase, collagen alpha-1(XII) chain, and zinc transporter 9, which are involved in energy metabolism, oxidative stress, gene transcription regulation, the vitellogenin interaction network, and zinc transportation. A functional analysis of these proteins indicated that BPA has different effects on gonadal development in male and female oysters.

Keywords: Bisphenol A; *Crassostrea angulata*; Label-free quantitative proteomics; Oyster; Endocrine disruptor; Reproductive toxicology

1. Introduction

Environmental contamination by endocrine-disrupting chemicals (EDCs) has been a major global issue for decades. EDCs enter the environment and the human food chain from plasticizers and can cause reproductive and teratogenic toxicity in animals (Kavlock et al., 1996; Diamanti-Kandarakis et al., 2009; Sweeney et al., 2015; Trasande et al., 2015). Consequently, many EDCs affect animal population sizes and sex ratios.

Xenoestrogens are an important type of EDC that mimic the natural steroid, 17 β -estradiol. They exhibit estrogenicity and can affect the physiological action of 17 β -estradiol when bound to estrogen receptors (Mills and Chichester, 2005; Campbell et al., 2006; Maqbool et al., 2016). Bisphenol A (2,2-Bis(4-hydroxyphenyl)propane; BPA) is a xenoestrogen and an important intermediate in the production of polycarbonate plastic resin and epoxy resin (Staples et al., 1998; Howdeshell et al., 1999; Campbell et al., 2006). Today, BPA is widely used in the polycarbonate plastics industry, with millions of tons of BPA being produced annually. The movement of BPA from plastic production to the environment has resulted in it becoming a ubiquitous global contaminant, particularly in the aquatic environment. For example, BPA concentrations of up to 193 ng/L have been detected in the surface waters of the Baltic Sea (Krishnan et al., 2011; Flint et al., 2012; Huang et al., 2012; Staniszewska, et al., 2015).

Much research has focused on the damage that BPA can cause to animal health and the environment, because it is a teratogen and endocrine disruptor in vertebrates (Sone et al., 2004; Yang et al., 2005). For example, seminiferous tubule histoarchitecture is disrupted in male *Caiman latirostris* embryos exposed to BPA, leading to male-to-female sex reversal

(Stoker et al., 2003). As an endocrine disruptor, BPA may induce changes in sex determination and gonadal function, during or after gonadal organogenesis (Crain et al., 1997). BPA has also been observed inducing changes in invertebrates: abnormal reproductive cells, organs, and imposex have been detected in freshwater snails, while superfemales and feminization have been reported in females of the freshwater prosobranch, *Marisa cornuarietis* (Oehlmann et al., 2000; Kang et al., 2007). Another adverse endocrine-disrupting effect in aquatic organisms is the inhibition of gonadal growth, which adversely affects the central nervous system and immune system (Sohoni et al., 2001; Mizuo et al., 2010; Nakamura and Kariyazono, 2010). However, the mechanisms involved are not known for certain (Crain et al., 2007).

Mollusks are sensitive to BPA, and they have frequently been used to investigate how BPA affects invertebrates (Jobling et al., 2003; Kaddar et al., 2009). The oyster is a typical mollusk that inhabits estuaries and coastal areas, and is consumed globally. Oysters are hermaphrodites and can change sex at almost any point in their development (Terje and Trygve, 2005); therefore, oysters should be a useful model species to investigate how different levels of BPA toxicity affect invertebrates. Here, *Crassostrea angulata* individuals were exposed to BPA for 16 days to determine the amount of BPA bioaccumulation in their gonads, the gonad-somatic index (GSI), the sex ratio, and protein profiles, in order to improve our understanding of BPA toxicity mechanisms in invertebrates, particularly with respect to the development of gonads. Our findings are important for companies that culture oysters in BPA-polluted estuaries and coastal areas, where oyster productivity may be negatively affected by the chemical.

2. Materials and methods

2.1. Chemicals and instruments

The following equipment was used: Sigma 3-18K refrigerated centrifuge (Sigma-Aldrich, USA), Milli-Q[®] water treatment system (Millipore, USA), gel imaging system (Bio-Rad, USA), Q Exactive[™] nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS) system (Thermo Fisher Scientific, USA), Agilent 7000B triple quadrupole gas chromatography-mass spectrometry (GC-MS/MS) system (Agilent Technologies, USA), ABI 7500 real-time polymerase chain reaction (PCR) system (Applied Biosystems, USA), and Infinite[®] M1000 PRO microplate reader (Tecan, Switzerland).

BPA (CAS 80-5-7, 99%), trifluoroacetic acid (CAS 76-05-1), dithiothreitol (DTT, CAS 3483-12-3), acrylamide (CAS 79-06-1), N,N'-methylenebisacrylamide (CAS 110-2-9), trimethylol aminomethane (Tris, CAS 77-86-1), sodium dodecyl sulfate (SDS, CAS 151-21-3), glycine (CAS 56-40-6), and acetonitrile (ACN, CAS 75-05-8) were obtained from Sigma-Aldrich. Iodopyridine (CAS 1120-90-7), ammonium persulfate (CAS 7727-54-0), and tetramethylethylenediamine (CAS 110-18-9) were obtained from Bio-Rad. RNAiso Plus Kit for Total RNA extraction, Takara PrimeScript[™] RT-PCR Kit, and SYBR[®] *Premix Ex Taq*[™] II (Perfect Real-Time) PCR Kit were obtained from Takara Bio co. LTD. China. Sequencing-grade trypsin (V5111) was obtained from Promega, USA. Bradford protein assay, total superoxide dismutase (SOD) assay with nitroblue tetrazolium, and catalase (CAT) assay

kits were obtained from Jiancheng Bioengineering Institute, China. All of the other chemicals used in the study were of analytically pure grade and supplied by Sigma-Aldrich.

2.2. Sampling and oyster culture

C. angulata individuals that weighed 5.02 ± 1.1 g (mean $\pm$ S.D., $n = 1000$, ~3 months old) were obtained from the Haicang Oyster Culture Co., Xiamen, Fujian Province, China. The oysters were cleaned of epiphytes and maintained in laboratory aquaria ($120 \times 120 \times 140$ cm) filled with seawater with $30.2 \pm 0.2\%$ salinity, pH 8.2 ± 0.1 , and 20–25 °C. The seawater was filtered using a 0.45- μ m filter membrane, and was changed every 24 h. After changing the seawater, *Chlorella* algal paste was fed to the oysters twice a day, at quantities representing 0.5% of the weight of the oysters cultured in the aquaria. The seawater was continuously aerated with air pumps.

The oysters were acclimated for 10 days in control tanks, and were then divided into eight groups of 120 animals each, which were further divided into three replicates. Three treatment groups were used for the BPA exposure experiments (control group, 1 mg.L⁻¹ BPA exposure for 16 days, and 2 mg.L⁻¹ BPA exposure for 16 days), while the other five groups were used to measure the median lethal concentration (LC₅₀) of BPA. The BPA concentration in the seawater was measured daily by GC-MS/MS (Supplementary Table 1). BPA concentrations in contaminated estuaries and BPA toxicity reports were used to determine the exposure levels (Honkanen et al., 2004; Sone et al., 2004; Yang et al., 2005).

