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Alternations in oxidative stress, apoptosis, and innate-immune gene expression at mRNA levels in subadult tiger puffer (*Takifugu rubripes*) under two different rearing systems

Yudong Jia[a,b,*], Qiqi Jing[a], Jieming Zhai[c], Changtao Guan[a], Bin Huang[a,**]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao Key Laboratory for Marine Fish Breeding and Biotechnology, Qingdao, 266071, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[c] Ming Bo Aquatic Co. Ltd., Laizhou, 261400, China

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ABSTRACT

Tiger puffer (*Takifugu rubripes*) is one of the major aquaculture fish species in China due to its high economic value. In this study, the transcriptions of hepatic antioxidant enzyme, stress, apoptosis, and immune-related genes of sub-adult tiger puffers (*Takifugu rubripes*) were evaluated under two different rearing systems [offshore sea cage aquaculture system (OSCS) and recirculating aquaculture system (RAS)]. Results showed that the mRNA expression levels of the antioxidant enzyme (*mn-sod*, *cu/zn-sod*, *gpx*, and *gr*) and stress-related (*hsp70* and *hsp90*) genes of male tiger puffers reared in the OSCS were significantly higher than female fish reared in the OSCS and fish reared in the RAS. The anti-apoptotic gene *bcl2* exhibited the similar results. By contrast, the mRNAs of the pro-apoptotic genes (*p53*, *caspase8*, *caspase9*, and *caspase3*) of male tiger puffers reared in the OSCS were significantly lower than female fish reared in the OSCS and fish reared in the RAS. Male tiger puffers reared in the OSCS displayed significantly higher complement components (*c3*) and inflammatory cytokine (*il-6*) mRNAs, whereas B-cell activating factor (*baf*) and tumor necrosis factor α (*tnf-α*) mRNAs remained unchanged. Meanwhile, the mRNA levels of pro-apoptotic (*bax*, *caspase8*) and immunity-related (*c3, il-6* and *il-7*) genes of female tiger puffers reared in the OSCS were significantly lower and higher than female fish reared in the RAS, respectively. In conclusion, the hepatic antioxidant, anti-apoptosis, and innate immunity of tiger puffers reared in the OSCS were better than fish in the RAS, male tiger puffer obtained the best values. These results expand the knowledge on the combined RAS and OSCS alternative aquaculture model for tiger puffers and aid in their management in captive.

1. Introduction

Tiger puffer (*Takifugu rubripes)* is an anadromous fish that is widely distributed in the Japan Sea, the East China Sea and the Yellow Sea. Given its excellent taste and abundant nutrients, this puffer species is the most commercially viable and highly traded [1]. However, the wild resource of pufferfish has sharply declined since the late 1980s because of overfishing [2]. To restore the population of this species and satisfy market demand, scientists have established techniques for broodstock management, artificial induction of maturation and ovulation, and seed production [3–5]. Tiger puffer have gradually become one of the major reared fish species because of its high-market value in China since the last century. The tiger puffer aquaculture is mainly distributed across the northern coast of China, and the annual production of farmed tiger puffers reaches approximately 5000 metric tons, and nearly all the produced tiger puffers are exported to Japan and South Korea as senior food [6].

An offshore sea cage aquaculture system (OSCS) and a recirculating aquaculture system (RAS) are two main aquaculture systems developed for tiger puffer aquaculture in the northern coast of China. In general, tiger puffers are cultured in an OSCS from June to November and then transferred to a RAS with heating equipment to overwinter. Tiger puffers are again switched back to the OSCS until the temperature of natural water increases to above 20 °C in June in the following year. The OSCS aquaculture that adopts natural ecological farm conditions can reduce marine organism mortality, improve growth rates, and

* Corresponding author. Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences No. 106 Nanjing Road, Qingdao, 266071, China.
** Corresponding author.
E-mail addresses: jiayd@ysfri.ac.cn, ydjia2011@hotmail.com (Y. Jia).

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decrease visceral fat [7–10]. The temperature, dissolved oxygen and water flow exchange of OSCS are different from those of RAS. As the important economic culture species in the OSCS, fish displays a wide variation in survival, physiological functions, and immune defense responses to various forms of environmental factors-that induce stress. On a molecular level, undesirable stress that is often manifested by excessive free radical production, is known as oxidative stress. However, stress does not necessarily imply pathology, and moderate stress can activate cell immunity, stimulate metabolism, and maintain homeostasis [11–13]. Our previous studies found the growth performance and metabolic state of subadult female tiger puffers reared in the OSCS are better than those of the fish reared in the RAS [14], suggesting that the stress resistance of the former is better than that of the latter. Liver is the central organ of metabolism with a considerable energy capacity and critical for animal health and body condition. In the current study, hepatic comparative transcriptomic profiles of tiger puffer reared in the RAS and OSCS found totally 703 common differentially expressed genes (DEGs), which were primarily enriched in Gene Ontology (GO) terms such as metabolism, single-organism, binding and catalytic activity etc., as well as in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways include metabolic pathway, microbial metabolism in diverse environment, phagosome and biosynthesis of antibiotic (supplemental data, S1). In addition, the apoptosis, immune and stress-related gens of tiger puffer manifest significantly difference of tiger puffer reared in the RAS and OSCS (supplemental data, S2). To fully understand the physiological differences of subadult tiger puffers reared in RAS and OSCS for short periods, while provide basic data for the design of combined land (RAS) and sea (OSCS) alternative aquaculture models in tiger puffer. Thus, we firstly investigated genes mainly related to antioxidant enzymes [superoxide dismutase (*mn-sod*, *cu/zn-sod*), catalase (*cat*), glutathione peroxidase (*gpx*), and glutathione reductase (*gr*)], apoptosis [*bax*, *bcl2*, *p53*, and caspases (*caspase8*, *caspase9*, and *caspase3*)], stress [heat shock proteins 70 and 90 (*hsp70*, and *hsp90*)], and innate immunity [complement (*c3*), B-cell activating factor (*baf*), tumor necrosis factor α (*tnfα*), and interleukin (*il-6*, *il-7*, *il-12*)] at the mRNA level in subadult tiger puffers under the two different rearing systems. This study may provide valuable data for further understanding of the stress tolerance and immune molecular mechanism of tiger puffer reared in OSCS and RAS.

