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Effects of formaldehyde on detoxification and immune responses in silver pomfret (*Pampus argenteus*)

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ABSTRACT

Formaldehyde can effectively control ectoparasites in silver pomfret (*Pampus argenteus*). However, there is limited information on the effects of formaldehyde treatment at a molecular level in fishes. In the present study, transcriptome profiling was conducted to investigate the effects of formaldehyde treatment (80 mg/L, bath for 1 h every day for three consecutive days) on the liver and kidney tissues of silver pomfret. A total of 617959982 clean reads were obtained and assembled into 265760 unigenes with an N50 length of 1507 bp, and the assembled unigenes were all annotated by alignment with public databases. A total of 2204 differentially expressed genes (DEGs) were detected in the liver and kidney tissues, and they included 7 detoxification- related genes and 9 immune-related genes, such as *CYP450*, *GST*, *MHC I & II*, and *CCR*. In addition, 1440 DEGs were mapped to terms in the GO database, and 1064 DEGs were mapped to the KEGG database. The expression of 4 detoxification-related genes and 6 immune-related genes in three days formaldehyde treatment were analyzed using RT-qPCR, and the antioxidant enzyme levels were also determined. The results indicate differential expression of detoxification- and immune-related genes during the three days formaldehyde treatment. Our data could provide a reference for the treatment of parasites to avoid high mortality and help in understanding the molecular activity in fishes after formaldehyde exposure.

1. Introduction

Formaldehyde interferes with the immune system, resulting in immune depression and alterations to host defense; however, the molecular mechanism is still unclear. In adverse (toxic) environments, levels of antioxidant enzymes such superoxide dismutase (SOD) and glutathione (GSH) would be significantly changed in fish tissues such as liver and kidney [1–3], and these enzymes have been widely used as sensitive bio-indexes to evaluate the environment. For detoxification, two types of gene families, cytochrome P450 and glutathione *S*-transferase (GST), play important roles in phase I and phase II detoxification, respectively [4–6]. The cytochrome P450 family mainly includes three subfamilies, CYP1, CYP2, and CYP3, that express the cytochrome P450 enzymes involved in detoxification [7,8]. GST is coded by a superfamily gene that could be divided into cytosolic, mitochondrial, and microsomal, in mammals [9] and Rho-class GSTs in fishes [10].

Despite formaldehyde is toxic for marine or estuarine species and is harmful to the immune system [11], it is wildly used to effectively control ectoparasites in aquaculture [12–14]. In 1986, FDA approved the use of formaldehyde for the treatment of finfish and their eggs against external parasites and bacterial and fungal infections [15], and many fishes have been demonstrated different levels of tolerance to formaldehyde [11,16,17]. Therefore, appropriate utilization of formaldehyde could avoid the high mortality and the damage of immune system, and it is important to determine the efficacy and safety of formaldehyde to control parasites of fishes.

Abbreviations: DEG, differentially expressed gene; GST, glutathione *S*-transferase; SOD, superoxide dismutase; GSH, glutathione; LC50, median lethal concentration; COG, Clusters of Orthologous Groups; GO, Gene Ontology; KO, KEGG orthology; BHH, bi-directional best hit; CAT, catalase; MDA, malondialdehyde; CYP450, Cytochrome P450; HSP70, Heat shock 70 kDa protein; GR, Glutathione reductase; CCR, C-C chemokine receptor; MHC, major histocompatibility complex

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The silver pomfret (*Pampus argenteus*) is one of the most preferred commercial marine fish species in many countries. Recently, the silver pomfret catch has greatly reduced because of overfishing, and culture and breeding of this fish are faced with many challenges. In 2018, we succeeded in breeding nearly a million young silver pomfret and found that the white spot disease was the main disease that affects the fish, resulting in an extremely high mortality rate in the fish whose gill and body are affected by parasites such as *Cryptocaryon irritans* and *Amyloodinium ocellatum* [18]. We found an effective and safe treatment for this disease, immersion of the fish in seawater with 60–80 mg/L formaldehyde for 1 h and repetition of the treatment for three consecutive days, that we have been using for the past five years. Many previous studies focused on the acute toxicity and tolerance of formaldehyde for fishes [11,17,19]. However, effects of the formaldehyde treatment at a molecular level are unclear.

Therefore, we performed transcriptome profiling to investigate the responses of detoxification- and immune-related genes to the formaldehyde treatment, and examined the related DEGs with RT-qPCR. Finally, we analyzed the antioxidant enzymes and detoxification- and immune-related genes during three days formaldehyde treatment. Our data could provide a reference for the treatment of parasites, reduce mortality, and help in understanding the molecular mechanisms underlying detoxification and immune reactions involved during formaldehyde exposure.

2. Materials and methods

2.1. Fish rearing, formaldehyde treatment and sampling

The silver pomfret (mean weight: 4.3 ± 0.15 g; mean body length: 5.3 ± 0.45 cm) were reared at Xiangshan Bay, Zhejiang, China. The fish were originated from the same batch with natural fertilization. All fish experiments were conducted in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Care and Use Committee of Ningbo University approved the protocols.