During BPA exposure, the number of dead oysters was recorded in each group daily. The sex of dead individuals was determined by the microscopic identification of gametes, and

dead oyster data from the three treatment groups were used to calculate the mortality rate and the male/female mortality ratio.

After 16 days of exposure to BPA, 30 oysters from each group were killed and the gonads removed for investigation. After determining the oysters' sex and rinsing their gonads with cold, distilled water, 10 males and 10 females were randomly selected and their gonads were weighed using an electronic analytical balance. The gonads were then separated into three subsamples (~50 mg each) for histological sections, protein analysis, and BPA accumulation measurements. Each subsample was pooled based on its treatment group and sex.

2.3. GSI determination

The GSI is an important index that is used to evaluate gonadal development in shellfish, and is determined by the weights of the gonads and soft body parts using the following formula (Grigorakis and Alexis, 2005):

$$\text{GSI} = (\text{gonad weight} / \text{soft tissue weight}) \times 100\%$$

2.4. Quantitative analysis of BPA concentrations in seawater and oyster samples by GC-MS/MS

BPA concentrations in purified and concentrated seawater and oyster tissues were measured by GC-MS/MS. Each 0.5 mL seawater sample was mixed with 0.5 mL acetone and 0.5 mL trichloromethane. The mixture was shaken for 30 min, stood at room temperature for

10 min, and then centrifuged at 12,000 g and 16 °C for 10 min to collect the organic phase. The samples were then dried with nitrogen. The extracted BPA samples were mixed with 100 µL N-methyl-N-trifluoroacetamide and 1% trimethylchlorosilane for 1 min before being incubated at 50 °C for 30 min in preparation for GC-MS/MS analysis.

A mixture of methanol-chloroform-acetone (600 µL volume; 1:1:1, v/v/v) was added to each 100 mg tissue sample. The samples were homogenized using an ultrasonic cell disruptor (Scientz-IID, Ningbo Scientz Biotechnology Co., LTD., Ningbo, China.) at 25 °C for 5 min. Then, 500 µL chloroform and water were added to the mixture, which was vortexed for 1 min before being centrifuged at 12,000 g and 16 °C for 10 min to collect the aqueous and lipid layers. Finally, the samples were dried with nitrogen. Derivatization was performed following the same methods.

A GC-MS/MS system equipped with an HP-5MS column (60 M × 0.32 mM × 0.25 µm) was used to measure the BPA concentration in comparison to a standard curve under the following conditions: electron ionization; ion source temperature, 230 °C; quadrupole radiation temperature, 150 °C; interface temperature, 250 °C; selected ion monitoring scan, 357.1 m/z ions, -70 eV, delay 6 min, and automatic tuning; helium as the carrier gas, 1.7 mL.min⁻¹; entrance temperature, 230 °C; splitless; and sample value, 1 µL. The oven was set at 100 °C for 1 min, increased to 150 °C (50 °C.min⁻¹) for 3 min, and further increased to 280 °C (40 °C.min⁻¹) for 14 min, until the end of the program.

2.5. SOD and CAT activity in *C. angulata* gonads

Each 100-mg gonad sample was mixed with 1.0 mL precooled phosphate-buffered saline

before homogenization. After centrifugation for 10 min at 12,000 g and 4 °C, the supernatant was removed and diluted 10 times. Then, SOD and CAT activity were measured following the instructions of the assay kits.

2.6. Histological sectioning and hematoxylin and eosin (H&E) staining

After being removed from the oysters, the gonads were cut into 0.5 cm² pieces and transferred to 2.0 mL centrifuge tubes, to which 1.0 mL Holland-Bouin liquid was added. After the gonads had been soaked for 3 days, the gonad block was washed three times with distilled water. The distilled water in the block was then completely replaced following dehydration, in which the tissue block was placed into the following solvents in this order: 30% alcohol for 30 min, 50% alcohol for 30 min, 75% alcohol for 30 min, 100% alcohol for 30 min (twice), 50% alcohol and 50% xylene for 30 min, 100% xylene for 23 min (twice), and 100% paraffin melted at 70 °C for 23 min (twice). The gonadal tissues were immersed in paraffin and cut into 5-µm slices using a rotary microtome. The paraffin rings were placed on glass slides and fixed using 50% polylysine solution. The rings were then dried and stained with H&E.

2.7. Label-free quantitative proteomics analysis

2.7.1. Preparation of the proteome

Each 100-mg tissue sample was mixed with 1.0 mL protein extract solution (50 mM Tris, 0.1% SDS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 6 M urea, 2 M thiourea, 20 mM DTT, and 2 mM ethylenediaminetetraacetic acid (EDTA, pH 8.1), to

which a 0.1% protease inhibitor cocktail was added (GE Healthcare, USA). The samples were then subjected to ultrasound for 5 min (on for 5 s, with 15-s breaks) at 16 °C. After standing the mixture for 10 min at 16 °C, it was then centrifuged at 12,000 g at 16 °C for 15 min to obtain the supernatant. Subsequently, 1.5 mL acetone solution (10% trichloroacetic acid (w/v) and 20 mM DTT dissolved in acetone) was added to the supernatant, and the protein was precipitated overnight at -20 °C. The mixture was then centrifuged at 17,000 g and 16 °C for 30 min to collect the protein sediment, which was washed five times with 1 mL -20 °C precooled acetone containing 20 mM DTT. The mixture was left to stand at 4 °C for 30 min, before being centrifuged at 17,000 g and 4 °C for 30 min to collect the sediment. Finally, the protein sediment was centrifuged and freeze-dried in a vacuum to completely eliminate the acetone solution, before being dissolved in 500 µL protein extract solution (50 mM Tris, 0.1% SDS, 10 mM HEPES, 6 M urea, 2 M thiourea, 20 mM DTT, and 2 mM EDTA, pH 8.1). The bicinchoninic acid method was used to measure the protein concentration, and 5.0µg protein samples were used for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis with silver staining. Ten biological replicates and three technical replicates were included.

2.7.2. Trypsin digestion of the proteome samples

A 50-µg proteome sample that had been dissolved in extract solution (50 mM Tris, 0.1% SDS, 10 mM HEPES, 6 M urea, 2 M thiourea, 20 mM DTT, and 2 mM EDTA, pH 8.1) was added to an iodoacetamide solution to obtain a final concentration of 40 mM iodoacetamide. The mixture was then stood in the dark for 30 min. Subsequently, the mixture was diluted with a threefold volume of 50 mM NH_4HCO_3 solution to reduce the concentration of urea to 2

M, before it was mixed with 0.5 µg trypsin. The mixture was then digested at 37 °C for 8 h. The digestion mixture was further diluted with a twofold volume of 50 mM NH₄HCO₃ solution, before being added to 0.5 µg trypsin followed by 8 h of digestion at 37 °C. Formic acid (FC) was then added to the enzyme digestion system to make a final concentration of 0.5% (pH 2–3) to terminate trypsin digestion.