2. Materials and methods

2.1. Animal rearing conditions and sampling schedule

Tiger puffer with a mean weight of 432.94 ± 21.29 g were obtained from Mingbo Aquatic Co., Ltd. (Laizhou, Shandong, China) and used in the present trial. Prior to the experiment, the fish were acclimatized to laboratory conditions for 14 days. Subsequently, fish were randomly distributed into the RAS (three tanks, 5 m × 5 m × 1.5 m per tank) with 100 individuals per tank and the OSCS (three sea cages, 10 m × 10 m × 7.5 m per sea cage) with 2000 individuals per sea cage. The fish were fed with frozen sand lance (*Ammodytes personatus*) twice a day at 07:00 and 18:00 until satiation in RAS and OSCS, respectively. The sand lance contained 73% moisture, 59% crude protein, 17% crude lipid, and 13% crude ash of dry matter. Water temperature, salinity, pH, dissolved oxygen, and ammonia nitrogen in the RAS and the OSCS were detected three times every week throughout the experimental period. The experimental period was from June 20, 2017 to August 29, 2017, totally 70 days. At the end of the experiment, the fish were fasted for 24 h and anesthetized with 100 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO) before harvest. The liver was collected from three females and three males in each trial tank and sea cage after the fish were sacrificed. The specimens were preserved in RNAstore reagent (Tiangen Biotech, Beijing, China) and then transfer to lab stored at −80 °C until RNA extraction.

2.2. RNA extraction and real-time quantitative polymerase chain reaction (RT-PCR)

A two-step, real-time RT-PCR was performed to detect the expression of antioxidant enzyme genes (*mn-sod*, *cu/zn-sod*, *cat*, *gpx*, and *gr*), apoptosis (*bax*, *bcl2*, *p53*, *caspase8*, *caspase9*, and *caspase3*), innate immunity-related (*baf*, *c3*, *tnfα*, *il-6*, *il-7*, and *il-12*) and heat shock proteins (*hsp70*, and *hsp90*). Total RNA was extracted from the liver of tiger puffers by use Trizol reagent (GIBCO-BRL, Carlsbad, CA, USA). The quality and quantity of total RNA were analyzed on 1% agarose gel electrophoresis and using a UV spectrophotometer. The Total RNA (1 μg) was DNase treated using the TURBO DNA-free Kit (Life Technology), and reverse transcribed with the Thermo One step RT-PCR kit according to the manufacturer's instructions. The mRNA expression levels of *mn-sod*, *cu/zn-sod*, *cat*, *gpx*, *gr*, *bax*, *bcl2*, *p53*, *caspase8*, *caspase9*, *caspase3*, *baf*, *c3*, *tnfα*, *il-6*, *il-7*, *il-12*, *hsp70*, and *hsp90* were assessed by real-time RT-PCR using TaKaRa RT-PCR Master Mix reagent and ABI StepOne Pluse Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A final volume of 20 μL, which contained 10 μL SYBR Green PCR mix, 6.0 μL sterile distilled water, 2.0 μL cDNA template, 0.4 μL of ROX dye and 0.8 μL each of forward and reverse primers. A no-primer control treatment for each template used 2.0 μL TE in the reaction mixture instead of primers. The thermal program used was 95 °C for 10 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Disassociation curves for each sample were analyzed on all plates. The primers for aforementioned genes were obtained from Cheng et al., 2015, 2017 [15,16] and listed in Table 1. Four commonly used housekeeping genes [18S ribosomal RNA (*18s*), beta-actin (*β-actin*), glyceraldehyde-3-phosphate-dehydrogenase (*gapdh*) and elongation factor 1-alpha (*ef1α*)] were evaluated to compare the CT values of a subset of samples. Evaluation revealed that *β-actin* is the most constantly expressed housekeeping gene and utilized as the endogenous control in this study. All samples were amplified in triplicates. The aforementioned genes expression was normalized to *β-actin* and expressed as a folded change relative to the expression level in the control according to the $2^{-\Delta\Delta CT}$ method [17].

2.3. Statistical analysis

Data were analyzed via one-way analysis of variance (ANOVA) and Tukey's multiple-range tests using the SPSS 16.0 software [18,19]. All data are presented as the means ± standard error of the mean. In all statistical tests used, $P < 0.05$ was considered significantly different.

3. Result

3.1. Water parameters in RAS and OSCS

The weekly average water temperature, salinity, pH, dissolved oxygen and ammonia nitrogen in RAS and OSCS were calculated and shown in Fig. 1. Water temperature was stable and showed no significantly change during 70-day short-term experiment in RAS (Fig. 1A, $P > 0.05$). However, water temperature significantly increased at four weeks later (Fig. 1A, $P < 0.05$; July 18) and remained unchanged until experiment complete (Fig. 1A, $P > 0.05$; from July 18 to August 29) in OSCS. Water salinity, pH, dissolved oxygen and ammonia nitrogen manifested no significantly difference in RAS and OSCS throughout 70-day short-term experiment (Fig. 1B, C, D, E, $P > 0.05$).

3.2. Antioxidant enzyme gene expression

The antioxidant enzyme-related genes manifested different expression patterns under the two different rearing systems (Fig. 2). The hepatic expression levels of *mn-sod*, *cu/zn-sod*, *gpx*, and *gr* of the male tiger puffers cultured in the OSCS were significantly higher than those of the female tiger puffers in the OSCS and the fish in the RAS (Fig. 2,

Table 1
Primers for real time RT-PCR.

