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The potential immune modulatory effect of chronic bisphenol A exposure on gene regulation in male medaka (*Oryzias latipes*) liver

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ABSTRACT

Bisphenol A (BPA) is a well-known estrogenic endocrine disrupting chemical (EDC) ubiquitously present in various environmental media. The present study aims to identify the responsive genes in male fish chronically exposed to low concentrations of BPA at the transcription level. We screened genes from a suppression subtractive hybridization library constructed from male medaka (*Oryzias latipes*) livers after 60-d exposure to 10 μg/L BPA under the condition at which changes of hepatic antioxidant parameters have been previously reported. The identified genes were predicted to be involved in multiple biological processes including antioxidant physiology, endocrine system, detoxification, notably associated with the immune response processes. With real time PCR analysis, the immune-associated genes including *hepcidin-like precursor*, *complement component and factors*, *MHC class I*, *alpha-2-macroglobulin* and *novel immune-type receptor 6* isoform were significantly up-regulated in a nonmonotonic dose response pattern in livers upon exposure to different concentrations of BPA (0.1, 1, 10, 100, 1000 μg/L). Our results demonstrated a negative impact on gene regulation in fish chronically exposed to relatively low and environmentally relevant concentrations of BPA, and suggested the potential immune modulatory effect of chronic EDC exposure on fish. The immunotoxicity of BPA and other EDCs should be much concerned for the health of human beings and other vertebrates exposed to it.

1. Introduction

Endocrine disrupting chemicals (EDCs) have been defined as exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development and/or behavior (USEPA, 2010). Bisphenol A (BPA), a well-known EDC and an estrogen-mimicking chemical, is a building block of polycarbonate plastic which has been widely employed in daily life such as in polycarbonate baby bottles, reusable water bottles, polyvinyl chloride stretch films and metallic food can inner coatings (Vandenberg et al., 2009). Since 2010, many countries including Canada, European countries, China and the USA have banned BPA from children's products due to its suspected toxicity during early development. As one of the highest volume chemicals produced worldwide, continuous exposure to BPA is evident base on the presence of detectable levels of BPA in more than 90% of the US population (Lang et al., 2008). The presence of BPA is also commonly identified at μg/L to even low mg/L or mg/kg in aquatic systems all around the world (Huang et al., 2012). Although BPA has a relatively short half-life in aquatic environment with low bio-accumulation to aquatic organisms, its detrimental effects to aquatic animals are still well documented by a series of investigations (Kang et al., 2007; Rogers et al., 2013).

Bisphenol A is classified as a slightly to moderately toxic substance based on its acute toxicity to adult fish, however, it can significantly impact the morphology, growth and sexual differentiation, as well as the behavior and histological structure of fish embryos/larvae/fry during their early life stages (Pastva et al., 2001; Lam et al., 2011; Kinch et al., 2015). The physiological endocrine disrupting effects are the most studied property of BPA. In reports, BPA is described as acting as an estrogen agonist, which produces potential harm to fish reproduction including alteration of sex determination, alteration of gonadal function and induction of hepatic vitellogen in production (Crain et al., 2007). However, interestingly, BPA has weak estrogenic potency and is approximately 15,000-fold less active in its affinity to estrogenic receptor α than an endogenous estradiol (E2), according to a yeast-based steroid hormone receptor gene transcription assay (Gaido et al., 1997). Since

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many studies conducted with both a low and a high dose of BPA show effects at the low dose that are not apparent after exposure to the high dose, and based on the observation of the nonmonotonic dose response curves of BPA, the low-dose effects of BPA have been proposed for further studies (Vandenberg, 2014). The results from genotoxicity and immunotoxicity studies show that low BPA exposure can cause chromosomal damage by significantly increasing micronuclei frequency in the erythrocytes of fish, as well as functional changes of macrophage and lymphocyte, such as enhanced bactericidal activity, which differ from the inhibitory effects of a higher dosage of BPA (Rezg et al., 2014; Yang et al., 2015). Furthermore, studies indicate that BPA exposure can lead to an alteration of oxidative stress indices and antioxidant parameters either in fish embryos/larvae and adult fish or in a rat model (Wu et al., 2011a; Xu et al., 2013; Hassan et al., 2012). Therefore, BPA effects, especially the subtle, low-dose effects of BPA, are not likely to be defined as just "classical" estrogenic effects. However, limited information on the action mechanism of BPA has been reported.

Japanese medaka (*Oryzias latipes*) is a small, oviparous, freshwater and seawater-tolerant fish, native to Asia, for which extensive information on physiology, embryology and genetics has been developed. As a benefit of its known genetic information, medaka provides us with a convenient basis to thoroughly investigate the effects of EDCs, especially at molecular levels. The fish liver, like the liver of other vertebrates, is a key organ which control many life functions and play a prominent role in fish physiology associated with digestion, storage, reproduction, blood turn-over, nitrogen catabolism, detoxication as well as immune defense (Brusle and Anadon, 1996). Our previous data reveal that 60-day exposure to BPA has changed antioxidant defense parameters in medaka livers responding to a series of BPA concentrations within an environmentally relevant range, indicating an induction of antioxidant system promotion at a low and environmentally relevant dose of 10 μg/L BPA exposure (Wu et al., 2011b). In the present study, we constructed a forward suppression subtractive hybridization (SSH) cDNA library from the livers of adult male medaka following 60-day exposure to 10 μg/L BPA for the purpose of screening and identifying the genes in fish in response to chronic BPA exposure. A series of selected genes were then separately assayed by real time PCR for expression patterns following various concentrations of BPA exposure. Our aim was to obtain a better understanding of the action mechanism of low doses of BPA's adverse effects at the transcriptional level.