After being completely digested by trypsin, the digestion product was desalted using a desalination column filled with a C-18 microsphere. The desalted peptides were collected, freeze-dried in a vacuum, and dissolved in a 50 µL solution with 0.1% FC and 2% ACN. Then, the mixture was passed through a 0.22-µm filter membrane for nano-LC-MS/MS analysis.

2.7.3. Label-free proteomics analysis by nano-ultra-performance (UP)LC-MS/MS

The proteome samples were separated without *a priori* fractionation using a Waters® nanoACQUITY® UPLC system equipped with a Trap nano-RPC18 column of Smartube C18 (5 µm, 15 cm × 75 µm inner diameter; Column Scientific, Xiamen, China) and an analysis nano-column of Smartube C18 (3 µm, 50 cm × 100 µm inner diameter; Column Scientific). The conditions for the nano-UPLC were as follows: A (mobile phase), with 98% ACN and 0.1% FC aqueous solution; B (mobile phase), with 2% ACN and 0.1% FC aqueous solution. A 5 µL volume of each peptide sample was injected followed by trapping for 5 min, with 5 µL per min of 3.0% A (mobile phase) mixed with 97% B (mobile phase). The analysis column was then maintained at 35 °C. Elution was conducted at an initial constant velocity of 0.3 µL.min⁻¹; the linearity of the A (mobile phase) was set as follows: 0 to 25 min, 3.0 to 8%; 25 to 135 min, 8 to 35%; 135 to 145 min, held at 35%; 145 to 180 min, 35 to 100%.

MS analysis of the proteome was performed using Q Exactive™, with the following conditions set: full scan/dd-MS² (top 15); spray voltage, 2.1 kV; capillary temperature, 250 °C; MS¹ 70000 full width at half maximum (FWHM), MS² 17500 FWHM; full scan automatic gain control (AGC) target 3e6, scan 350–1750 m/z; higher-energy collisional dissociation energy, 30%; intensity threshold, 1.00 e+04; dynamic exclusion, 30; MS² Max IT, 100 ms; and MS² AGC, 1.00 e+05.

The proteomic MS data were identified and analyzed using Proteome Discoverer™ (version 1.4) in the Swiss-Prot database (Source Organism: Oyster). The key retrieval parameters in Proteome Discoverer™ were set as follows: enzyme named as trypsin (full); precursor ion mass tolerance, ± 15 ppm; fragment ion mass tolerance, ± 20 mmu; static modification of carbon terminal (carbamidomethyl/+57.021 Da), variable modification of nitrogen terminal (Gln→pyro-Glu/-17.027 Da), and variable modification of carbon terminal (oxidation/+15.995 Da(M)); most missed cleavage site, 2; and false discovery rate of all peptide and protein identifications, <1%. After protein identification, differentially expressed proteins were analyzed using SIEVE™ 2.0 software.

Pathway and interactions analysis of the differentially expressed proteins was conducted using the Search Tool for Interactions of Chemicals (STITCH, http://stitch.embl.de/cgi/show_input_page.pl?UserId=tBhJnETw4sy&sessionId=EqdIX7fPWY5H&input_page_type=multiple identifiers) and the online tool STRING Version 10.0 of ExPASy Bioinformatics Resource Portal (<http://string-db.org/>). Moreover, due to most differently expressed proteins in oyster were lack functional annotations and not available to establish the interaction network, the homologous

proteins from *Mus musculus* was employed to establish the interaction network and evaluated the connectivity of the differentially expressed proteins and the gene products.

2.8. Quantitative real-time PCR analysis

Total RNA was prepared using a RNAiso Plus Kit, and its quality was checked by electrophoresing ~5 µg of each total RNA sample on a denaturing formaldehyde/agarose gel. Approximately 1 µg total RNA was reverse transcribed to the cDNA (RT-PCR) based on the instructions provided in the Takara PrimeScriptTM RT-PCR kit manual. RT-PCR was performed following the instructions in the SYBR[®] Premix Ex TaqTM II (Perfect Real-Time) kit manual. β-Actin was used as an internal control gene, and three independent replicates of each gene were included. The relative number of copies of each mRNA was calculated using the average of each value, and the ratio of mRNA transcript fold changes to protein expression fold changes was analyzed by correlation index analysis. ABI 7500 Series software was used to analyze the amplification and melting curves. The genes selected for the relative quantification of their mRNA copies, and the primers that were designed according to the cDNA sequences from the transcriptome, are listed in Table 1.

3. Results

3.1. BPA bioaccumulation in gonads

BPA accumulation in the gonads was time-dependent in both male and female oysters (Fig. 1). Sixteen days after the oysters were exposed to 2 mg.L⁻¹ BPA, the gonads of males and females had accumulated 351.56 ± 17.82 and 329.51 ± 13.07 µg/g BPA, respectively, which was over 165- and 220 fold higher, respectively, than that in control oysters.

Furthermore, more BPA accumulated in female than male gonads. Notably, BPA bioaccumulation in the gonads did not reach equilibrium, even after 16 days.

3.2. Sex ratio and the GSI after BPA exposure

The sex ratio of each group was measured after identifying the oysters' sex after the completion of the BPA exposure experiments (Table 2). Exposure to higher concentrations of BPA induced higher mortality rates in the oysters. Significantly more male than female oysters died during BPA exposure; therefore, BPA was more toxic to male than female oysters, which affected the sex ratio (Table 3). However, sex-changed and hermaphroditic oysters were not observed.

Gonadal development was not synchronized and varied among individuals (Fig. 2). BPA clearly affected gonadal development; most of the control male oysters had GSI values of 40–60, whereas males in the BPA exposure groups had GSI values of 20–40. Exposure to high BPA concentrations resulted in more male oysters with GSI values of 20–40, and fewer male oysters with GSI values of 40–60 and >60. Most of the control female oysters had a GSI value of 20–40, with most of the others having GSI values of 40–60. Most females in the 1 mg.L⁻¹ BPA exposure group had GSI values of 40–60, whereas most females in the 2 mg.L⁻¹ BPA exposure group had GSI values of 20–40. Compared to the control females, 2 mg.L⁻¹ BPA exposure resulted in fewer females having GSI values of 40–60. Exposure to 2 mg.L⁻¹ BPA concentrations resulted in fewer oysters having GSI values of >60.

Our results confirm that the GSI is a reliable index with which to evaluate the level of gonadal development in bivalves, and high GSI values indicate good gonadal development.

BPA exposure changed the GSI, and significantly inhibited gonadal development in male oysters. In contrast, exposure to low BPA concentrations could enhance early gonadal development in females. The different changes in the GSI between male and female oysters suggest that differences exist between the sexes in how BPA affects gonadal development.

3.3. Histological analysis

The gonads of control males (Fig. 3A) consisted of dense follicles with abundant radially attached sperm cells. Consequently, the connective tissues between the follicles had almost disappeared, and the follicles were uniformly stained. In the gonads of male oysters exposed to 1.0 mg.L⁻¹ BPA, abundant connective tissue was found between the follicles, and the reproductive cells were not uniformly stained (Fig. 3B). In the gonads of male oysters exposed to 2 mg.L⁻¹ BPA, the follicles were significantly smaller than the control males (Fig. 3C), indicating toxicity in male oysters exposed to 1 mg.L⁻¹ and 2 mg.L⁻¹ BPA.