Genes	Accession No	Primer sequence (5′ to 3′)	Product length (bp)
BAF	XM_011607861.1	CCTTCCTCTCAGCAGTGTCC CCGCCTCAAAGACAGAAAAG	108
TNF-α	NM_001037985.1	TCGTGGTGGTCCTCTGTTGC CTTGGCTTTGCTGCTGATGC	98
IL-6	NM_001032722.1	GCTGGAAAACAAGGTGAGGG TGTGGAAGGTGTCGGGGTAGT	127
IL-7	NM_001136148.1	CGCAGCTGAGATTCAAGGCA TTCTGCAGGCTAGCTTGTGC	106
IL-12	AB096268.1	AGACGGACGGGAGCAGTGGC GGTCTGGCTGTGGCAGGTGT	158
C3	XM_003972086.2	ATTCGTGGCTGCTACTGTGCT GGGCTGCTGTCAGGATTCAA	145
Mn-SOD	EF667049.1	AGATGTCCGCCGCTACAGTTGC GCCAAGGAGCGGGATGAGACC	121
Zn-SOD	EF667050.1	GCCGACAGGCATGTTGGAGACC CAGGTCGTCGGCCTTCTCGTG	125
CAT	EF667050.1	TGAGCCAAGCCCTGACAAGATGC GGTAGTTGGCCACACGGGTTCTGTGT	111
GPX	FJ418581.1	TGTCCGTCCTGGAGGTGGTTTCG ACTTTGGGTCAGCCATGAGAGC	104
GR	EF667051.1	CTCACACCTGTTGCCATTGCTGC GCCTCCTCTTCTGTTAGTCCCAC	135
Bax	XM_003964782.2	GGAGGAGGCGATCAAGGCAATTC TGCTGTCTCCATGTCGTTGAACAC	126
Bcl-2	KP898414.1	GCGTCTCCATCCGCAGGTGC TGCCGCGGCGTCGTCCCC	117
P53	XM_011606669.1	GCTTGGAAAATGAGCAATGGCACTC CTCGGAGTAGGTGGAGGTGACG	135
Caspase-9	XM_003978475.2	ATCGTCCAGTTATCCAACCCCTTC GGCTTCAGTCTCATGTACTCCCGC	110
Caspase-8	XM_011616711.1	GCTGCTCCACACTATCCATCGAAA AGACCCTTCTTTTCCATTTCAGTAA	104
Caspase-3	NM_001032699.1	CGAGGGCGTGTTTTTTGGT GGGATCTTGGTGGTGCTGC	105
HSP90	EU853673.1	TTTGGTGTGGGATTTTACTCAGCCTAC TTGTCCGTCCTGACTGTAAATGAACCT	119
HSP70	FJ429326.1	GCAGAAGCCTACCTCGGAAAGAC CGCCAAGATCAAAAATCAACACG	101
β-actin	U37499.1	CAGGGAGAAGATGACCCAGA CATCACCAGAGTCCATGACG	125

BAF: B-cell activating factor; TNF-α: tumor necrosis factor α; IL-6: interleukin 6; IL-7: interleukin 7; IL-12: interleukin 12; C3: complement component 3; Mn-SOD: manganese-superoxide dismutase; Cu/Zn-SOD: cooper/zinc superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; Bax: Bcl2-associated X protein; Bcl-2: B-cell lymphoma-2; P53: tumor protein p53; Caspase-9: cysteinyl aspartate specific proteinase 9; Caspase-8: cysteinyl aspartate specific proteinase 8; Caspase-3: cysteinyl aspartate specific proteinase 3; HSP70: heat shock protein 70; HSP90: heat shock protein 90.

$P < 0.05$), whereas the *cat* mRNA levels of the tiger puffers were not significantly different between the RAS and the OSCS (Fig. 2C, $P > 0.05$). The hepatic mRNA levels of *mn-sod*, *cu/zn-sod*, and *gr* of the female tiger puffers in the OSCS were remarkably higher than those of the female tiger puffers in the RAS (Fig. 2A, B, E, $P < 0.05$). The hepatic mRNA levels of *gpx* of the female tiger puffers were not significantly different between the RAS and the OSCS (Fig. 2D, $P > 0.05$).

3.3. Apoptosis-related gene expression

The mRNA levels of the main genes involved in cell apoptotic signaling processes in the tiger puffer liver were altered under the two different rearing systems (Fig. 3). The hepatic expression levels of *bax*, *caspase8*, and *caspase3* of the tiger puffers cultured in the RAS were significantly higher than those of the tiger puffers cultured in the OSCS (Fig. 3B, D, F, $P < 0.05$). By contrast, the hepatic *bcl2* mRNA levels of the tiger puffers cultured in the RAS were significantly lower than those of the male tiger puffers cultured in the OSCS (Fig. 3C, $P < 0.05$). The hepatic mRNA levels of *bax*, *caspase8*, and *caspase3* of the male and female tiger puffers reared in the RAS and the OSCS showed no significant changes (Fig. 3B, D, F, $P > 0.05$). The hepatic *p53* and *caspase9* mRNA levels of the male tiger puffers cultured in the OSCS were significantly lower than those of the female tiger puffers in the OSCS and those of the fish cultured in the RAS (Fig. 3A, E, $P < 0.05$). However, no significant differences in the hepatic mRNA levels of *p53* and *caspase9* were observed between the female tiger puffers cultured in the OSCS and the fish cultured in the RAS (Fig. 3A, E, $P > 0.05$).

3.4. Immune-related gene expression

The mRNA levels of the innate immune system-related genes displayed different expression patterns under the two different culture systems. The hepatic mRNA levels of *c3* and *il-6* of the male tiger puffers cultured in the OSCS were significantly higher than those of the fish in the RAS (Fig 4A, D, $P < 0.05$), whereas the *baf* and *tnf-α* mRNA levels of the tiger puffers were not significantly different between the RAS and the OSCS (Fig. 4B, C, $P > 0.05$). The mRNA levels of *il-12* exhibited no significant differences between the same gender (female vs. female, male vs. male) in the RAS and the OSCS (Fig. 4F, $P > 0.05$). The hepatic *il-7* mRNA levels of the male and female tiger puffers were not significantly different in the RAS and the OSCS, respectively (Fig. 4E, $P > 0.05$).