2. Materials and methods

2.1. Chemicals

Bisphenol A was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 10 g/L which was then stored at 4 °C for subsequent exposure experiments.

2.2. Animals

Medaka eggs (*Oryzias latipes*, orange red strain) were kindly provided by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. They were cultured to adults in our laboratory using aerated dechlorinated tap water at 25 ± 1 °C in a static-renewal system under a 14 h light: 10 h dark cycle. The fish were fed with brine shrimp *(Artemia nauplii*) twice daily. Adult male medaka fish were used in BPA exposure experiments. The experiments were conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shanghai University, China.

2.3. BPA exposure and sample collection

Adult male medaka fish (nine-month old) were randomly distributed and incubated in 5 mL glass tanks containing different concentrations of BPA for 60 d. Six fishes were placed in each tank. A blank control group and a vehicle control group (0.005% DMSO) were set in parallel and the test was replicated at least thrice for each treatment group. BPA solutions were completely replaced daily with fresh ones. The BPA concentrations for exposure were 0.1, 1, 10, 100 and 1000 μg/L, based on results obtained from the 72 h-LC_{50} for male medaka (Kashiwada et al., 2002) and from previous studies involving fathead minnow (Sohoni et al., 2001) and C. riparius (Lee and Choi, 2007). Bisphenol A did not degrade to an appreciable level under the conditions of these experiments as measured in our previous study (Wu et al., 2015). The fish were fed normally during the exposure period except for the last day prior to sampling. After 60-day exposure, body length and weight were determined. Livers from six fish randomly selected from each treatment group were individually collected and frozen in liquid nitrogen, and stored at −80 °C.

2.4. Subtractive cDNA library construction

The total RNA was extracted from each collected liver sample using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Equal aliquots of RNA from six fish livers were mixed as one sample pool for each treatment group to lower the individual difference within groups. The cDNA synthesis was performed from 1 μg of total RNA using a SMARTer PCR cDNA Synthesis Kit (Clontech) following the manufacturer's protocol. Suppression subtractive hybridization was performed using a PCR-select cDNA Subtractive Kit (Clontech) following the manufacturer's instructions. In brief, the cDNA synthesized from the fish livers exposed to 10 μg/L BPA was chosen to be a tester pool, while an equal quantity of cDNA synthesized from the fish livers exposed to solvent was used as a driver pool, which was subtractively hybridized by the tester to isolate genes which expression was induced after BPA exposure. The concentrations of 10 μg/L was chose based on the information of BPA concentration in the water streams (Kolpin et al., 2002) and the threshold value (10 μg/L) for response of adult medaka suggested in our previous study (Wu et al., 2011b). Briefly, SMART cDNA from the control (the driver) and the 10 μg/L BPA treatment (the tester) was digested with Rsa I to generate short, blunt-ended double-stranded cDNA. After phenol extraction and ethanol precipitation, the Rsa I-digested Tester was ligated with Adaptor 1 and Adaptor 2 at the 5′-end of each strand of the tester cDNA. The adaptor 1-ligated and the adaptor 2-ligated tester cDNAs were then separately hybridized with excess driver cDNA at 68 °C for 8 h after denaturation at 98 °C for 90 s. The sequences of adaptor 1 and adaptor 2 are as follows:

Adaptor 1: 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCG-CCCGGGCAGGT-3′.

3′-DGGCCCGTCCA-5′

Adaptor 2: 5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGG-CCGAGGT-3′.

3′-GCCGGCTCCA-5′.

After the first and the second subtractive hybridization processes, the differentially expressed gene fragments were obtained, and then amplified by two rounds of suppressive PCR for enrichment. The amplicons were purified by size using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol and ligated into a T/A cloning vector PMD18-T (TaKaRa), and then transformed into competent *Escherichia coli* TOP10 cells. The recombinant clones containing differentially expressed gene fragments were plated onto Luria-Bertani agar plates supplemented with 100 μg/mL ampicilin and incubated at 37 °C overnight.

2.5. Gene sequencing and annotation

The recombinant clones were randomly selected from the agar plates. Polymerase chain reactions (PCRs) were performed with vector primers to identify the positive clones, which contained the plasmids with the inserted fragment of about 400–2000 bp and these were then sequenced using ABI 3730 automated sequencers (Applied Biosystems) at Shanghai Jie Li Biotechnology Co. Ltd, China. The gene sequences obtained were trimmed for vector and adaptor sequences and quality assessed using DNAssist 2.0 software. Homology searches were performed against the non-redundant database of the National Center for Biotechnology Information using BLASTn, BLASTx and BLASTp programs (www.ncbi.nlm.nih.gov). A significant match was selected based on a reliable expectation (E) value of $\leq 10^{-5}$ between our sequences and the NCBI database match. Go annotations for putative proteins were obtained employing AmiGo against the GO database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) or using the QuickGo browser searching from the EBI database (Binns et al., 2009). Proteins (genes) were categorized via their associated Go information using STRAP software (Bhatia et al., 2009), and then pie charts were created to interpret the variety of biological process, cellular component, and molecular function GO terms in our protein list obtained from the SSH library.