The gonads of control female oysters contained distinct follicles filled with oocytes, with no visible connective tissue between the follicles (Fig. 3D). The gonads of female oysters exposed to 1 mg.L⁻¹ BPA appeared to be more developed than those of the controls, supporting the GSI results. Large oocytes filled the inner part of the follicular wall, with some developed oocytes being released from the follicular wall (Fig. 3E). However, the 2 mg.L⁻¹ BPA treatment significantly inhibited gonadal development in females. Histological sections of gonads from female oysters exposed to 2 mg.L⁻¹ BPA for 16 days contained shrunken oocytes, and had significantly lower gamete densities than those of the controls. The follicular walls were also partly degraded with many cavities, and follicular connective tissue was

present (Fig. 3F). Therefore, while exposure to low BPA concentrations enhanced gonadal development in female oysters, high BPA concentrations inhibited it.

3.4. Differentially expressed proteins induced by BPA exposure

More than 1100 proteins were identified in each gonadal sample. 245 and 230 differentially expressed proteins were detected within the gonads of oysters exposed to 2 mg.L⁻¹ BPA and control oysters, respectively. Compared to the controls, 33 and 32 proteins were 1.5-fold-upregulated and 0.5-fold-downregulated, respectively (Table 4).

3.5. Real-time PCR analysis of differentially regulated genes

A summary of the denaturing formaldehyde agarose gel electrophoresis results is presented in Supplementary Fig. 1. Distinct 28s RNA, 18s RNA, and 5.8s RNA bands and no smeared bands indicated that the total RNA was of high quality. To verify the reliability of the quantitative proteomics analysis, seven differentially expressed proteins were selected for further quantitative analysis by real-time PCR (Fig. 4). The correlation index of the ratio of the mRNA transcript fold change to the protein expression fold change was 0.989, validating the proteomics results.

4. Discussion

Both male and female oysters quickly accumulated large quantities of BPA in their gonads following exposure, indicating that the oyster's metabolic pathways are inefficient in removing BPA from the gonads. The LC₅₀ (96 h) value was 26.86 ± 3.92 mg.L⁻¹, indicating

that BPA has low acute toxicity in oysters; however, the LC_{50} value from 24 h to 96 h exposure sharply decreased, indicating that long-term exposure to BPA may causes major damage to oysters (Supplementary Table 1). The histological analysis revealed that female gonads were less affected than male gonads, despite female gonads accumulating more BPA. In addition, significantly more male oysters died during BPA exposure than female oysters, so BPA exhibits differential toxicity in male and female oysters, which may change the sex ratio.

BPA-affected proteins clearly differed between male and female oysters, an analysis of which using the online servers of the STITCH workstation (Supplementary Fig. 2) indicated that differentially expressed proteins in male and female gonads are involved in important bioprocesses including energy metabolism, oxidative stress response, gene transcription, protein translation, and the vitellogenin-adjusting process. Increased oxidative stress in the gonads is an important mechanism of BPA-related reproductive toxicity in oysters. BPA induced significantly different expression levels of oxidative response proteins, including Cu/Zn SOD and SOD. Following BPA exposure, SOD and CAT activity recovered more rapidly in female gonads than in male gonads, suggesting that BPA exposure caused lower oxidative stress in female gonads than in male gonads (Fig. 5). Therefore, females may be able to adapt to BPA stress more rapidly than males.

Energy metabolism proteins in the gonads were also affected by BPA exposure, with significant differences between males and females. The differentially expressed proteins induced by BPA exposure that participate in energy metabolism were mainly involved in glycolysis and the tricarboxylic acid cycle in male oysters. The upregulation of these enzymes in male gonads indicates that males need to invest more energy than females to respond to

BPA exposure. In contrast, only 78 kDa of glucose-regulated proteins and adenosine triphosphate (ATP) synthase were upregulated in female gonads, indicating that their energy metabolism was less affected by BPA exposure than the males'. Several histone and ribosomal proteins that are critical for gene transcription and protein translation were significant downregulated in male gonads exposed to 2 mg.L⁻¹ BPA, indicating that BPA noticeably inhibits gene transcription in male gonads, which, in turn, affects gonadal development.

BPA exposure upregulated the expression of transcription elongation factors in the gonads of female oysters but not in male oysters, suggesting that BPA acts as an estrogen-like hormone and regulates gene translation in female gonads. BPA may act as an oxidative stimulus in males by affecting energy metabolism and causing oxidative stress. The positive transcription elongation factor β (P-TEF β) is a cyclin-dependent kinase that controls the elongation phase of transcription by RNA polymerase II (RNAPII). Translation elongation factors are important for the regulation of gene expression (Peterlin and Price, 2006), and are involved in rapid responses to developmental processes or stressors by controlling protein expression (Bar-Nahum et al., 2005).

BPA exposure upregulated vitellogenin (Vtg) expression by more than 20-fold in female gonads but only by 2-fold in male gonads, indicating that Vtg plays a key role in oyster gonads by responding to BPA-induced stress. Vtg is the major precursor of vitellin, which is the egg-yolk protein responsible for energy storage during embryonic development in oviparous organisms. In general, males do not synthesize Vtg, but do produce and release Vtg during exposure to estrogens (Wallace et al., 1985). Vtg is a biomarker of xenoestrogen

exposure in aquatic invertebrates (Matozzo et al., 2008), and is produced and released into the bloodstream of mature females before being stored in developing oocytes. This process is induced by the effects of endogenous estrogens. However, the Vtg gene is usually silent in males until it is activated by (xeno)-estrogens, such as β -estradiol (Matozzo et al., 2008). BPA has a similar molecular structure to (xeno)-estrogens like β -estradiol, and can directly bind to estrogen receptors. BPA may also have estrogenic effects, and can activate Vtg expression (Masaki et al., 2005). BPA exposure sharply upregulated Vtg expression in female gonads, with its effects being more moderate in male gonads. BPA may increase the number of estrogen receptors in female gonads, so sex may affect the efficacy of Vtg as a biomarker when assessing estrogen-like chemicals in pollutants.

Voltage-dependent anion-selective channel protein 2 (VDAC2) participates in the formation of hydrophilic pore structures in membranes, and its expression was upregulated in the gonads of male oysters. VDACs are involved in the flow of particles and metabolic molecules that are transported through the mitochondrial membrane, and interact with Ca^{2+} , ATP, and glutamic acid to regulate the permeability of the outer membrane of the mitochondrion. Consequently, VDACs can adjust mitochondrial functioning (Hinsch et al., 2004; Abu-Hamad et al., 2006). VDAC2 overexpression inhibits Bcl-2 homologous antagonist/killer (BAK) activity, preventing the operation of the mitochondrial apoptotic pathway. Therefore, by regulating BAK activity, VDAC2 affects mitochondrial physiology and the apoptotic pathway (Cheng et al., 2003). Our results confirm that increased VDAC2 expression could interfere with the mitochondrial apoptotic pathway, and prevent the cell apoptosis that is caused by BPA-related damage to the mitochondrion.