3.5. Heat shock protein gene expression

The hepatic mRNA levels of *hsp70* and *hsp90* of the tiger puffers

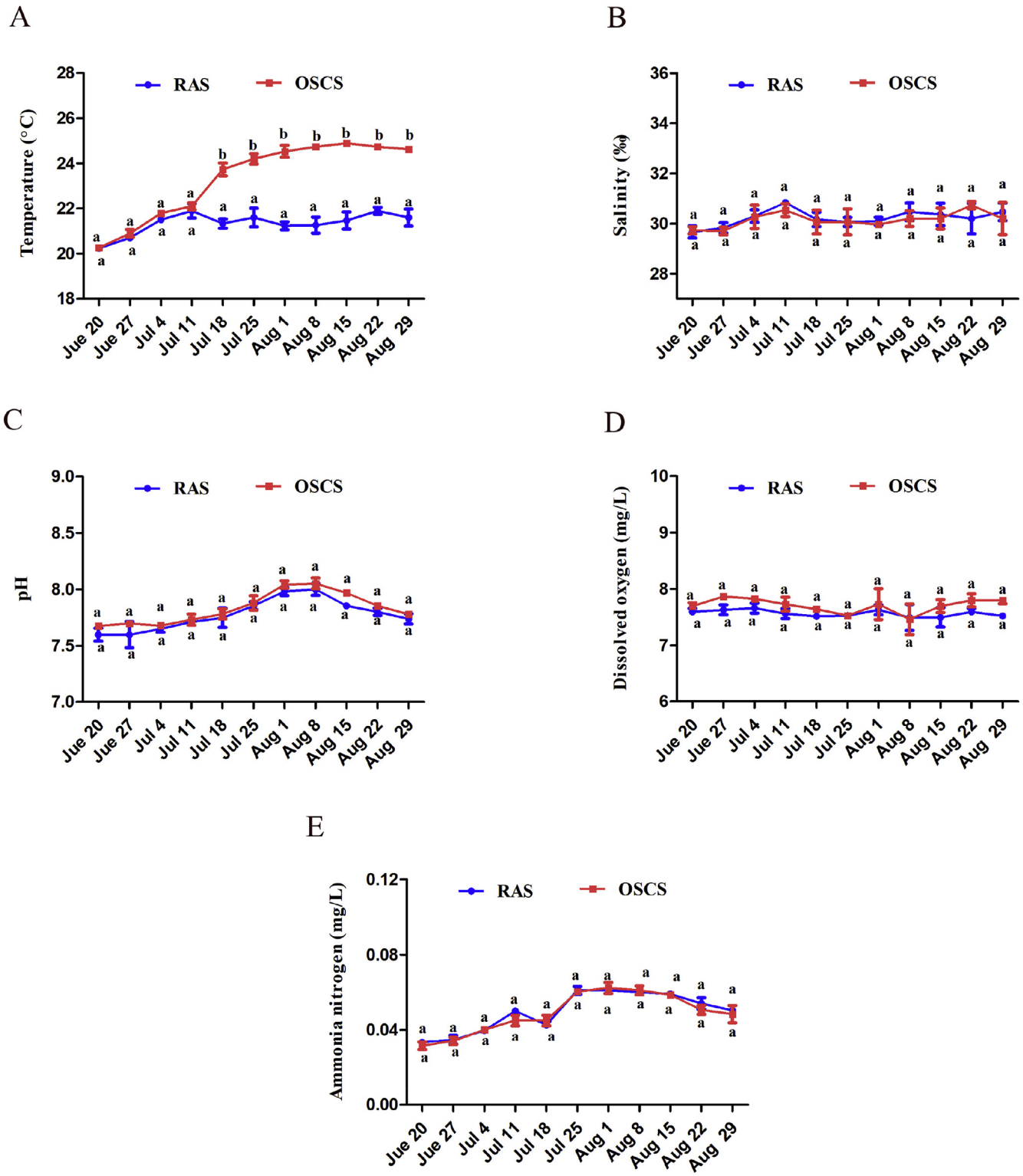

Fig. 1. Comparison of water temperature (A), salinity (B), pH (C), dissolved oxygen (D) and ammonia nitrogen (E) in RAS and OSCS throughout experiment period. Bars with different superscripts are statistically different ($P < 0.05$, n = 3).

cultured in the OSCS were significantly higher than those of the fish in the RAS (Fig. 5, $P < 0.05$). Similarly, the hepatic mRNA levels of *hsp70* and *hsp90* of the male tiger puffers were significantly higher than those of the female tiger puffers cultured in the RAS and the OSCS (Fig. 5, $P < 0.05$).

4. Discussion

Our recent study has identified that the growth performance and metabolic state of the female tiger puffer reared in the OSCS are superior to those of the male fish in the OSCS and the fish in the RAS [14]. Wang et al. (2016) found the male fish grew better than the female in open-indoor combination aquaculture system, however the trend was