Healthy silver pomfret were cultured in 500 L ponds containing sand-filtered seawater (25 ± 0.5 °C; pH, 8.01 ± 0.3; dissolved oxygen, 7.35 ± 0.05 mg/L; salinity, 24 ± 0.8‰) and fed with a commercial feed (larve love 5#, Hayashikane Sangyo Co., Ltd, Japan) for four days before experiments. The total amount of feed provided was not less than 2–3% of their body weight per day, and the fish were starved for 12 h before the experiments.

To investigate the effects of the formaldehyde treatment at a molecular level, we used 80 mg/L of formaldehyde (purity, 37–40%; SINOPHARM, China) to simulate the highest concentration of the formaldehyde treatment for three consecutive days. The healthy fish were divided into the control group without formaldehyde and formaldehyde treatment (FA) groups and cultured in 500 L ponds (10 individuals in each pond and six ponds in each group). The fish of FA group were immersed in seawater with 80 mg/L formaldehyde for 1 h bathing in one day treatment, and the water was change totally after treatment. The treatment was duplicated for three consecutive days. The fish were starved for 12 h before the next day treatment. Every day, three fish from each pond of two group were captured immediately after the treatment, and liver and kidney tissues were extracted, and each type of tissues were pooled in one 1.5 mL tube (RNase-Free; Axygen, CA, USA) for one independent biological replicate, and stored in liquid nitrogen (liver of Control group-GC, kidney of Control group-SC, liver of treatment group-GT, kidney of treatment group-ST). The samples of two groups collected in the third day were used for the RNA-Seq and verification experiments, and the samples of two groups collected in the first two days were used for the verification experiments (three independent biological replicates for each experiment).

2.2. RNA quantification and qualification

Total RNAs were extracted using the TRIzol reagent (Invitrogen, CA, USA). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit and Qubit® 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.3. Library preparation for transcriptome sequencing

A total of 1.5 μg RNA per sample was used as the input material. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), according to the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from the total RNA by using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under high temperature in NEBNext First Strand Synthesis Reaction Buffer (5 ×). First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA was synthesized using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of the DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated for hybridization. To select cDNA fragments of preferentially 250–300 bp, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 μL of the USER Enzyme (NEB, USA) was added to the size-selected, adaptor-ligated cDNA at 37 °C for 15 min, followed by 5 min at 95 °C. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers, and Index (X) Primer. The PCR products were purified (AMPure XP system), and library quality was assessed using the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed using the cBot Cluster Generation System and TruSeq PE Cluster Kit v3-cBot-HS (Illumina), according to the manufacturer's instructions. After cluster generation, the libraries were sequenced using the Illumina Hiseq platform, and paired-end reads were generated.

2.4. Data analysis

2.4.1. Quality control

Raw data (raw reads) in the fastq format were first processed through in-house Perl scripts, and clean data (clean reads) were obtained by removing reads containing the adapter and ploy-N and low-quality reads; simultaneously, Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on high-quality clean data.

2.4.2. Transcriptome assembly and gene functional annotation

To obtain unigenes, transcriptome *de novo* assembly was conducted using Trinity (Grabherr et al., 2011, release-20121005). The assembled unigenes of transcriptome were input into BLASTx searches and annotated against the NCBI non-redundant protein sequence database (NR-NCBI), using an e-value cut-off of 0.00001. BLASTx alignments (e-value < 0.00001) were then performed between the unigenes and several protein databases, including Swiss-Prot and the Clusters of Orthologous Groups (COG) database. With NR-NCBI annotation, the Blast2GO program was used to predict Gene Ontology (GO) terms related to molecular functions, cellular components and biological processes. After obtaining GO annotations for every unigene, we used the GOseq R package and Wallenius noncentral hyper-geometric distribution to conduct GO functional classification of all unigenes and to understand the overall distribution of gene functions in this species. We

used KOBAS software to test the statistical enrichment of differentially expressed genes among KEGG pathways for the assigned carp orthologs. KEGG orthology (KO) assignments were performed based on the bi-directional best hit (BHH) of the BLAST.

2.4.3. Differential expression analysis

Gene expression levels in each sample were estimated using RSEM (Li et al., 2011). Differential expression analysis of two groups (three biological replicates per condition) was performed using the DESeq R package (1.18.0). The resulting P-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P value < 0.05 were identified as differentially expressed.

2.5. Determination of antioxidant enzyme activities

The liver and kidney tissues were collected and homogenized in physiological saline and centrifuged at $500 \times g$, and the supernatant was collected. The levels of SOD, catalase (CAT), GSH, and malondialdehyde (MDA), as an index of oxidative damage, were determined. Commercial assay kits for SOD, CAT, GSH, and MDA were provided by Jiancheng Biotechnology Research Institute (Nanjing, China). The statistical analysis was performed using Duncan's multiple range test followed by one-way ANOVA (SPSS, version 16.0).

2.6. RT-qPCR analysis

The total RNA was reverse-transcribed into cDNA by using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). The primers were designed using Primer 5.0 software (Table S1). β-actin was used as the internal control, and the reaction volume contained 2 μL cDNA, 1 μL forward and reverse primers, 10 μL SYBR Green I Master Mix (TaKaRa), and 6 μL PCR-grade water. RT-qPCR was performed using an Eppendorf PCR machine (Mastercycler ep Realplex, Hamburg, Germany) with one cycle at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. Dissociation curve analysis was performed to determine target specificity. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method, and statistical analysis was performed using Duncan's multiple range test followed by one-way ANOVA (SPSS, version 16.0). The qPCR was performed in triplicate to confirm the expression patterns.