2.6. Quantitative Reverse Transcriptase–PCR (qRT-PCR)

The genes, which were screened from the SSH library in the male medaka liver with increased expression levels induced after the 10 μg/L BPA exposure, were tested for their expression profiles responding to different concentrations of BPA exposure using a relatively quantitative RT-PCR method. The RT reactions were performed with 100 ng of total RNA from a six fish liver RNA mixture using an iScript™ cDNA Synthesis kit (Bio-Rad, USA) following the manufacturer's instructions, and qRT-PCR was conducted using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, USA) in an iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad). The endogenous reference gene 18 s rRNA which has been validated as a suitable control gene for expression profiling in studies on the EDCs issue using medaka (Zhang and Hu, 2007), was used for normalizing the target gene expression for each tested sample. The sample from the solvent control liver was used as the calibrator sample and, as a result, the relative quantity of the target genes were presented as the fold difference to solvent control and calculated by the formula $2^{-\Delta\Delta Ct}$. The PCR reaction cycle was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and the fluorescent signals were measured at the annealing/extension step. After reaction, the PCR specificity was checked by performing the melting curves of amplicons and agarose gel electrophoresis. For each target gene, qRT-PCRs were performed in triplicate. The sequences of the primers are shown in the Supplementary Data (Table S1).

2.7. Statistical analysis

Gene expression in each treatment group was indicated as the fold difference to the solvent control group, i.e. the mean fold changes ± standard error of the mean. SPSS Statistics software (version 18.0) was used for statistical analysis. All data were checked for normality and homogeneity of variance based on the Kolmogorov–Smirnov one-sample test and Levene's test. One-way analysis of variance (ANOVA) followed by Duncan's test was used to evaluate the significance of differential gene expression, and the level was set at $p < 0.05$ or 0.01 shown as "*" or "**", respectively.

3. Results

3.1. Effects of BPA exposure on survival of fish

There were no marked effects of BPA on survival of adult fish. In all groups, the length and weights of fish after 60-day exposure to BPA were also not significantly affected. No differences were observed between the blank and solvent control group in any of the cases, and thus the solvent group was used as the control.

3.2. cDNA sequencing and annotation summary

A total of 355 among the randomly selected clones from the SSH library were confirmed as positive recombinants. Among them, 79.2% had 500–1000 bp inserts, and 12.4% had fragments over 1000 bp, and 8.45% with fragments lower than 500 bp. After a medium-scaled sequencing program, a total of 344 cDNA sequences were obtained. Among them, 255 sequences were homologous to the known genes released in the NCBI database with an E-value under 10^{-5}, and corresponded to 63 genes. These genes were considered as candidates responsible for susceptibility to BPA exposure. Using AmiGO online analysis software against the Gene Ontology database, these genes are categorized into 11 groups (Table 1). Of the responding genes, 11 genes are involved in the oxidation-reduction process including two genes, catalase (*cat*) and Glutathione S-transferase (*gst*) which are directly related to oxidative stress. Ten genes are associated with the developmental and sexual reproduction processes and 10 genes encode components associated to the immune system. In addition, 12 genes have biological regulatory activity including six genes which had enzyme regulator activities, and eight genes as binding proteins.

Furthermore, with the use of STARP software, the genes were divided into more detailed categories in accordance with different biological processes, cellular components and molecular functions (Fig. 1). Consistent with the above AmiGo online analysis results, the identified genes are potentially involved in multiple biological processes, notably associated with immune system processes, and achieved 23%. While of the molecular functions, binding proteins are the largest category with 41% possession.

3.3. Gene expression profiles using qRT-PCR

To identify and confirm genes in response to chronic BPA exposure, the expression patterns of a series of selected genes which were screened from the SSH library were quantitatively determined in male medaka livers after exposure to different concentrations of BPA. Compared to the control group, most selected genes showed up-regulated expression after different concentrations of BPA exposure, but linear dose-response curves were lacking and the folds induced varied. Based on the highest fold induced by BPA exposure, the mRNA transcripts of 20 genes were significantly up-regulated more than two fold, of which 13 genes were increased more than five fold after exposure to different concentrations of BPA (Table 2). Interestingly, a group of dioxin-responsive genes including *cytochromeP450 1A* (*cyp1a*), *hepcidin-like precursor*, *L-FABP*, *ependymin*, *tributyltin-binding protein type 1* were significantly up-regulated upon 60-d exposure to BPA as shown in Table 3 In particular, the expressions of a *hepcidin-like precursor* gene and a gene encoding for liver-type fatty acid-binding protein (L-FABP) were dramatically induced up to 100 fold in the 0.1 or 100 μg/L of BPA-treated fish livers. Moreover, a group of immune response related genes were also significantly up-regulated upon different concentrations of BPA exposure (Table 3), including three complement genes (*complement factor properdin-like*, *complement factor H* and *complement component C9*), two major histocompatibility complex (MHC) genes (*classical MHC*

Table 1
List of genes identified from a suppression subtractive hybridization cDNA Library from male medaka livers exposed to 10 μg/L bisphenol A for 60 days.