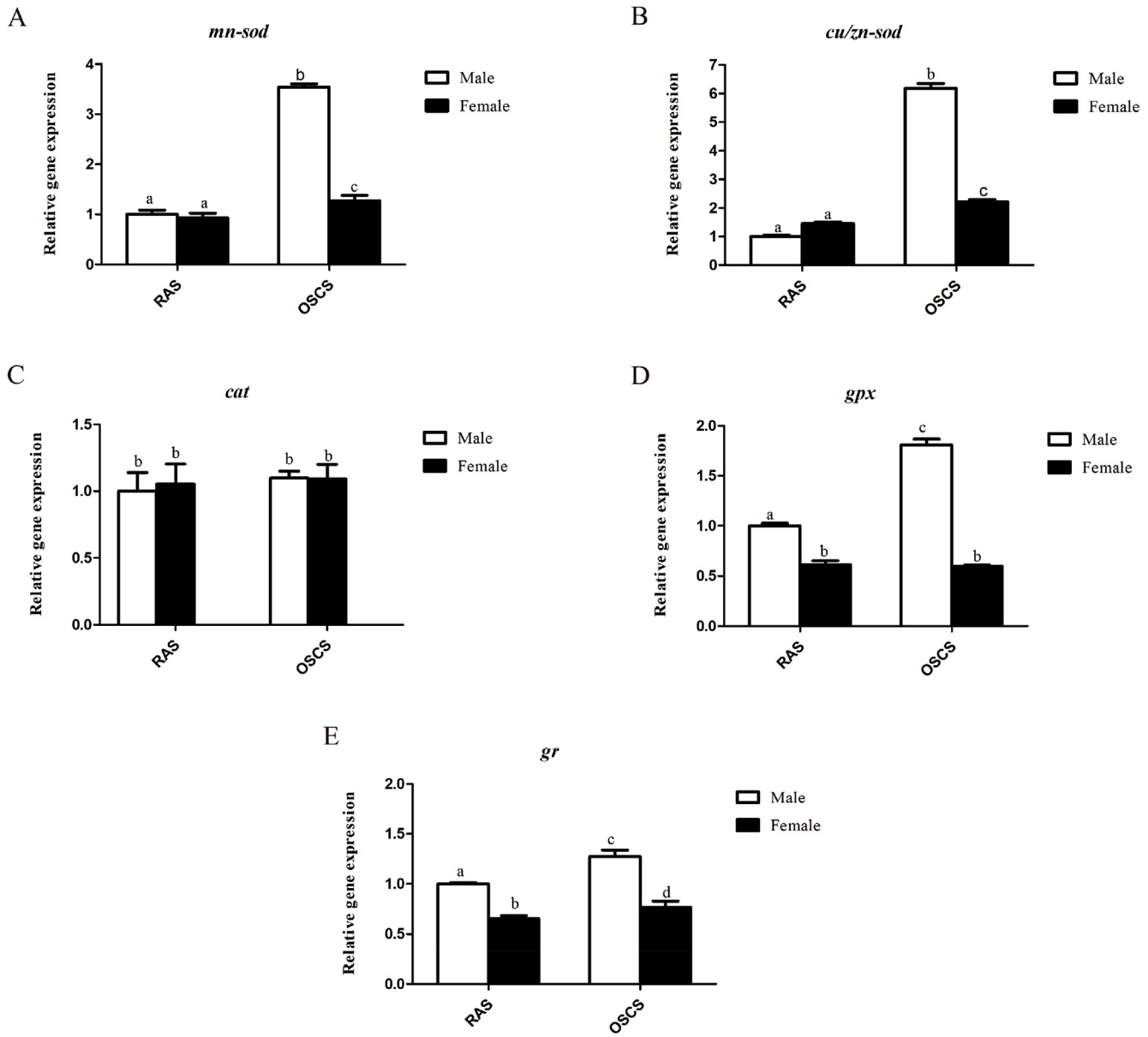

Fig. 2. Relative mRNA expression levels of hepatic antioxidant-related genes *mn-sod* (A), *cu/zn-sod* (B), *cat* (C), *gpx* (D) and *gr* (E) in tiger puffers reared in RAS and OSCS. Data are presented as means ± SEM. Bars with different superscripts are statistically different ($P < 0.05$, n = 3).

reverse in RAS [6]. Thus, it is important for aquaculture enterprise design and adjust culture strategy to satisfy market supply by fully understanding the mechanism of the physiological differences in subadult tiger puffers reared in these two culture systems. Based on the hepatic transcriptome assay data. The present study further investigates the hepatic antioxidant enzyme, apoptosis, innate immune, and stress-related genes expression of the subadult tiger puffers cultured in these two culture systems.

Antioxidant enzymes play a key role in response to oxidative stress during balance intracellular redox status and maintain homeostasis in vertebrate include fish. The antioxidant enzymes SOD, CAT, GPx, and GR participated in regulating the cellular defense against stress damage and played key roles in maintaining homeostasis during the life cycle of vertebrates, including fish. SOD can convert intracellular oxygen free radicals into hydrogen peroxide [20]. Subsequently, CAT and GPx eliminate hydrogen peroxide and reduce its toxic effect [21]. Glutathione is another effective antioxidant that can directly scavenge singlet oxygen and hydroxyl radicals. GR can catalyze the reduction of oxidized glutathione back to glutathione and maintain the redox balance of cells [22]. Nutrients and environmental factors, such as salinity, ammonia, and temperature, markedly affect the expression and activity of the antioxidant enzymes in aquatic animals [16,23–26]. In the present study, the mRNA expression levels of *mn-sod*, *cu/zn-sod*, *gpx*, and *gr* of the fish cultured in the OSCS were higher than those of the fish cultured in the RAS, and the highest values were observed in the male tiger puffers reared in the OSCS. These results indicate that the elevation of antioxidant enzymes of tiger puffer reared in the OSCS can confer protection against the detrimental effects induced by environmental stress and maintain homoeostasis. The water parameters include pH, salinity, ammonia nitrogen and dissolved oxygen of OSCS that were comparable with the RAS, whereas the temperature is significantly higher in the OSCS than in the RAS at the later period of experiment (Fig. 1A). Temperature is known to be an important factor affect development, metabolic rates and mortality in marine teleost [27]. Kikuchi et al. (2006) found the optimal growth temperature for tiger puffer is between 20 °C and 25 °C in the RAS [28]. High temperature can induce the up-regulation of CAT and SOD in tiger puffer [29]. Similar results were reported in estuarine fish [30] and antarctic fish