Periostin was significantly downregulated in the gonads of male oysters exposed to BPA. Periostin was originally identified as a putative bone adhesion protein in mice, and is mainly involved in regulating the adhesion and differentiation of osteoblasts (Kudo, 2007; Andrew-Priestley et al., 2012). By combining periostin with integrin $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$ increases cell adhesion and spread (Shao et al., 2004). Periostin also activates the Akt/PKB signaling pathway, and it works with $\alpha\text{v}\beta 3$ to increase cell survival (Bao et al., 2004). Moreover, periostin is important for maintaining the balance between cell survival and apoptosis, and for responding to environmental stress (Baril et al., 2007). The downregulation of periostin in the gonads of male oysters exposed to BPA indicates that BPA facilitates apoptosis in gonadal cells, which is consistent with the changes observed in the gonadal tissue that were caused by BPA exposure (Fig. 3C). Periostin also interacts with collagens; Sidhu et al. (2010) reported that periostin regulates the expression of collagens by activating TGF- β to directly upregulate collagen expression. This mechanism involves TGF- β activity in the fibroblast that upregulates collagen expression. Collagens are involved in the development of the testes, particularly type VI collagen (Nakamoto et al., 2004). Therefore, the downregulation of periostin may cause TGF- β activation to decrease, further downregulating the expression of collagens such as type VI collagen. Collagen deficiency may cause testicular dysontogenesis in male oysters exposed to BPA.

The expression levels of several zinc transporter proteins were upregulated in the gonads of both female and male oysters after exposure to BPA. Zinc transporter proteins are important in the intake and outtake of Zn^{2+} and other bivalent metal ions, and maintain Zn^{2+} homeostasis in cells (Sawada and Yazama, 1994). Zinc accumulation in the gonads was

measured using inductively coupled plasma mass spectrometry, which revealed that long-term BPA exposure significantly reduced zinc accumulation in the gonads of both male and female oysters (Supplementary Table 2 and Supplementary Fig. 3.). Therefore, Zn^{2+} homeostasis in the gonads is affected by exposure to BPA, and zinc is an essential metal ion for gonadal development in oysters. Zinc accumulation is dependent upon the developmental stage of the gonad (Adjei-Boateng et al., 2011).

In conclusion, exposure to BPA affects gonadal development differently in male and female oysters, and Zn^{2+} may be involved in the gonadal response to BPA toxicity. To prevent increased BPA bioaccumulation in oysters, BPA concentrations in estuaries and coastal areas where oysters are cultured should be monitored to guarantee food safety. Moreover, BPA concentrations in estuaries, along the coast, and in nursery ponds should be monitored by the companies that culture the oysters, because productivity may be negatively affected by the differential effects of BPA on the development of oyster gonads between the sexes. In order to furtherly reveal the different reproductive toxicity and the mechanisms of BPA on male and female oysters, we should furtherly investigate the chronic toxicity of BPA on oysters and their offspring.

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454 **References**

455 Abu-Hamad, S., Sivan, S., Shoshan-Barmatz, V., 2006. The expression level of the voltage-dependent
456 anion channel controls life and death of the cell. *P. Natl. Acad. Sci.* 103, 5787-5792.

457 Adjei-Boateng, D., Obirikorang K.A., Amisah, S., Madkour, H.A., Otchere, F.A., 2011. Relationship
458 between gonad maturation and heavy metal accumulation in the clam, *Galatea paradoxa* (Born
459 1778) from the Volta estuary, Ghana. *B. Environ. Contam. Tox.* 87, 626-632.

460 Andrew-Priestley, M.N., O Connor, W.A., Dunstan, R.H., Van Zwieten, L., Tyler, T., Kumar, A.,
461 MacFarlane, G.R., 2012. Estrogen mediated effects in the Sydney rock oyster, *Saccostrea*
462 *glomerata*, following field exposures to sewage effluent containing estrogenic compounds and
463 activity. *Aquat. Toxicol.* 120, 99-108.

464 Bao, S., Ouyang, G., Bai, x., Huang, Z., Ma, C., Liu, M., Shao, R., Anderson, R.M., Rich, J.N., Wang,
465 X.F., 2004. Periostin potently promotes metastatic growth of colon cancer by augmenting cell
466 survival via the Akt/PKB pathway. *Cancer Cell* 5, 329-339.

467 Baril, P., Gangeswaran, R., Mahon, P.C., Caulee, K., Kocher, H.M., Harada, T., Zhu, M., Kalthoff, H.,
468 Crnogorac-Jurcevic, T., Lemoine, N.R., 2007. Periostin promotes invasiveness and resistance of
469 pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k
470 pathway. *Oncogene* 26, 2082-2094.

471 Bar-Nahum, G., Epshtein, V., Ruckenstein, A.E., Rafikov, R., Mustaev, A., Nudler, E., 2005. A ratchet
472 mechanism of transcription elongation and its control. *Cell* 120, 183-193.

473 Campbell, C.G., Borglin, S.E., Green, F.B., Grayson, A., Wozel, E., Stringfellow, W.T., 2006.
 474 Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine
 475 disrupting compounds in water: A review. *Chemosphere* 65, 1265-1280.

476 Cheng E.H., Sheiko, T.V., Fisher, J.K., Craiqen, W.J., Korsmeyer, S.J., 2003. VDAC2 inhibits BAK
 477 activation and mitochondrial apoptosis. *Science* 301, 513-517.

478 Crain, D.A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G.A., Guillette Jr., L.J., 2007. An
 479 ecological assessment of bisphenol-A: Evidence from comparative biology. *Reprod. Toxicol.* 24:
 480 225-239.

481 Crain D, Aa Jr LJG, 1997. Endocrine-disrupting contaminants and reproduction in vertebrate wildlife.
 482 *Rev Toxicol.* 1:207–31.

483 Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller,
 484 R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement.
 485 *Endocr. Rev.* 30, 293-342.

486 Flint, S., Markle, T., Thompson, S., Wallace, E., 2012. Bisphenol A exposure, effects, and policy: A
 487 wildlife perspective, *J. Environ. Manag.* 104, 19-34.

488 Grigorakis K., Alexis M.N., 2005. Effects of fasting on the meat quality and fat deposition of
 489 commercial-size farmed gilthead sea bream (*Sparus aurata*, L.) fed different dietary regimes.
 490 *Aquacult. Nutr.* 11, 341-344.

491 Hinsch, K.D., De Pinto, V., Aires, V.A., Schneider, X., Messina, A., Hinsch, E., 2004.
 492 Voltage-dependent Anion-selective Channels VDAC2 and VDAC3 Are Abundant Proteins in
 493 Bovine Outer Dense Fibers, a Cytoskeletal Component of the Sperm Flagellum. *J. Biol. Chem.*
 494 279, 15281-15288.

495 Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenberg, J.G., vom Saal, F.S., 1999.