Gene description	Gene symbol	Taxonomy	Accssion no.	Length (aa)	E-value	Clone amount
Oxidation-reduction process						
Cytochrome b (complex III)	cytb	*Oryzias latipes*	BAH84905.1	380	0	4
Cytochrome b-c1 complex subunit 7 (complex III)	QCR7	*Esox lucius*	ACO13731.1	110	2.00E−39	1
Cytochrome c oxidase subunit I (complex IV)	COI	*Oryzias latipes*	BAH84895.1	518	0	2
Cytochrome c oxidase subunit II (complex IV)	COII	*Oryzias latipes*	BAH84896.1	230	0	7
Cytochrome c oxidase subunit III (complex IV)	COIII	*Oryzias latipes*	BAC23845.1	261	0	5
Cytochrome P450 1A	CYP1A	*Oryzias latipes*	NP_001098557.1	521	0	1
NADH dehydrogenase 1 alpha subcomplex subunit 4 (complex I)	NDUFA4	*Anoplopoma fimbria*	ACQ58140.1	82	6.00E−12	1
NADH dehydrogenase subunit 4 (complex I)	ND4	*Oryzias latipes*	BAE72156.1	460	0	1
Tumor suppressor candidate 4 (Nitrogen permease regulator 2)	tusc3	*Salmo salar*	ACI34281.1	379	3.00E−63	2
Response to oxidative stress						
Catalase	N/A	*Oplegnathus fasciatus*	AAU44617.1	527	2.00E−67	2
Glutathione S-transferase	N/A	*Oplegnathus fasciatus*	AAU44618.1	223	2.00E−31	1
Biosynthetic process						
60S ribosomal protein L17	RL17	*Oncorhynchus mykiss*	ACO07686.1	184	8.00E−14	1
Mitochondrial nucleoside diphosphate kinase	Nme4	*Perca flavescens*	ADX97129.1	75 (partial)	2.00E−06	1
Ubiquitin	Ubq	*Siniperca chuatsi*	ADK27292.1	128	0	1
Catabolic process						
Chitinase3	fChi3	*Paralichthys olivaceus*	BAD15061.1	477	4.00E−27	1
Uridine phosphorylase 2	Upp2	*Hypophthalmichthys nobilis*	ACO51107.1	150	1.00E−61	2
Other metabolic process						
ATP synthase subunit O, mitochondrial precursor	ATP50	*Anoplopoma fimbria*	ACQ58932.1	207	2.00E−173	1
Apolipoprotein A-I	N/A	*Rachycentron canadum*	ACV50420.1	263	3.00E−18	13
Mitochondrial uncoupling protein 1	N/A	*Siniperca chuatsi*	ACI32421.1	313	1.00E−89	1
System development						
Alpha-2-macroglobulin	A2m	*Perca flavescens*	ACO36147.1	521	3.00E−86	7
Ependymin	epd	*Perca flavescens*	ABU49423.1	215	8.00E−52	4
Gamma-aminobutyric acid receptor-associated protein	GBRAP	*Salmo salar*	NP_001136189.1	122	2.00E−16	1
Inter-alpha (globulin) inhibitor H4 isoform 1	ITIH4	*Danio rerio*	AAH96879.1	915	4.00E−18	1
Novel immune-type receptor 6 isoform 1	NITR6	*Oryzias latipes*	EU419362.1	285	1.00E−124	1
Precerebellin-like protein	CBLNL	*Oncorhynchus mykiss*	AAF04305.2	182	1.00E−30	1
Serotransferrin precursor	tf	*Oryzias latipes*	NP_001116384.1	690	2.00E−165	3
Transgelin 2	N/A	*Sparus aurata*	ADO67627.1	198	2.00E−84	1
Sexual Reproduction						
Choriogenin H	Chg H	*Oryzias latipes*	BAA13994.1	591	0	1
Female expressed transcript 1	N/A	*Gallus gallus*	AAM52407.1	434	1.00E−07	1
Immune system						
Beta-2-microglobulin	Orla-B2m	*Oryzias latipes*	NP_001098130.1	116	5.00E−60	2
Classical MHC class I molecule, alpha-chain	Orla-UAA	*Oryzias latipes*	NP_001098573.1	356	6.00E−56	1
Complement component C3-1/C3-2	Orla C3-1/C3-2	*Oryzias latipes*	NP_001098552.1/ NP_001098553.1	1658/1657	7E−21/1E−58	40
Complement component 4 (within H-2S)	Orla C4	*Oryzias latipes*	NP_001098167.1	1718	4.00E−75	9
Complement component C9	N/A	*Oncorhynchus mykiss*	CAJ01692.1	601	1.00E−57	1
Complement factor B (bf/c2 protein)	Bf/C2	*Oryzias latipes*	NP_001098275.1	754	1.00E−92	5
Complement factor H	cfh	*Oncorhynchus mykiss*	AJ627206.1	827	6.00E−07	5
Complement factor properdin-like precursor	si:dkeyp-22b2.3	*Danio rerio*	NP_001153598.1	437	1.00E−09	1
Complement regulatory plasma protein	N/A	*Paralabrax nebulifer*	AAA92556.1	1053	2.00E−16	55
Hepcidin 5 precursor	N/A	*Acanthopagrus schlegelii*	AAU00798.1	86	3.00E−07	4
Biological regulation						
Annexin max2	N/A	*Oryzias latipes*	CAA72123.1	317	0	1
Apolipoprotein A-I	apoa1	*Rachycentron canadum*	ACV50420.1	263	3.00E−18	13
ChaC, cation transport regulator homolog 1	CHAC1	*Xenopus (Silurana) tropicalis*	AAI67509.1	196	1.00E−35	2
Cyclin G1	ccng1	*Danio rerio*	AAQ97992.1	299	1.00E−132	1
Fibrinogen, gamma polypeptide	fgg	*Danio rerio*	AAH45868.1	431	2.00E−42	1
Rho GTPase-activating protein 7-like	N/A	*Danio rerio*	XP_001921774.2	1091	4.00E−13	1