3. Results

3.1. Transcriptome sequencing and assembly

For 12 libraries, a total of 628596728 raw reads were generated, and 617959982 clean reads (98.31%) were obtained. The mean Q30 and GC content were 93.56% and 49.66%, respectively (Table S2). A total of 265760 unigenes, which ranged from 201 to 23343 bp and N50 length of 1507 bp, were detected in these libraries. The length distribution revealed that most of the unigenes (93913, 35.34%) were between 201 and 500 bp, followed by 500–1000 bp (76871 unigenes, 28.92%) and 1–2 kbp (63114 unigenes, 23.75%).

3.2. Gene functional annotation

The unigenes were annotated by alignment with public protein databases, such as NR-NCBI, Swiss-Prot, KEGG, COG, and GO (Table 1).

The E-value distribution of the top matches showed that 70.9% of the NR-mapped sequences were in the range of 0–1.0 e−30, and 61.5% had high E-value scores (E-value $<$ 1.0 e−45; Fig. S1A). These results reflect the validity and reliability of our *de novo* assembly. A substantial majority (94.6%) of the NR-annotated sequences exhibited 60–100% similarity to known sequences (Fig. S1B). Additionally, we compared the unigenes to sequences from other fish species; 35.5% unigenes were best matched to sequences from *Larimichthys crocea*, followed by those from *Stegastes partitus* (24.6%), *Oreochromis niloticus* (5.4%), *Notothenia coriiceps* (4.3%), *Maylandia zebra* (3%), and others (27.1%) (Fig. S1C).

Table 1
Summary of functional annotation of unigenes.

Database	Number of Unigenes	Percentage (%)
NR -NCBI	135727	48.93
NT-NCBI	179340	67.48
KO	67607	25.43
Swiss-Prot	105725	39.78
PFAM	99838	37.56
GO	99956	37.61
KOG	48225	18.14

The unigenes (99956) were classified into 1742 functional groups of three main categories of GO classifications, which are biological processes, cellular components, and molecular functions (Fig. S2). For biological processes, cellular process (GO:0009987), single-organism process (GO:0044699), and metabolic process (GO:0008152) were highly represented. For cellular components, cell part (GO:0044464), followed by cell (GO:0005623), was highly represented. Finally, the most well-represented molecular functions were binding (GO:0005488) and catalytic activity (GO:0003824). In these functional groups, immune system process (GO:0002376, 1419 unigenes) and detoxification (GO:0098754, 32 unigenes) may have been involved in the response to formaldehyde exposure of the fish.

Next, 48225 unigenes were annotated and classified into 26 categories (Fig. S3). Signal transduction mechanisms (9037, 18.74%) was the largest group, followed by General function prediction only (7638, 15.84%) and Posttranslational modification, protein turnover, chaperones (4951, 10.27%). In addition, 3068 unigenes (6.36%) were classified as Function unknown, and only five unigenes were assigned to the unnamed protein classification. In 26 categories, Defense mechanisms (390 unigenes) may have been involved in protecting the fish from formaldehyde exposure.

The KEGG pathway analysis identified the functions and interactions of the unigenes. A total of 67607 unigenes were mapped to 239 pathways in five main categories: Cellular Processes, Environmental Information Processing, Environmental Information Processing, Metabolism, and Organismal Systems (Fig. S4). In the five main categories, most annotated genes showed PI3K-Akt signaling pathway (ko04151, 3.44%), followed by pathways related to Endocytosis (ko04144, 3.33%) and Rap1 signaling pathway (ko04015, 3.01%). The immune system was involved in the response to the formaldehyde exposure because, in Organismal Systems, Chemokine signaling pathway (ko04062, 2.19%) was highly enriched by annotated unigenes, followed by Platelet activation (ko04611, 2.13%), Leukocyte transendothelial migration (ko04670, 1.64%), T cell receptor signaling pathway (ko04660, 1.62%), and Fc gamma R-mediated phagocytosis (ko04666, 1.56%).

3.3. Analysis of DEGs

A total of 2204 DEGs were detected, including 1243 upregulated and 961 downregulated genes, in the liver and kidney tissues of the FA and control groups (GT vs. GC and ST vs. SC): 536 DEGs (291 upregulated and 245 downregulated) in the liver tissues (GT vs. GC) and 1668 DEGs (952 upregulated and 716 downregulated) in the kidney tissues (ST vs. SC). In the two tissue types, 16 detoxification- and immune-related genes were detected (Table 2), and the significant DEGs were analyzed with RT-qPCR (low adjusted P value).

3.4. GO and KEGG analyses of DEGs

According to the GO of analysis, a total of 1440 DEGs were mapped

Table 2
Differential expressed genes (DEGs) involved in detoxification and immune.