496 Environmental toxins: Exposure to bisphenol A advances puberty, *Nature* 401, 763-764.

497 Huang, Y.Q., Wong, C.K.C., Zheng, J.S., Bouwman, H., Barra, R., Wahlström, B., Neretin, L.,

498 Wong, M.H., 2012. Bisphenol A (BPA) in China: A review of sources, environmental levels, and

499 potential human health impacts, *Environ. Int.* 42, 91-99.

500 Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawłowski, S.,

501 Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of mollusks and fish to

502 environmental estrogens and estrogenic effluent. *Aquat. Toxicol.* 65, 205-220.

503 Kang, J.H., Asai, D., Katayama, Y., 2007. Bisphenol A in the aquatic environment and its endocrine-

504 disruptive effects on aquatic organisms. *Crit. Rev. Toxicol.* 37, 607-625.

505 Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster,

506 M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T.,

507 Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of

508 endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.*

509 104, 715-740.

510 Nakamura, K., Kariyazono, H., 2010. Influence of endocrine-disrupting chemicals on the immune

511 system. *J. Health Sci.* 56, 361-373.

512 Kaddar, N., Bendridi, N., Harthé, C., de Ravel, M.R., Bienvenu, A.L., Cuilleron, C.Y., Mappus, E.,

513 Pugeat, M., Déchaud, H., 2009. Development of a radioimmunoassay for the measurement of

514 bisphenol A in biological samples. *Anal. Chim. Acta* 645, 1-4.

515

516

517 Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D., 2011. Bisphenol-A: an estrogenic
 518 substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132,
 519 2279-2286.

520 Kudo, Y., 2007. Periostin: novel diagnostic and therapeutic target for cancer. *Histol. Histopathol.* 22,
 521 1167-1174.

522 Maqbool, F., Mostafalou, S., Bahadar, H., Abdollahi, M., 2016. Review of endocrine disorders
 523 associated with environmental toxicants and possible involved mechanisms. *Life Sci.* 145,
 524 265-273.

525 Masaki, N., Shiroyama, K., Inoue, M., Hara, A., Takao, Y., Kohra, S., Ishibashi, Y., Tominaga, N.,
 526 Yoshihara, S., Arizono, K., 2005. Estrogenic Potency of a Bisphenol A Metabolite on
 527 Vitellogenin Synthesis in Medaka, *Oryzias latipes*. *J. Health Sci.* 51, 93-95.

528 Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a biomarker of
 529 exposure to estrogenic compounds in aquatic invertebrates: a review. *Environ. Int.* 34, 531-545.

530 Mills, L.J., Chichester, C., 2005. Review of evidence: Are endocrine-disrupting chemicals in the
 531 aquatic environment impacting fish populations? *Sci. Total Environ.* 343, 1-34.

532 Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., Markert, B., 2000. Effects of endocrine disruptors
 533 on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and
 534 octylphenol as xeno-estrogens. *Ecotoxicology* 9, 383-397.

535 Peterlin, B.M., Price, D.H., 2006. Controlling the elongation phase of transcription with P-TEFb. *Mol.*
 536 *cell* 23, 297-305.

537 Nakamoto, T., Shiratsuchi, A., Oda, H., Inoue, K., Matsumura, T., Ichikawa, M., Saito, T., Seo, S.,
 538 Maki, K., Asai, T., Suzuki, T., Hangaishi, A., Yamagata, T., Aizawa, S., Noda, M., Nakanishi, Y.,

539 Hirai, H., 2004. Impaired spermatogenesis and male fertility defects in CIZ/Nmp4-disrupted mice.
540 GENES to Cells, 9, 575-589.

541 Sawada, H., Yazama, F., 1994. Type VI collagen in the rat testis: monoclonal antibody, isolation, and
542 localization during development. Biol. Reprod. 50, 702-710.

543 Shao, R., Bao, S., Bai, X., Carrie, B., Ryan M., A., Dang, T., Mikhail L., G., Jeffrey R., M.,
544 Wang,X.F., 2004. Acquired Expression of Periostin by Human Breast Cancers Promotes Tumor
545 Angiogenesis through Up-Regulation of Vascular Endothelial Growth Factor Receptor 2
546 Expression. Mol. Cell Biol. 24, 3992-4003.

547 Sidhu, S.S., Yuan, S., Innes, A.L., Kerr, S., Woodruff, P.G., Hou, L., Muller, S.J., Fahy, J.V., 2010.
548 Roles of epithelial cell-derived periostin in TGF- β activation, collagen production, and collagen
549 gel elasticity in asthma. Proc. Natl. Acad. Sci. U. S. A. 107, 14170-14175.

550 Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy,
551 R., Gargas, M., 2001. Reproductive effects of long-term exposure to Bisphenol A in the fathead
552 minnow (*Pimephales promelas*). Environ. Sci.Technol. 35, 2917-2925.

553 Staniszewska M, Koniecko I, Falkowska L, and Krzemyk E. 2015. Occurrence and distribution of
554 bisphenol A and alkylphenols in the water of the gulf of Gdansk (Southern Baltic). Mar Pollut
555 Bull,91, 372-379.

556 Staples, C.A., Dome, P.B., Klecka, G.M., Oblock, S.T., Harris, L.R., 1998. A review of the
557 environmental fate, effects, and exposures of bisphenol A. Chemosphere 36, 2149-2173.

558 Stoker, C., Rey, F., Rodriguez, H., Ramos, J.G., Sirosky, P., Larriera, A., Luque, E.H., Muñoz-de-Toro,
559 M., 2003. Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of
560 the xenoestrogen bisphenol A. Gen. Comp. Endocr. 133, 287-296.

561 Sweeney, M.F., Hasan, N., Soto, A.M., Sonnenschein, C., 2015. Environmental endocrine disruptors:
 562 Effects on the human male reproductive system. *Rev. Endcr. Metab. Disord* 16, 341-357.
 563 Terje, R., Trygve, G., 2005. Selection and breeding programs in aquaculture, Springer Netherlands.
 564 Trasande, L., Zoeller, R.T., Hass, U., Kortenkamp, A., Grandjean, P., Myers, J.P., DiGangi, J.,
 565 Bellanger, M., Hauser, R., Legler, J., Skakkebaek, N.E., Heindel, J.J., 2015. Estimating Burden
 566 and Disease Costs of Exposure to Endocrine-Disrupting Chemicals in the European Union. *J. Clin.*
 567 *Endocrinol. Metab.* 100, 1245–1255.
 568 Mizuo, K., Narita, M., Miyagawa, K., Suzuki, T., 2010. Effects of prenatal and neonatal exposure to
 569 bisphenol A on the development of the central nervous system. *Biomol. Ther.* 18, 125-134.
 570 Wallace R. Vitellogenesis and oocyte growth in non-mammalian vertebrates. In: Browder LW, editor.
 571 Developmental biology. New York: Plenum Press; 1985. p. 127–77.
 572 Sone K, Hinago M, Kitayama A, Morokuma J, Ueno N, Watanabe H, et al. Effects of 17beta-estradiol,
 573 nonylphenol, and bisphenol-A on developing *Xenopus laevis* embryos. *Gen Comp Endocrinol*
 574 2004;138:228–36.
 575 Yang FX, Xu Y, Wen S. Endocrine-disrupting effects of nonylphenol, bisphenol A and *p,p'*-DDE on
 576 *Rana nigromaculata* tadpoles. *Bull Environ Contam Toxicol* 2005;75:1168–75.
 577 Honkanen JO, Holopainen IJ, Kukkonen JV. , 2004. Bisphenol A induces yolk sac edema and other
 578 adverse effects in landlocked salmon (*Salmo salar m. sebago*) yolk-sac fry. *Chemosphere*
 579 55:187–96.
 580
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A **B** **C** **D**

The different effect of the BPA exposure on the gonad development of male and female oyster were observed

BPA were significantly accumulated in the gonad of oysters after exposed to BPA

Male

Female

Label free qualitative and quantitative proteomics discovered that BPA exposure induce the different reproduction toxicities in male and female gonads of oysters.