Table 1 (*continued*)

Gene description	Gene symbol	Taxonomy	Accssion no.	Length (aa)	E-value	Clone amount
Enzyme regulator activity						
Inter-alpha trypsin inhibitor	N/A	*Fundulus heteroclitus*	AAU21481.1	201	6.00E−77	1
Probable N-acetyltransferase camello	CML	*Dicentrarchus labrax*	CBN80767.1	223	9.00E−08	2
Protein Z-dependent protease inhibitor	serpina1l	*Danio rerio*	XP_003200452.1	429	8.00E−45	1
Serine peptidase inhibitor, clade F, member 2	serpinf2	*Oncorhynchus mykiss*	NP_001185743.1l	437	3.00E−60	1
Serine/Cysteine proteinase inhibitor	N/A	*Xiphophorus helleri*	ACO40442.1	401	7.00E−122	1
Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	serpina7	*Danio rerio*	NP_001005972.1	372	3.00E−30	2
Binding protein						
26S proteasome non-ATPase regulatory subunit11	PSD11	*Salmo salar*	ACN58663.1	413	3.00E−101	1
Coagulation factor V precursor	F5	*Takifugu rubripes*	AAO33375.1	1802	1.00E−54	1
Insulin-like growth factor I	N/A	*Paralichthys olivaceus*	AAC62228.1	186	5.00E−63	1
Liver-type fatty acid-binding protein	N/A	*Epinephelus coioides*	ADG29164.1	126	3.00E−118	17
Tributyltin binding protein type 2-like protein	N/A	*Epinephelus coioides*	ACJ26846.1	231	4.00E−19	1
Tributyltin-binding protein type 1	TBT-bp1	*Oryzias latipes*	AB568120.1	218	2.00E−29	3
Vertebrate human immunodeficiency virus type I enhancer binding protein 2 (HIVEP2)-like	CH211-232D9.5-001	*Danio rerio*	XP_696438.4	1123	1.00E−22	1
Zinc finger protein 36, C3H type-like 1 like	zfp36l1b	*Danio rerio*	AAH63991.1	348	3.00E−44	3
Others						
Erythroblast membrane-associated protein	N/A	*Danio rerio*	XP_696219.3	1192	1.00E−32	2
Tetraodon nigroviridis full-length cDNA	N/A	*Tetraodon nigroviridis*	CR690467.2	618mRNA	1.00E−29	2
Uncharacterized protein	N/A	*Dicentrarchus labrax*	CBN80766.1	183	3.00E−22	1
Unnamed protein product	N/A	*Tetraodon nigroviridis*	CAF91835.1	180	6.00E−06	3

class I molecule, *α-chain* and *beta-2-microglobulin*, which are the two subunits of *MHC class I*), two acute phase protein (APP) genes (*alpha-2-macroglobulin* and *fibrinogen*, *gamma polypeptide*), as well as a *novel immune-type receptor* (*NITR*) *6 isoform*, which has been proposed to be a "functional orthodox" of mammalian natural killer receptor in teleost species and functions within innate immunity to regulate cell mediated cytotoxicity (Yoder, 2009).

4. Discussion

In the present study, to identify responsive genes to chronic exposure of BPA and understand the potential mechanism of low dose effect of BPA, an SSH library was constructed from male adult medaka liver following 60-day exposure to a low and environmentally relevant dose of BPA exposure. Our results showed that the identified genes from SSH cDNA library were involved in multiple biological processes, providing abundant information about the negative impacts of chronic low concentrations of BPA exposure on gene expression regulation in male madaka. Since the fish we used for SSH cDNA library construction were supposed to be under oxidative stress caused by chronic BPA exposure according to our previous study (Wu et al., 2011b), 11 genes involved in the oxidation-reduction process were identified from the SSH library. Among them two antioxidant enzymes which are vital at first-line defense against oxygen toxicity and directly respond to oxidative stress, *cat* and *gst* were identified from the BPA-treated fish livers. These observations, in turn, implied the occurrence of oxidative stress in fish liver, which are in accordance with the previous observation on the BPA's effects (Wu et al., 2011a, 2011b; Xu et al., 2013).

Also, a number of estrogen-responsive genes reported previously were also recognized from our SSH library. Of those genes, *choriogenin H* is a sensitive biomarker for monitoring estrogenic EDCs in the aquatic environment. Lee et al. (2002a, 2002b, 2007) demonstrate that *choriogenin H* transcript level can be induced in a dose-dependent manner upon 6-day treatment with BPA, nonylphenol (NP), and 17-α-ethinylestradiol (EE_2). Similarly, consistent with previous studies on the gene screening in fish tissues after acute or short-term treatment with environmental estrogens (EEs), e.g. in 28-h E_2-treated sea bream (*Sparus aurata*) (Pinto et al., 2006) and in 4-day E2, NP, 2-chlorophenol (2CP) and BPA treated medaka (Pham et al., 2011), we obtained identical genes in BPA-treated medaka after 60-day exposure, including *choriogenin H*. Moreover, consistent with the study on E_2-treated sea bream Pham et al. (2011), according to the molecular functions, we found that binding protein genes, enzyme regulator activity genes and system development genes are the largest group of identified genes in BPA-treated male medaka livers.