Gene-id	Up/down	P-value (adjusted)	Description
Liver (GT vs GC)			
C109559	down	4.24E-08	Cytochrome P450
C86612	up	1.36E-17	Glutathione S-transferase theta b
Kidney (ST vs SC)			
C117945	down	2.96E-13	MHC class I antigen
C78631	down	1.33E-06	MHC class II antigen
C110181	down	1.14E-05	MHC II invariant chain
C130975	down	5.68E-05	MHC class II antigen alpha chain
C98046	up	0.034403	Cytochrome P450 2K1 a
C81330	up	0.036366	Cytochrome P450 2K1 b
C109559	up	0.042068	Cytochrome P450
C185244	down	5.12E-08	C-C chemokine receptor type 9 a
C185238	down	0.017352	C-C chemokine receptor type 9 b
C185239	up	0.020825	C-C chemokine receptor type 9 c
C6101	up	0.0054431	C-C chemokine receptor type 7-like protein, partial
C163966	down	0.023141	Atypical chemokine receptor 3
C118979	up	0.0013097	Heat shock 70 kDa protein 4-like
C54763	up	5.35E-10	Glutathione reductase, mitochondrial

to terms in the GO database. Ten highly enriched categories of GO ($P < 0.05$) are listed in Table S3, for example, tetraterpenoid metabolic process (GO:0016108), tetraterpenoid biosynthetic process (GO:0016109) in liver tissues (GT vs. GC), and apelin receptor binding (GO:0031704) and neuropeptide receptor binding (GO:0071855) in kidney tissues (ST vs. SC).

According to the KEGG pathway analysis, 1064 DEGs were mapped to the KEGG database. Ten highly enriched categories of KEGG ($P < 0.05$) are listed in Table 3, for example, *Staphylococcus aureus* infection (ko05150), Citrate cycle (TCA cycle) (ko00020) in liver tissues (GT vs. GC) and Butirosin and neomycin biosynthesis (ko00524), and Peroxisome (ko04146) in kidney tissues (ST vs. SC). There pathways may play potential roles in assisting the detoxification. For example, Drug metabolism - cytochrome P450 (ko00982) was involved in the response to formaldehyde exposure and degrade the toxic substances in phase I detoxification, and Citrate cycle (TCA cycle) (ko00020) involved in the energy metabolism process may provide many energy substances (ATP/GTP) for the Drug metabolism to relieve toxicity.

Table 3
KEGG pathway analysis of the 10 highly enriched categories.

Pathway term	ID	P-value
Liver (GT vs GC)		
Staphylococcus aureus infection	ko05150	0.000773812
Citrate cycle (TCA cycle)	ko00020	0.004175648
Tryptophan metabolism	ko00380	0.008889423
Drug metabolism - cytochrome P450	ko00982	0.017825442
alpha-Linolenic acid metabolism	ko00592	0.021200672
Arachidonic acid metabolism	ko00590	0.021951231
Linoleic acid metabolism	ko00591	0.032253298
Glycosaminoglycan biosynthesis - HS/Hep	ko00534	0.042379507
Bile secretion	ko04976	0.046036305
Vasopressin-regulated water reabsorption	ko04962	0.048323734
Kidney (ST vs SC)		
Butirosin and neomycin biosynthesis	ko00524	0.001004511
Peroxisome	ko04146	0.011158052
Viral myocarditis	ko05416	0.015359324
Starch and sucrose metabolism	ko00500	0.01868724
Intestinal immune network for IgA production	ko04672	0.018923331
Influenza A	ko05164	0.022201071
Biosynthesis of unsaturated fatty acids	ko01040	0.023737062
Type I diabetes mellitus	ko04940	0.025613311
Carbohydrate digestion and absorption	ko04973	0.02596693
Ribosome biogenesis in eukaryotes	ko03008	0.026354226

3.5. RT-qPCR analysis of the detoxification- and immune-related genes

We selected 10 significant DEGs, namely, *CYP450* (C109559) and *GST* (C86612) in the liver tissues and *MHC I* (C117945), *MHC II* (C78631), *CYP450 2K1* (C98046), *CCR9 a* (C185244), *CCR7* (C6101), *ACR3* (163966), *HSP70* (C118979), and *GR* (C118979) in the kidney tissues, for the qPCR analysis. The results were mostly consistent with those of the RNA-seq analysis (Figs. 1 and 2).

In the liver tissues of the fish (Fig. 1), *CYP450* was upregulated in the first day but significantly downregulated in the last two days, and the expression of *GST* increased in all three days of the formaldehyde treatment ($P < 0.05$).

In the kidney of the fishes (Fig. 2), *MHC I*, *MHC II*, *CCR9 a*, and *ACR3* were all upregulated in the first two days but significantly downregulated in the last day; *CYP450 2K1*, *CCR7*, and *GR* were all upregulated in three days. *HSP70* showed no significant changes in the first day, but it was upregulated in the last two days ($P < 0.05$).

3.6. Analysis of antioxidant enzymes in silver pomfret during the three days of formaldehyde treatment

The antioxidant enzyme activities and MDA content in the liver and kidney tissues of the silver pomfrets during three days of formaldehyde exposure are summarized in Figs. 3–6.