Table 1: Primers Sequences for the real-time PCR in the BPA-exposure experiment

No.	Gene name	Direction	Primer(5'→3')
1	Vitellogenin	Forward	CAGACTCCCAGTCAAGCCTA
		Reverse	TGACGTCGGACTTCGTTTTTC
2	Superoxide dismutase [Cu-Zn]	Forward	GACGGTGTCGCTAAAATCAG
		Reverse	AACCACGGTTCTTCCAATG
3	14-3-3 protein epsilon	Forward	GACAGGGCTTGTAGGTT
		Reverse	TCAGAGGTCCATAGTGTTA
4	Major egg antigen	Forward	CACCTGGTTGTTGGCATTG
		Reverse	GGGACGGGATTTTGACTG
5	Cavortin	Forward	TTATGCTCAATGCGAGATGGA
		Reverse	GTCTCCCCGTTGATGCATTT
6	78 kDa glucose -regulated protein	Forward	AACGGTGAGGAGAAAGT
		Reverse	ATTGAAGTAAGCAGGGAC
7	Elongation factor 1 alpha	Forward	CGCTCTATTGGCTTTCA
		Reverse	ACCATAACCAGGCTTCATA
8	Beta-actin	Forward	GGTGTAATGGTTGGTATGGGTC
		Reverse	ACGATGCCGTGTTCAATG

Table 2. Mortality rate of oysters caused by BPA exposure, and the male ratio of mortality

	Mortality rate during BPA exposure	Male ratio of the mortality oysters
Control	0%	0
1 mg/L BPA group	7.81%	74.28%
2 mg/L BPA group	13.75%	83.33%

Control: Oysters reared in laboratory under control conditions for 16 days;

1 mg/L BPA group: Oysters exposed to 1 mg/L BPA for 16 days;

2 mg/L BPA group: Oysters exposed to 2 mg/L BPA for 16 days.

Table 3. The death number and change to the gender ratio of oysters following BPA-exposure

		Male		Female		Female-biased ratio (%)		
		After BPA exposure	Total dead number	After BPA exposure	Total death number	After BPA exposure	Total	After BPA exposure
Control	182	182	0	140	140	0	43.48	43.48
1 mg/L								
BPA	165	146	19	155	149	6	48.44	50.51
group								
2 mg/L								
BPA	173	136	37	147	140	7	45.94	50.73
group								

Control: Oysters reared in laboratory under control conditions for 16 days;
1 mg/L BPA group: Oysters exposed to 1 mg/L BPA for 16 days;
2 mg/L BPA group: Oysters exposed to 2 mg/L BPA for 16 days;
Total number: the number of oysters employed for BPA exposure.

Table 4: Significantly regulated proteins in the gonads of oysters exposed to 2 mg/L BPA for 16 days

N o.	Accession ID	Protein name	Source organism	2 mg/L BPA vs Control			
				Male		Female	
				(Folds, mean $\pm$ SD)	<i>P</i> value	(Folds, mean $\pm$ SD)	<i>P</i> value
1	40642728	Phosphoglucumutase	<i>Crassostrea</i> <i>gigas</i>	26.95 $\pm$ 5.03	0.006	-	-
2	405958915	Arginine kinase	<i>Crassostrea</i> <i>gigas</i>	17.17 $\pm$ 2.83	0.004	-	-
3	333449475	Tubulin beta-2B chain-like protein- partial	<i>Crassostrea</i> <i>ariakensis</i>	10.62 $\pm$ 2.26	0.008	-	-
4	117956110	Histone 4	<i>Crassostrea</i> <i>virginica</i>	6.3 $\pm$ 1.49	0.010	-	-
5	405974703	ATP synthase subunit alpha- mitochondrial	<i>Crassostrea</i> <i>gigas</i>	3.64 $\pm$ 0.72	0.007	2.84 $\pm$ 0.56	0.007
6	405962350	Histone H3	<i>Crassostrea</i> <i>gigas</i>	3.61 $\pm$ 0.49	0.002	-	-
7	405970598	Citrate synthase- mitochondrial	<i>Crassostrea</i> <i>gigas</i>	3.44 $\pm$ 0.41	0.002	-	-
8	405945214	Zinc transporter ZIP12	<i>Crassostrea</i> <i>gigas</i>	3.24 $\pm$ 0.08	0.000	-	-
9	405974790	ATP synthase subunit beta- mitochondrial	<i>Crassostrea</i> <i>gigas</i>	2.52 $\pm$ 0.43	0.005	-	-
10	405976072	Actin-binding LIM protein 1	<i>Crassostrea</i> <i>gigas</i>	2.08 $\pm$ 0.27	0.001	-	-
11	24475539	Vitellogenin	<i>Crassostrea</i> <i>gigas</i>	2.02 $\pm$ 0.07	0.001	20.86 $\pm$ 0.76	0.000
12	117956112	Histone 3	<i>Crassostrea</i> <i>virginica</i>	1.75 $\pm$ 0.09	0.002	-	-
13	405963686	Calmodulin	<i>Crassostrea</i> <i>gigas</i>	1.72 $\pm$ 0.24	0.002	-	-
14	405961646	ADP-ATP carrier protein	<i>Crassostrea</i> <i>gigas</i>	1.67 $\pm$ 0.11	0.001	-	-
15	405978841	Voltage-dependent anion-selective channel protein 2	<i>Crassostrea</i> <i>gigas</i>	1.67 $\pm$ 0.19	0.000	-	-
16	405971021	Major egg antigen	<i>Crassostrea</i> <i>gigas</i>	1.63 $\pm$ 0.36	0.014	-	-
17	405963427	Malate dehydrogenase- mitochondrial	<i>Crassostrea</i> <i>gigas</i>	1.63 $\pm$ 0.36	0.015	-	-
18	405963773	ATP synthase subunit	<i>Crassostrea</i>	1.6 $\pm$ 0.04	0.008	-	-