On the other hand, it should be noted that many immune system associated genes were identified from the SSH library by chronic BPA exposure. Among them, several genes encode complement components and factors, as well as regulatory proteins. A group of immune response associated genes including *complement component and factors*, *MHC class I*, *APPs* and *NITR* were significantly induced in fish liver upon chronic BPA exposure. Our results implied that chronic low concentration of BPA exposure might have a significant impact on the modulation of immune response related gene expression in fish, particularly on the regulation of acute phase response (APR)-associated genes. Fish are the earlier vertebrate group to display a full immune response but are very dependent on innate immune mechanisms (Magnadóttir, 2006). Complements are essential components of the innate immune system and provide an important link between adaptive and innate immune responses (Iwasaki and Medzhitov, 2015). MHCs produce the primary response that slows down invading pathogens, and they also play an important role in initiating the adaptive immune response by presenting foreign antigens at the cell surface for recognition by cytotoxic T cells (Dixon and Stet, 2001). Acute phase proteins are blood proteins primarily synthesized by hepatocytes participating in the acute phase response, which is a prominent systemic reaction of the organism to local or systemic

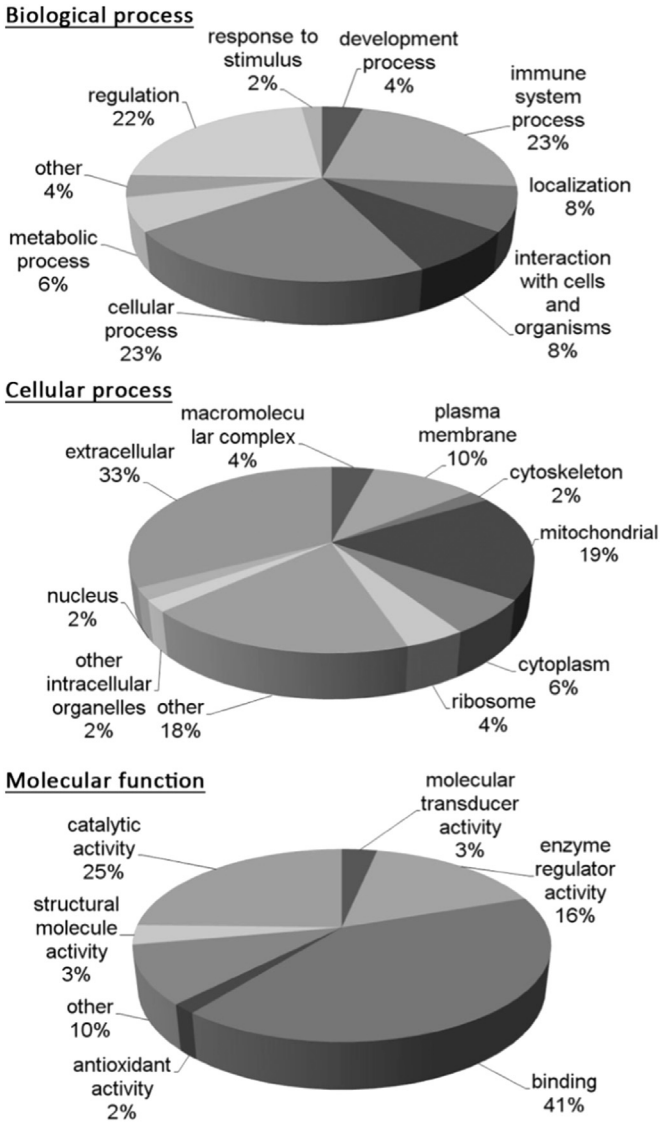

Fig. 1. Distribution of differentially expressed genes identified from a suppression subtractive hybridization cDNA Library constructed from male medaka livers exposed to 10 μg/L bisphenol A for 60 days. Genes were categorized via their associated Go information using STRAP software, and percentages of genes in each of functional category are listed to interpret the variety of biological process, cellular component, and molecular function.

disturbance in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders (Gruys et al., 2005). The acute phase response in fish is proposed to be more robust than that in mammals for the compensation of a less-evolved adaptive immune response (Cray et al., 2009). In the present study, *hepcidin precursor*, *complement component C9,* α*-2-macroglobulin* and *fibrinogen*are all positive APPs, and they have multiple functions including modulating the immune system and tissue protection from damage by the inflammatory process.