The SOD activity was significantly increased in the first two days but decreased in the third day in the liver tissues ($P < 0.05$), and it was significantly increased in the first day but decreased in the third day in the kidney tissues ($P < 0.05$). The SOD activity decreased gradually during the three days of treatment in both liver and kidney tissues of the FA groups (Fig. 3).

The CAT activity was significantly increased in the first two days but decreased in the third day in the liver tissues ($P < 0.05$), and it no significant changes were observed in the kidney tissues ($P < 0.05$). The CAT activity decreased gradually during the three days of treatment in both liver and kidney tissues of the FA groups (Fig. 4).

The GSH activity was significantly increased in the first day but decreased in the last two days in the liver tissues ($P < 0.05$), and it was significantly increased in the first day but did not significantly change in the last two days in the kidney tissues ($P < 0.05$). The GSH activity decreased in the first two days and increased in the third day in the liver of the FA groups, and it decreased gradually during the three days of treatment in the kidney tissues of the FA groups (Fig. 5).

The MDA content was significantly increased in the second and third days in the liver and kidney tissues ($P < 0.05$). The MDA content increased in the liver tissues during the three days of treatment, and it increased in the last two days but decreased in the first day in the kidney tissues of the FA groups (Fig. 6).

4. Discussion

4.1. Transcriptome analysis of detoxification- and immune-related genes

To understand effects of formaldehyde exposure at a molecular level, we used RNA-seq to analyze the transcriptomic profiles of the liver and kidney tissues of silver pomfret. After three days formaldehyde treatment, we found 536 DEGs (291 upregulated and 245 downregulated) in the liver tissues (GT vs. GC) and 1668 DEGs (952 upregulated and 716 downregulated) in the kidney tissues (ST vs. SC). Sixteen DEGs were involved in the detoxification and immune activity, for example, cytochrome P450, GSH, MHC class I & II genes, C-C chemokine receptor, heat shock protein (HSP) 70, and glutathione reductase (GR).

For detoxification activity, cytochrome P450 is the key enzyme for drug metabolism in phase I detoxification, and it plays a vital role in the biosynthesis or oxidative metabolism of many endogenous and exogenous compounds [4,7,20]; cytochrome P450 genes can be easily

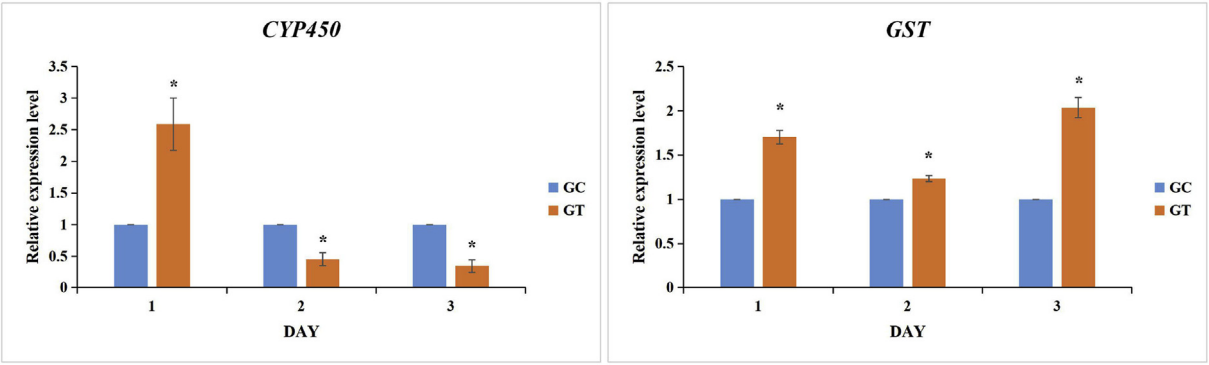

Fig. 1. Expression of detoxification- and immune-related genes in the liver during the three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); P < 0.05; GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

activated or inhibited by toxic substances [8,21]. In this study, cytochrome P450 genes (*CYPs*) were significantly downregulated in the liver tissues but upregulated in the kidney tissues during formaldehyde exposure. Previous studies have reported that *CYP* was induced significantly in the liver of rabbitfish (*Siganus oramin*) after exposure to a neurotoxin [22]; however, *CYP* expression was reduced in the liver cells of trout (*Oncorhynchus mykiss*) exposed to heavy metals [23]. The expression varieties of CYP in the kidney tissues of fishes exposed to different toxic substances was reported to be similar in the liver tissues [24–26]. Tissues have been suggested to have different detoxification abilities, but toxic substances often inhibited *CYPs* in many tissues [27,28], which might be due to the damage of drug metabolism by high level or strong toxicity of toxin. *GST* and *GR* also play important roles in phase II detoxification, which could catalyze the metabolites from phase I [29,30]. *GST* has been widely reported to show a significant response after toxin exposure in many fishes, such as the river pufferfish (*Takifugu obscurus*) [31], pinfish (*Lagodon rhomboides*) [32], and plaice (*Pleuronectes platessa*) [33], and many studies have found that GR can degrade the drug metabolites in phase II [34,35]. In the present study, *GST* and *GR* were significantly upregulated in the liver and kidney tissues, respectively, which suggests that the two genes may play key roles in different tissues after formaldehyde treatment. Toxic substances are metabolized by the CYP 450 enzyme system in phase I detoxification, and the intermediate metabolites from phase I are further degraded by the GST enzyme system in phase II [36,37]. Thus, the results indicate that *CYP 450* was downregulated by formaldehyde, but the intermediate metabolites from phase I may still activate the GST enzyme system to protect the tissues of the fish.