		gamma- mitochondrial	<i>gigas</i>				
19	253769246	Malate dehydrogenase	<i>Crassostrea ariakensis</i>	1.52 ± 0.03	0.012	-	-
20	405968607	78 kDa glucose-regulated protein	<i>Crassostrea gigas</i>	-	-	2.55 ± 0.51	0.008
21	194360223	Elongation factor 1 alpha	<i>Ostrea edulis</i>	-	-	2.23 ± 0.22	0.000
22	48476113	Cavortin	<i>Crassostrea gigas</i>	-	-	0.16 ± 0.02	0.006
23	405960663	Extracellular superoxide dismutase	<i>Crassostrea gigas</i>	-	-	0.16 ± 0.02	0.005
24	313585713	Cu/Zn SOD	<i>Crassostrea hongkongensis</i>	0.46 ± 0.02	0.012	-	-
25	48476115	Cyclophilin	<i>Crassostrea gigas</i>	0.46 ± 0.06	0.007	-	-
26	405954824	Severin	<i>Crassostrea gigas</i>	0.42 ± 0.03	0.009	1.77 ± 0.15	0.027
27	405963584	14-3-3 protein epsilon	<i>Crassostrea gigas</i>	0.42 ± 0.05	0.007	-	-
28	405970466	Periostin	<i>Crassostrea gigas</i>	0.42 ± 0.05	0.007	-	-
29	405965439	40S ribosomal protein S3a	<i>Crassostrea gigas</i>	0.38 ± 0.06	0.005	2.75 ± 0.59	0.009
30	405957670	Peroxiredoxin-6	<i>Crassostrea gigas</i>	0.35 ± 0.02	0.014	-	-
31	405965392	Actin-3	<i>Crassostrea gigas</i>	0.34 ± 0.06	0.019	0.32 ± 0.04	0.006
32	405954419	Collagen VI alpha chain	<i>Crassostrea gigas</i>	0.33 ± 0.03	0.007	-	-
33	405972837	Retinal dehydrogenase 1	<i>Crassostrea gigas</i>	0.25 ± 0.03	0.006	0.02 ± 0.01	0.005
34	405968440	Collagen VI alpha-6 chain	<i>Crassostrea gigas</i>	0.18 ± 0.03	0.009	1.99 ± 0.14	0.000
35	405949998	Fatty acid-binding protein-adipocyte	<i>Crassostrea gigas</i>	0.17 ± 0.03	0.009	-	-
36	405968439	Collagen alpha-1(XII) chain	<i>Crassostrea gigas</i>	0.1 ± 0.05	0.009	3.98 ± 0.81	0.008
37	405953462	ATP synthase subunit delta	<i>Crassostrea gigas</i>	0.07 ± 0.06	0.010	2.84 ± 0.28	0.001
38	405960643	Glutamate receptor	<i>Crassostrea gigas</i>	-	-	0.01 ± 0.01	0.005
39	405973234	40S ribosomal protein S3	<i>Crassostrea gigas</i>	-	-	0.02 ± 0.00	0.005
40	387598255	Heterogeneous nuclear ribonucleoprotein a/b- partial	<i>Ostrea edulis</i>	-	-	0.02 ± 0.01	0.006
41	405974623	26S proteasome non-ATPase regulatory subunit 12	<i>Crassostrea gigas</i>	-	-	0.03 ± 0.01	0.005
42	405968273	Blastula protease 10	<i>Crassostrea gigas</i>	-	-	0.03 ± 0.00	0.005
43	405967949	Elongation factor 1-alpha	<i>Crassostrea gigas</i>	-	-	0.04 ± 0.01	0.005
44	405964146	Protein disulfide-isomerase	<i>Crassostrea gigas</i>	-	-	0.06 ± 0.01	0.005
45	33337655	Collagen receptor related protein	<i>Crassostrea gigas</i>	-	-	0.07 ± 0.03	0.005
46	3668408	Tropomyosin	<i>Crassostrea virginica</i>	-	-	0.12 ± 0.05	0.004
47	405965590	Tubulin beta chain	<i>Crassostrea gigas</i>	-	-	0.21 ± 0.02	0.006
48	405950944	Phospholipase D1	<i>Crassostrea gigas</i>	-	-	0.27 ± 0.04	0.005
49	405962093	Calreticulin	<i>Crassostrea gigas</i>	-	-	0.27 ± 0.06	0.004
50	405961012	Superoxide dismutase	<i>Crassostrea gigas</i>	-	-	0.39 ± 0.07	0.004
51	405951433	Tubulin beta chain	<i>Crassostrea gigas</i>	-	-	0.39 ± 0.12	0.034

52	56603668	Alpha-tubulin	<i>Crassostrea gigas</i>	-	-	0.39 ± 0.04	0.007
53	32968056	Superoxide dismutase	<i>Crassostrea gigas</i>	-	-	0.50 ± 0.06	0.009
54	405973740	Translationally-controlled tumor protein	<i>Crassostrea gigas</i>	-	-	1.86 ± 0.14	0.000
55	405971213	Zinc transporter 9	<i>Crassostrea gigas</i>	-	-	5.75 ± 0.63	0.002
56	357529498	Vitellogenin 2	<i>Crassostrea gigas</i>	-	-	20.86 ± 2.17	0.002

-: No significant regulation was observed in the gonad of BPA exposed oysters compared to the control.

^a: Change to protein abundance was expressed as the average ratio of BPA exposure test/control (mean ± SD, n = 3).

A value >1.5 represents upregulation, whereas a value <0.5 represents downregulation.

^b: The statistical significance in the change of abundance was assessed using **one way ANOVA test**. p < 0.05 was considered significant.

Figures captions

Fig.1. Amount of BPA bioaccumulation in the gonads of *Crassostrea angulata* oysters (2mg/L group). *: T-test showed a significant difference in BPA accumulation between the gonads of male and female oysters with $p < 0.05$ (n=3).

Fig. 2. Changes to the gonad-somatic index (GSI) in *Crassostrea angulata* oysters exposed to BPA. Control, 1 ppm, and 2 ppm represent oysters exposed to no BPA, 1 mg/L BPA, and 2mg/L BPA, respectively, for 16 days. GSI = Gonad weight/soft body weight $\times 100\%$ (n=90).

Fig. 3. Histological observations of the gonads of *Crassostrea angulata* oysters exposed to BPA (Hematoxylin and eosin staining, $\times 200$). A. male control group; B. male 1 mg/L group; C. male 2mg/L group; D. female control group; E. female 1mg/L group; F. female 2mg/L group.

Fig. 4. Difference in the abundance of mRNA abundance in the gonads of male and female *Crassostrea angulata* oysters exposed to 2 mg/L BPA for 16 days versus no exposure (control) revealed by Real-time PCR. T-test was used to assess significance at $*P < 0.05$ and $**P < 0.01$ (n=3).

Fig. 5. Variation in SOD and CAT activity in the gonads of *Crassostrea angulata* oysters when exposed to 1 mg/L and 2 mg/L BPA. A: SOD activity in the gonads of male oysters; B: SOD activity in the gonads of female oysters; C: CAT activity in the gonads of male oysters; D: CAT activity in the gonads of female oysters. The average of the SOD and CAT activities are used (mean $\pm$ SD, n = 3)