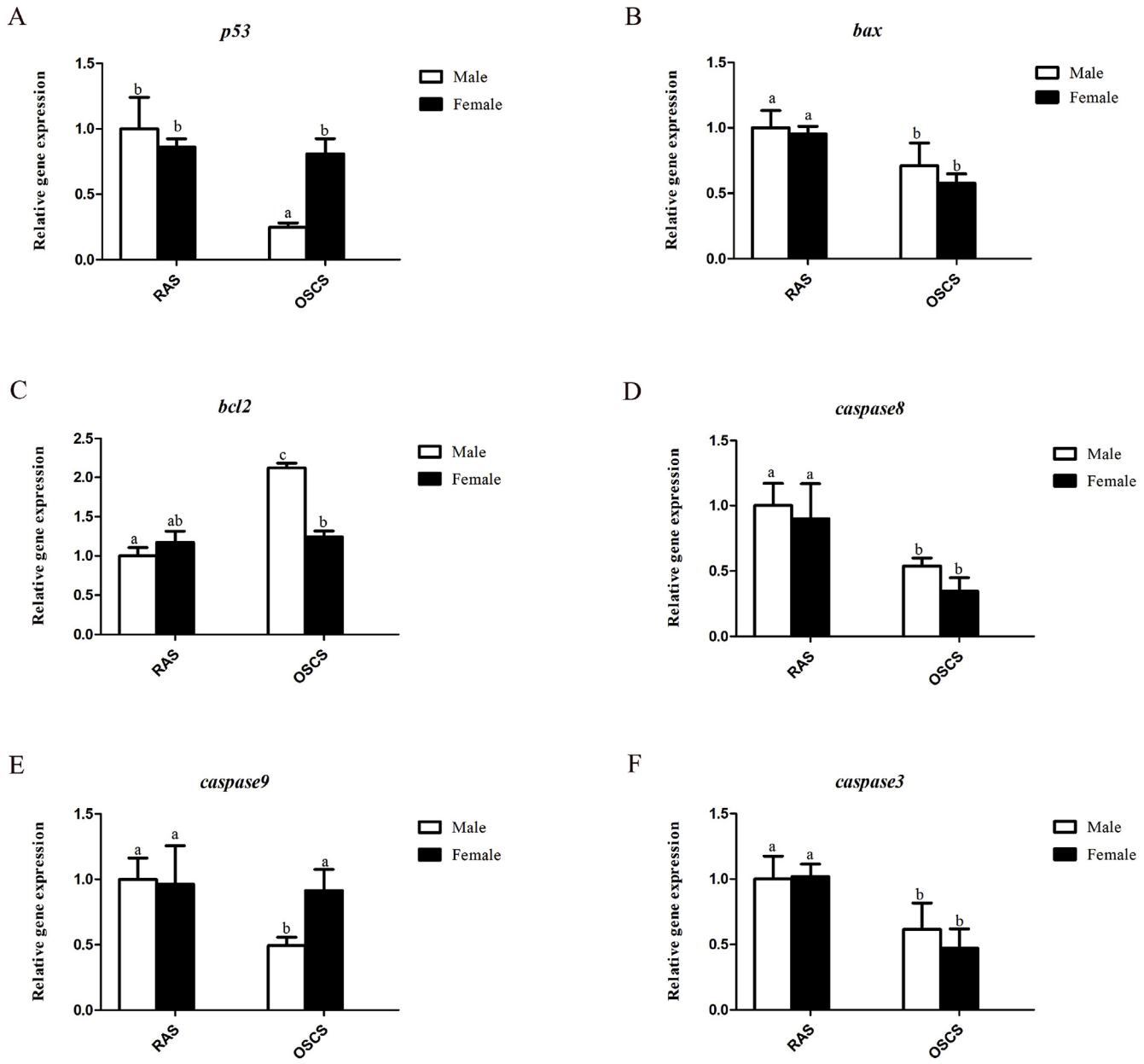

Fig. 3. Relative mRNA expression levels of hepatic apoptosis-related genes *p53* (A), *bax* (B), *bcl2* (C), *caspase8* (D), *caspase9* (E) and *caspase3* (F) in tiger puffers reared in RAS and OSCS. Data are presented as means ± SEM. Bars with different superscripts are statistically different ($P < 0.05$, $n = 3$).

[31]. Meanwhile, other environmental factors include ammonia exposure can induce hepatic oxidative stress, cause the up/down-regulation of antioxidant enzymes genes in tiger puffer [15] and yellow catfish [32]. Thus, we speculate that the antioxidant enzyme-related gene expression of the tiger puffers reared in the OSCS being higher than that in the RAS possibly because of the higher optimal culture temperature or other uncertain reasons. However, the detailed regulatory mechanism needs to be further studied.

Apoptosis is a highly organized form of cell death that is important for tissue homeostasis, organ development and senescence during the life cycle of vertebrates. To date, the extrinsic (death receptor mediated) and intrinsic (mitochondria derived) apoptotic pathways have been widely characterized [33,34]. The extrinsic death receptor pathway is modulated by the recognition of extracellular ligands with transmembrane receptors, which directly activate the initiator caspase8 [35]. The intrinsic mitochondrial pathway releases proapoptotic molecules to activate caspase-dependent or caspase-independent mechanisms that promote apoptosis. In caspase-dependent signaling, mitochondria release proapoptotic molecules cytochrome *c* and form cytochrome *c*/apaf-1/caspase 9-containing apoptosome via the active caspase9 [36]. Caspase3 executes the final steps of apoptosis by the cleavage of a series of proteins in various cell types. In addition, the BCL2 family is involved in the regulation of the mitochondrial apoptosis pathway by regulating BCL2 or BAX expression. BAX and BCL2 are located on the membrane of the mitochondria, BAX can induce the release of cytochrome *c* into the cytosol, and BCL2 can inhibit the release of cytochrome *c* from the mitochondria. The tumor suppressor P53 can induce apoptosis by up-regulating the transcription of BAX [20,37]. Cheng et al. (2018) found caspase dependent and p53 signaling pathways could play important roles in thermal stress-induced apoptosis in tiger puffer [38]. Meanwhile, c-Myc-Bax-Caspase9 apoptosis pathway involved in the regulation of hepatic apoptosis induced by ammonia stress in grass carp [39]. Accumulating evidences suggest up-regulation of p53-related apoptotic genes associated with zebrafish