Exposure to toxic substances often interferes with the immune system, leading to immune depression and alterations to the host defense [38–40]. In the present study, two types of immune-related genes, MHC and chemokine receptor, were detected to be significantly expressed in the kidney tissues of silver pomfret after three days formaldehyde exposure. The MHC family is divided into class I and class II, which are central to specific responses of the acquired immune system in vertebrates [41]. We found that MHC class I and II genes were all downregulated, which indicates that formaldehyde may be able to damage the immune system and inhibit MHC family genes in the kidney tissues. Chemokine receptors are involved in inflammation and infection [42], and the FA groups showed three downregulated and two upregulated chemokine receptors after formaldehyde treatment. Formaldehyde can damage the DNA and proteins in cells [43,44], resulting in cell death. Thus, high formaldehyde concentration may induce cell damage and death, leading to immune responses such as inflammation and inhibition of the expression of some genes. Moreover, numerous studies have reported that immune responses are connected to oxidative stress [38,41,45], so formaldehyde may induce ROS and the immune response.

HSP 70 represents the biggest subfamily and most highly conserved HSP family, and it plays an essential role in cell protection, antioxidation, and stress control [46,47]. HSP 70 is a type of molecular chaperone involved in protein folding and transportation across membranes [48]. In the present study, one HSP 70 gene was upregulated in the kidney after three days formaldehyde exposure, which suggests that HSP 70 may be involved in both detoxification and immune responses, and many studies have also reported similar findings [49–51]. Further, HSP 70 may help fold and transfer some factors that ameliorate toxicity and control the stress induced by formaldehyde.

4.2. Effects of formaldehyde treatment on detoxification and immune responses

The expression of detoxification-related genes was significantly different in the liver and kidney tissues after three days formaldehyde treatment. In the liver tissues, *CYP450* was upregulated in the first day but significantly downregulated in the last two days, and the expression of *GST* increased in all three days. This result indicates that the formaldehyde concentration may decrease phase I detoxification, but intermediate metabolites from phase I still induced the high expression of *GST* in phase II in the liver tissues; this is consistent with the results of some studies [52,53]. However, *CYP450 2k1* and *GR* were both upregulated during the three days, which suggests that the kidney tissues of silver pomfret may be more capable of dealing with formaldehyde. Most of the immune-related genes were upregulated in the first two days but downregulated in the last day. The relative expression of *CCR7* was not lower in the third day than in the first two days, which demonstrates that formaldehyde could induce the immune reaction in the beginning but may decrease it with exposure time. Similar results have been reported in the common carp (*Cyprinus carpio* L.) after exposure to chlorpyrifos and cadmium [38,39]. It was normal for *HSP70* to be highly expressed during the three days of treatment because the genes may have been involved in the detoxification and immune responses. The results showed these genes had nearly similar expression trends, which increased first, followed by a decrease; this suggested that the tolerance of the fishes for formaldehyde had been weak in the later period.

Toxic substances can induce changes in ROS, leading to the loss of normal physiological functions and even high mortality in fishes [54,55], and numerous studies have reported that SOD, CAT, and GSH play important roles in antioxidant defense and detoxification [1,2,38]. In the present study, the levels of almost all three antioxidant enzymes increased in the first day but decreased in the last two days of the treatment in the two tissues. The results suggest that formaldehyde may have inhibited the antioxidant defense of silver pomfret in the last two days of the treatment, which is similar to hormesis [56]. Moreover, MDA, an end product of lipid peroxidation [57], increased progressively during the treatment period, which demonstrated the negative effect of formaldehyde on antioxidant enzymes. The significant changes