Since the close bidirectional interactions between the endocrine system and immune system, it is reasonable to propose that EDCs may also interfere with the immune response considering their disruption to the endocrine system. Evidence is accumulating that BPA interferes with immune functions in rodent and fish models, as well as *in vitro* immune cells (Youn et al., 2002; Ohnishi et al., 2008; Milla et al., 2011; Yang et al., 2015). Jin et al. (2010) and Xu et al. (2013) indicate that exposure to BPA and some other EDCs can significantly induce the gene expression levels of several pro-inflammatory cytokines and chemokines and it is accompanied by an occurrence of oxidative stress caused by chemical exposure. Oxidative stress may be induced by a diverse source of environmental stress when the balance between the production and the depletion of reactive oxygen species (ROS) is broken in biological systems. The immune system is vulnerable to oxidative stress due to the increase of ROS levels, and thus, oxidative stress has been hypothesized to be associated with environmental stress-induced inflammation and plays important roles in the etiology of chronic diseases (Lubrano et al., 2013). Regulated by various pro-inflammatory cytokines, APPs are also excellent non-specific markers of diverse inflammatory etiologies. From our results, APR-associated genes including *hepcidin-precusor*, *complement components and factor*, α*-2-macroglobulin* and other factors associated with non-specific and specific immune response were all significantly up-regulated upon chronic BPA exposure, the condition under which oxidative stress has been reported previously (Wu et al., 2011a). Taken together, our observations on the APR-associated gene up-regulation implied a link of oxidative stress with inflammation in the effects of BPA. The chronic low concentrations of BPA exposure might promote inflammatory response reactions in fish, and we proposed that it might be associated with the regulation of cytokine and chemokine synthesis or release which was possibly correlated with BPA-induced ROS pathways. Similarly, Yang et al. (2009) also found a significant association between BPA exposure and oxidative stress and inflammation markers in postmenopausal women by a cross-sectional study.

Table 2
The highest fold changes of responsive genes in male medaka liver upon various concentrations of bisphenol A exposure after 60 days.[a]

> 50 fold	20–50 fold	5–20 fold	2–5 fold	1–2 fold
Hepcidin 5 precursor	*Zinc finger protein 36, C3H type-like 1*	*Novel immune-type receptor 6 isoform 1*	*Tumor suppressor candidate 4*	*Fibrinogen, gamma polypeptide*
Liver-type fatty acid-bingding protein			*Alpha-2-macroglobulin*	*Glutathione S-transferase*
	Mitochondrial uncoupling protein 1	*Complement factor H*	*Beta-2-microglobulin*	*Inter-alpha (globulin) inhibitor H4 isoform 1*
	Complement factor properdin-like	*Cytochrome P450 1A*	*Tributyltin binding protein type 2-like protein*	*ATP synthase subunit O, mitochondrial precursor*
		Ependymin	*Complement component C9*	
		Protein Z-dependent protease inhibitor	*Ubiquitin*	
		Classical MHC class I molecule, alpha-chain	*26S proteasome non-ATPase regulatory subunit11*	
		Catalase		
		Cytochrome oxidase subunit I		

[a] The quantity of mRNA was normalized by reference gene *18s rRNA* using comparative quantitative real-time PCR.

Table 3
The expression of selected genes in male medaka livers identified from SSH library in response to various concentrations of bisphenol A exposure after 60 days using comparative quantitative real-time PCR.

Genes	The exposure concentrations of bisphenol A (μg/L)				
	0.1	1	10	100	1000
Cytochrome P450 1A	19.87 ± 3.84 **	0.87 ± 0.25	7.22 ± 2.03	12.07 ± 0.84**	4.72 ± 0.56
Hepcidin 5 precursor	84.13 ± 16.75**	2.09 ± 0.44	9.60 ± 3.65	129.26 ± 19.48**	2.25 ± 0.42
Liver-type fatty acid-binding protein	111.56 ± 18.87**	6.09 ± 1.25	4.86 ± 0.98	47.93 ± 9.42*	13.77 ± 3.53
Ependymin	8.35 ± 0.45 **	2.57 ± 0.54	7.68 ± 1.37**	10.74 ± 1.96**	2.26 ± 0.39
Tributyltin binding protein type 2-like protein	2.80 ± 0.41**	3.18 ± 0.19**	1.35 ± 0.39	3.17 ± 0.13**	0.68 ± 0.02
Complement factor properdin-like	1.99 ± 0.28	24.41 ± 0.58**	7.52 ± 0.52**	1.33 ± 0.03	0.63 ± 0.09
Complement factor H	5.55 ± 0.82	0.10 ± 0.06	12.83 ± 4.77**	1.27 ± 0.03	0.15 ± 0.01
Complement component C9	1.16 ± 0.09	3.32 ± 0.77**	0.73 ± 0.35	0.78 ± 0.07	0.40 ± 0.01
Classical MHC class I molecule, alpha-chain	4.98 ± 2.09	5.14 ± 0.85	1.25 ± 0.44	3.00 ± 0.96	2.87 ± 0.47
Beta-2-microglobulin	0.87 ± 0.07	1.89 ± 0.13	3.08 ± 0.47**	1.19 ± 0.43	1.12 ± 0.09
Alpha-2-macroglobulin	4.22 ± 0.85**	4.31 ± 0.17**	0.77 ± 0.12	1.88 ± 0.19	1.96 ± 0.29
Fibrinogen, gamma polypeptide	0.42 ± 0.07*	0.20 ± 0.00**	1.76 ± 0.25**	0.48 ± 0.04*	0.43 ± 0.14*
Novel immune-type receptor 6 isoform 1	6.17 ± 2.80*	0.97 ± 0.12	1.73 ± 0.42	2.55 ± 0.09	0.66 ± 0.06

Data are expressed as a mean ± S.D. (n = 3) of the fold changes to the control. '*' and '**' indicate significant difference between the treatment group and the control group, representing $p < 0.05$ and $p < 0.01$, respectively (one way ANOVA followed by Duncan's test).