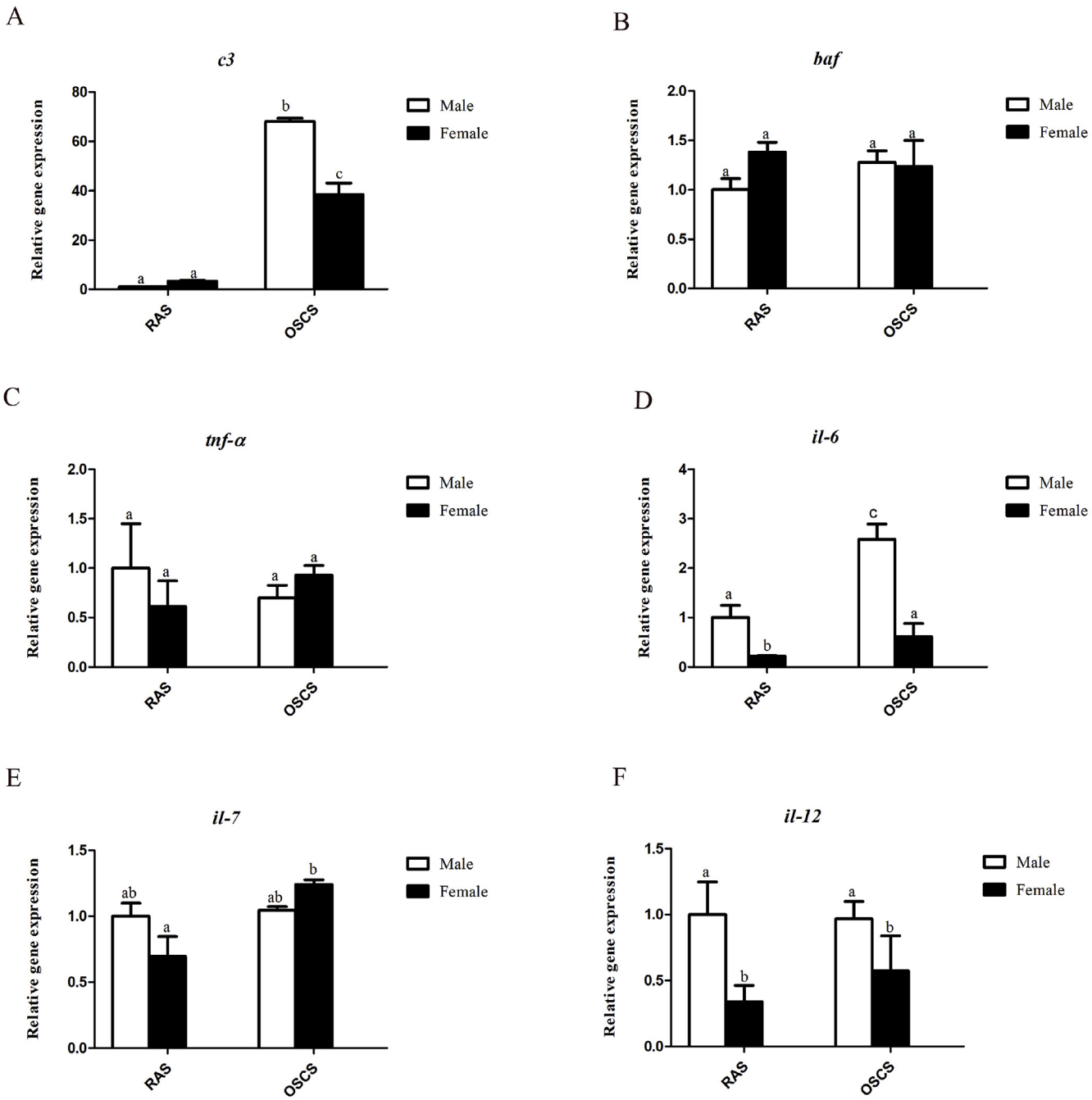

Fig. 4. Relative mRNA expression levels of hepatic immune-related genes *c3* (A), *baf* (B), *tnfα* (C), *il-6* (D), *il-7* (E) and *il-12* (F) in tiger puffers reared in RAS and OSCS. Data are presented as means ± SEM. Bars with different superscripts are statistically different ($P < 0.05$, $n = 3$).

development [40,41]. In the present study the mRNA levels of the anti-apoptotic gene *bcl2* of the fish cultured in the OSCS were higher than those of the fish cultured in the RAS, whereas the mRNA levels of proapoptotic genes (*bax*, *p53*, *caspase8*, *caspase9*, and *caspase3*) of the former were lower than those of the latter. The highest values of *bcl2* and the lowest values of the proapoptotic genes were observed in the male tiger puffers reared in the OSCS. Hence, different rearing systems can affect the hepatocyte apoptosis of tiger puffers, and the anti-apoptotic physiological functions of the fish cultured in the OSCS were superior to those of the fish cultured in the RAS.