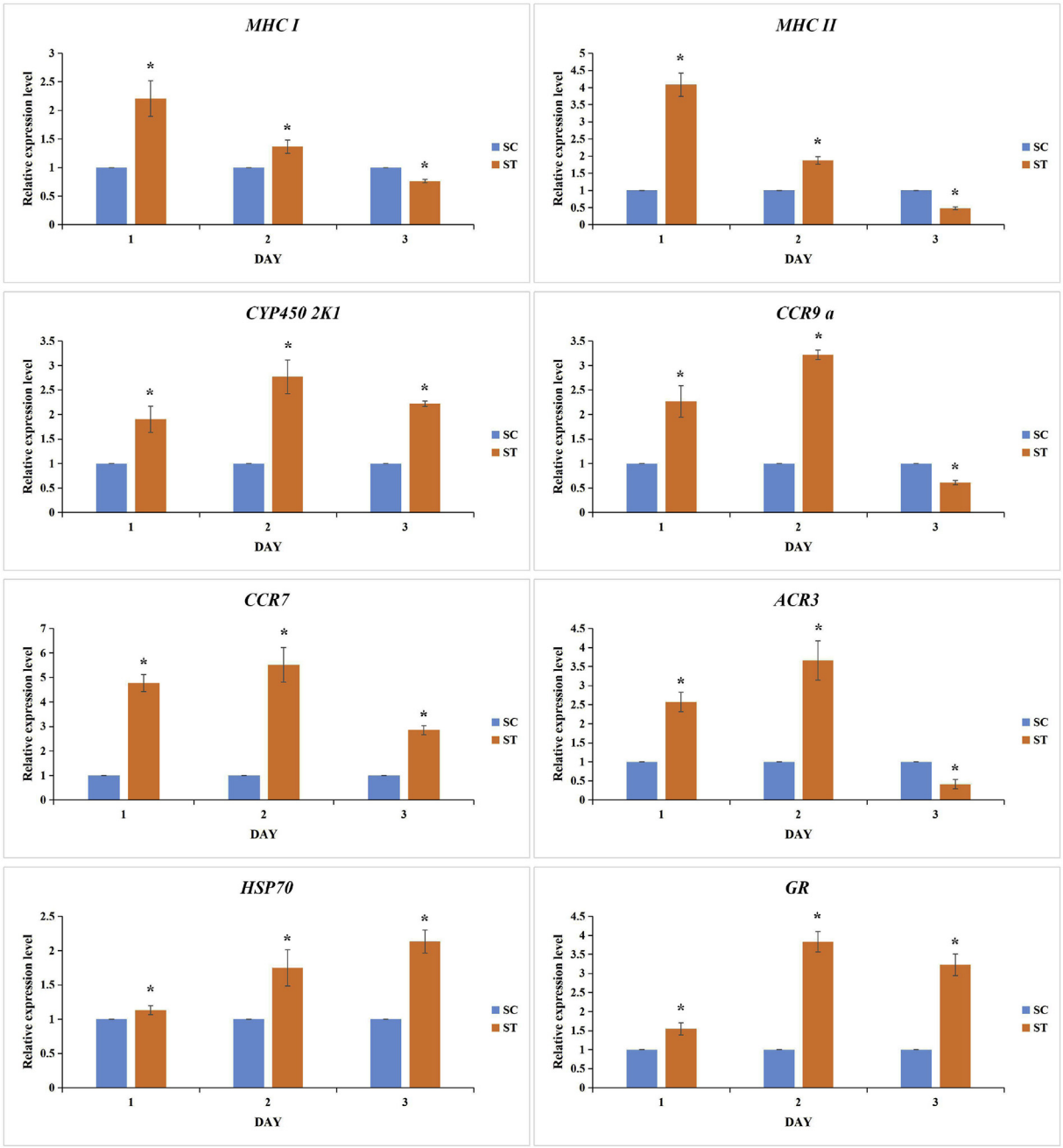

Fig. 2. Expression of detoxification- and immune-related genes in the kidney during the three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); P < 0.05; GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

in ROS induced by formaldehyde proved our inference for the differential expression of immune-related genes.

No mortality was observed in the control and FA groups during the three days of formaldehyde treatment; however, negative effects on most of the detoxification- and immune-related genes and enzymes were observed in the liver and kidney tissues on the second day. Therefore, the formaldehyde treatment could be shortened to two days, the third day should be used to relieve the toxicity and damage caused by formaldehyde. To avoid severe toxicity and immune damage, it should depend on the condition of parasitic diseases whether the treatment needs to be continued. Further experiments need to be performed to investigate the recovery of silver pomfret after formaldehyde treatment.

5. Conclusions

In the present study, we performed transcriptome profiling of silver pomfret to investigate the responses of detoxification- and immune related genes to formaldehyde exposure and examined related DEGs, such as *CYP450*, *MHC I* & *II*, and *CCR*, with RT-qPCR. And we analyzed the antioxidant enzymes during three days of the treatment. The results indicated differential expression of the detoxification- and immune-related genes in three days formaldehyde treatment of the fish. Our data could provide a reference for the treatment of parasites to avoid mortality and help in understanding the molecular activity in fishes after formaldehyde exposure.

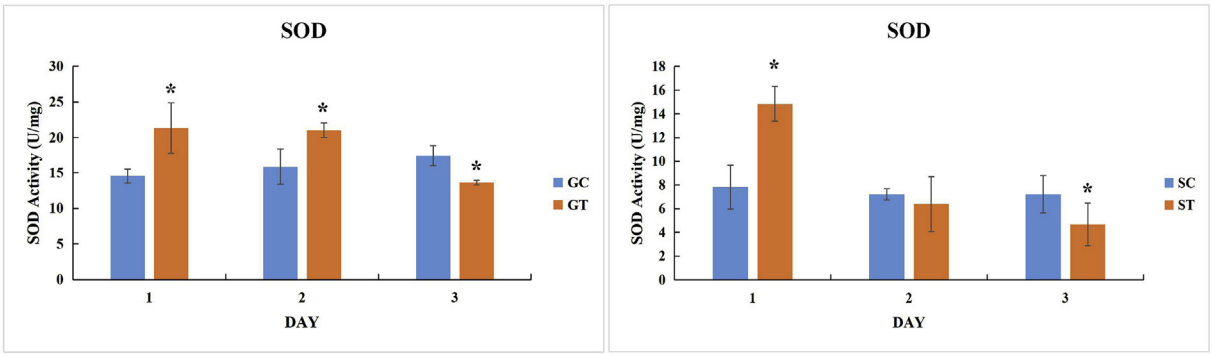

Fig. 3. SOD activity in the liver and kidney tissues of silver pomfret during three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