CytochromeP450 proteins (CYPs) are a superfamily of heme-containing monoxygenases that are responsible for metabolism of most xenobiotics and required for the efficient elimination of foreign chemicals from the body. Previous studies reveal that CYPs are associated with the effects of some EDCs on fish, such as NP and EE2 (Ishibashi et al., 2004; Zhang et al., 2008; Qiu et al., 2015). Consistent with those studies, a *cyp1a* gene was identified in the present study, and the up-regulation of *cyp1a* expression upon different concentrations of BPA exposure was confirmed. The expression of *CYP1A* was significantly up-regulated in a non-monotonic dose-response pattern upon BPA exposure, indicating a stimulating trend in those with relatively low and BPA treatment groups. This finding is consistent with previous work showing that non-linear dose responses are commonly observed for hormones and endocrine-disrupting chemicals (Vandenberg, 2014; Iwanowicz et al., 2014). We interpreted these findings to indicate that the effects of BPA on the expression of CYP1A depends on the duration of exposure and exposure concentrations. CYP1As are involved in phase I xenobiotic and drug and steroid (especially estrogen) metabolism. The expressions of xenobiotic metabolizing enzymes such as CYP1As and some other oxidation-reduction enzymes are directly controlled by AHR. Nishizawa et al., (2005a, 2005b) reported that exposure *in utero* to BPA at even extremely low-dose (0.02 μg/kg/day, 1/100 of the dose of environmental exposure) increases *AHR* mRNA expression in the cerebra, cerebella, and gonads (testes and ovaries) of male and female mid- and late-developmental stage murine embryos, and they revealed that BPA exposure *in utero* disrupts the expression of *AHR* and related factors (*AHR repressor* and *AHR nuclear translocator*, *ARNT*) and the expression and hepatic protein levels of xenobiotic metabolizing enzymes including cyp1a1. Similarly, our results indicated a dramatic *cyp1a* mRNA induction after BPA exposure, especially upon the relatively lower concentration of BPA exposure at 0.1 μg/L; and the highest induction of *cyp1a* mRNA reached up nearly 10 fold of that in the control group. Also, Bonefeld-Jorgensen et al. (2007) have reported that the effects of weak estrogenic EDCs, such as BPA, BPA dimethacrylate, NP and OP can be mediated via several cellular pathways, including AHR pathway. These findings suggested that chronic low concentration of BPA exposure might disturb cellular metabolism as a result of xenobiotic metabolizing, which might directly or indirectly activate AHR pathway.

Hepcidin is a β-defensin-like antimicrobial peptide of the innate immune defense system and also a principal regulator of systemic iron homeostasis. In concordance with this dual function its expression is modulated by systemic iron requirements, and in response to infectious and inflammatory stimuli (Verga Falzacappa and Muckenthaler, 2005). Hepcidin genes have been widely identified in fish in recent years. Generally, fish hepcidins are regarded as antimicrobial peptides due to their broad spectrum antimicrobial activity *in vitro* and their dramatically induced expression patterns after bacterial or lipopolysaccharide challenge (Yang et al., 2007). Meanwhile, Wang et al. (2009) indicate that exposure to benzo[*a*]pyrene, another AhR-dependent carcinogen, can also increase *hepcidin* expression in fish embryos/larvae and adult livers, and the induced expression pattern of hepcidin is comparable to that of *cyp1a1*. Interestingly, our results showed similar comparable induced expression patterns between *hepcidin-like* and *cyp1a* in medaka livers exposed to 0.1 μg/L and 100 μg/L BPA. Since *hepcidin* and *cyp1a* in medaka both contain putative AHREs in the upstream of their transcriptional start sites (Volz et al., 2006), the observation on the joint induction of these genes further suggested that AHR pathway might be involved in the effects of BPA.

In short, this study revealed the immunomodulatory effect of BPA on male Japanese medaka at a relatively low but environmentally relevant concentration. Our findings provide further evidence that BPA interferes with the immune regulation of fish following chronic exposure to low concentrations in *in vivo* conditions. Adverse effects on the immune system upon BPA exposure were observed and the direct or indirect involvement of an activated AHR pathway was suggested for its mechanism of action. The data provided in the study shall strengthen our understanding of BPA immunotoxicity in fish.

5. Conclusion

In summary, the present study demonstrated that the immune components, especially those involved in the innate immune system response in fish were highly susceptible to BPA exposure, and even low concentrations of 0.1 and 1 μg/L BPA at environmental relevant levels could modulate the immune gene expression after long-term exposure. According to Volz et al. (2006), most toxicant-induced gene expression changes occur early could precede hepatocellular toxicity and inflammation in the liver afterwards, and these transcriptional events upon the toxicant exposure are significantly associated with the downstream toxicant-induced hepatic alterations observed at the histologic level. Therefore, based on our results, we presumed that the inflammatory response reactions might be induced in fish livers following chronic low concentrations

of BPA exposure; and as these reactions are persistently induced, the inflammatory response could possibly lead to chronic local inflammation eventually. Since BPA is a common contaminant surrounding our daily life and it is ubiquitous in the environment, the adverse effects of BPA and other EDCs on the immune function and their immunotoxicity should not be ignored for the health concerns of human beings and other vertebrates exposed to it.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.04.015.

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