Similar to the immune system of mammals, the immune system of teleosts consists of innate and adaptive immunity. Innate immunity is an essential component in combating pathogens because of the limitations of the adaptive immune system for poikilothermic nature, limited repertoire of antibodies, and slow proliferation, maturation, and memory of lymphocytes in teleosts [42,43]. Complement proteins are the major soluble components of the innate immune system, which consists of approximately 30 proteins that collectively constitute classical, lectin, and alternative complement pathways [44]. C3 is the central component in all pathways of complement activation and has various biological activities, including promotion of inflammatory responses, tagging of foreign organisms, and stimulation of B lymphocytes. Interleukins (ILs) and tumor necrosis factors (TNFs) are important cytokines involved in regulating immune responses through an autocrine or paracrine manner in the fish immune system. IL-6 is a pleiotropic cytokine that implicate in host defense [45]. IL-7 is essential for lymphocyte development and plays a central role in the survival, proliferation, and maturation of T and B cells [46]. IL-12 stimulates the

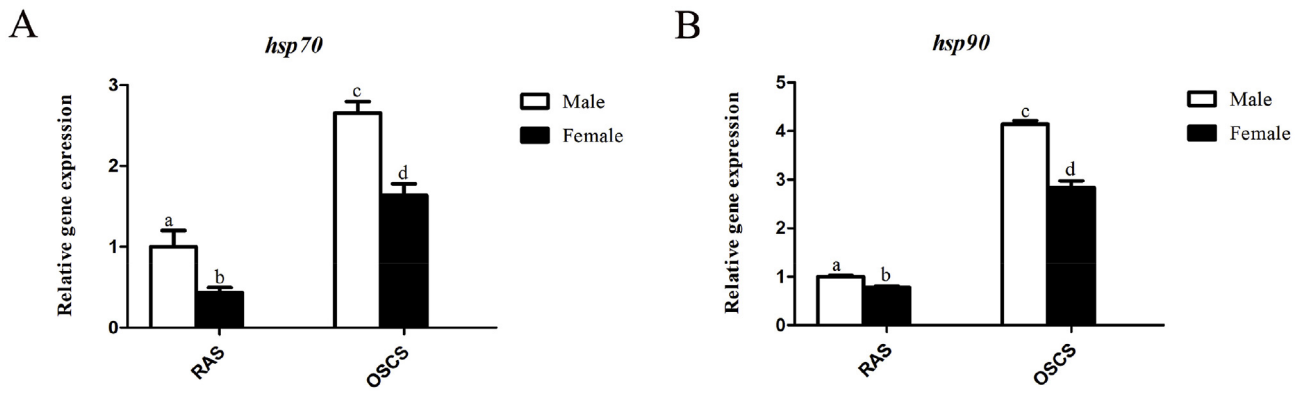

Fig. 5. Relative mRNA expression levels of heat shock proteins genes *hsp70* (A) and *hsp90* (B) in tiger puffer reared in RAS and OSCS. Data are presented as means ± SEM. Bars with different superscripts are statistically different ($P < 0.05$, $n = 3$).

production of interferon from T cells and natural killer cells and enhances the generation and cytolytic function of cytotoxic T cells [47]. BAFF and TNFα are considered key members of the TNF family of cytokines because they regulate the innate immune system against infectious and inflammatory diseases [48,49]. It has been identified natural environment have profound impacts on teleost immune function [50]. OSCS aquaculture is in the open sea and with the complex and changeable ecological environment. In general, immunity system was activated via external stimuli and caused up/down-regulation of aforementioned immunity-related gene expression in fish [51,52]. Ammonia stress could induce up-regulation of inflammatory cytokines BAFF, TNF-α, IL-6, IL-12 transcription in tiger puffer [15] and TNF, IL-1 and IL-8 in yellow catfish [32]. Furthermore, similar results were reported induced by thermal-stress in tiger puffer [16,38], grass carp [53] and Atlantic cod [54]. In the present study, the mRNA levels of *c3* and *il6* of the fish cultured in the OSCS were higher than those of the fish cultured in the RAS, and the highest values were observed in the male tiger puffers reared in the OSCS. However, the mRNA expression levels of *il7*, *baff*, and *tnfα* remained unchanged. The mRNA expression levels of *il12* exhibited no significant differences between the same gender (female vs. female, male vs. male) in the RAS and the OSCS. Overall, the innate immune responses of the tiger puffers cultured in the OSCS were superior to those of the fish cultured in the RAS.

HSPs belong to a family strongly related to the functioning of unstressed and stressed cells, and they are responsible for promoting higher cell resistance and tolerance against several aggressor agents and homeostasis alterations [55–57]. Among HSPs, HSP70 and HSP90 are well known for their interaction with other intracellular proteins that are involved in many cell functions. HSP90 works as chaperone proteins that support various components of the cytoskeleton and steroid hormone receptors [58,59]. HSP70 assists the folding of nascent polypeptide chains, mediates the import of proteins into organelles and facilitates the dissociation of aggregated proteins [60]. Different rearing environments alter the physiological status and affect *hsp70* and *hsp90* gene expression levels in fish. The enhanced levels of *hsp70* and *hsp90* reflected a protective response against environmental stress to maintain the homeostasis of fish. Thermal-stress and ammonia exposure induce up-regulation of HSP70 and 90 in puffer fish [15,16,38]. Similar results were found in other fish species include turbot [61], olive flounder [62] and lake trout [63]. Our results showed that different rearing environments changed the transcription of *hsp90* and *hsp70*, indicating that they may be involved in regulating protein functions to adopt rearing environmental changes caused by homeostasis alterations in tiger puffer.

In conclusion, this study is the first work to demonstrate the effects of two different rearing systems on the antioxidant enzyme, apoptosis, innate immunity, and stress-related gene expression in subadult tiger puffers. Our results indicate that the antioxidative, antiapoptotic, and innate immune response activities of the male tiger puffers reared in the OSCS are higher than those of the fish reared in the RAS. Further studies should explain why the growth performance of female tiger puffers reared in the OSCS is better than that of males and why the transcription of antioxidative, antiapoptotic, and innate immune response genes in males is higher than that fish reared in the RAS. We speculate that moderate environmental stress and sexual dimorphism may lead to the aforementioned differences in subadult tiger puffers reared in the OSCS and the RAS during our short-term experiment. Nonetheless, the precise mechanism of this discrepancy remains unclear and requires further investigation. The information presented in the current study provides fundamental data and give a new insight about the combined land (RAS) and sea (OSCS) alternative aquaculture model of tiger puffers in captivity.

Ethics and conflicts of interest statement

Fish rearing and handling procedures were conducted according to the guidelines established by the Institutional Animal Care and Use Committee at Yellow Sea Fisheries Research Institute. The studies did not involve in any endangered or protected species. All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.016.

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