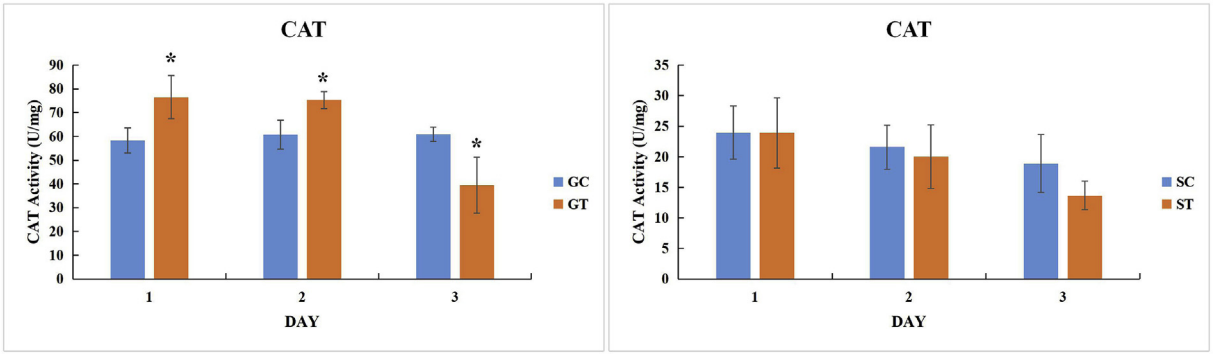

Fig. 4. CAT activity in the liver and kidney tissues of silver pomfret during three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

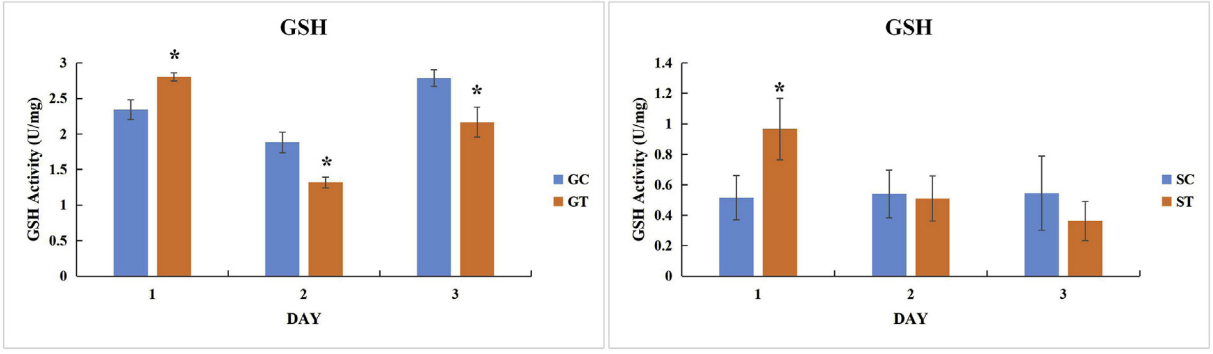

Fig. 5. GSH activity of in the liver and kidney tissues of silver pomfret during three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

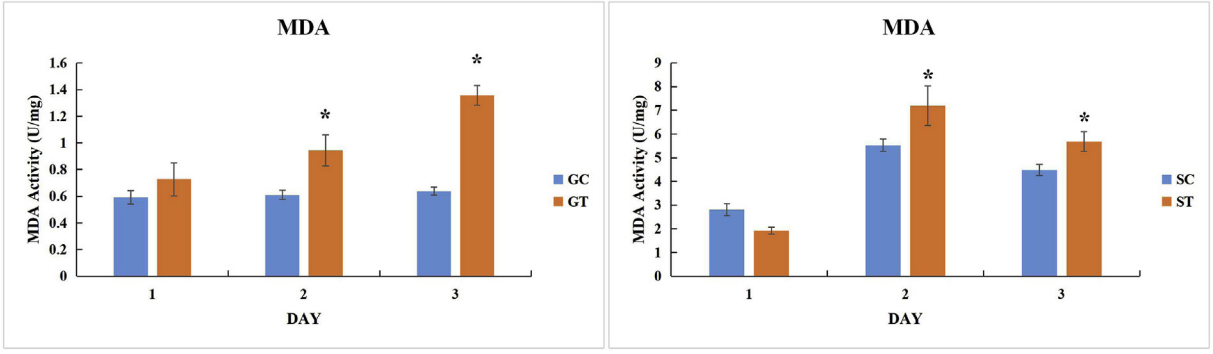

Fig. 6. MDA content in the liver and kidney tissues of silver pomfret during three days of formaldehyde treatment. Each bar represents mean ± SD (n = 3); GC/GT: liver tissues of control group/liver tissues of FA group, SC/ST: kidney tissues of control group/kidney tissues of FA group.

Availability of supporting data

The raw data files were uploaded to NCBI′ s Sequence Read Archive (BioProject accession numbers: PRJNA508497).

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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.2019.03.019.

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