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Short communication

Effects of cyclical short-term food deprivation and refeeding on compensatory growth and gene expression of SOD, GPX and HSP70 in *Schizothorax wangchiachii*

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ABSTRACT

The present study evaluated the effects of cyclical short-term food deprivation and refeeding on growth performance, body composition, and gene expression of SOD, GPX and HSP70 in *Schizothorax wangchiachii*.The experimental design included four feeding protocols for eight weeks: feeding every day of the week (control), starvation for one day and refeeding for six days per week (S1F6 treatment), starvation for two days and refeeding for five days per week (S2F5 treatment), and starvation for three days and refeeding for four days per week (S3F4 treatment). The results showed that no significant difference in final body weight, specific growth rate and feed conversion efficiency were observed among the treatments ($P > 0.05$).The feeding rate significantly increased with the duration of food deprivation per week compared to the control ($P < 0.05$). The expression levels of HSP70 showed no significant differences in the gill, liver and spleen of *S.wangchiachii* subjected to different feed restriction regimes($P > 0.05$), but in the kidney, the expression levels of HSP70 were significantly downregulated in S1F6 and S2F5 compared to the control($P < 0.05$). The expression levels of SOD and GPX in the examined tissues were not affected by the different feed restriction regimes($P > 0.05$). In conclusion, full compensatory growth was observed in *S.wangchiachii* under eight cycles of food deprivation and refeeding. Hyperphagia was the main mechanism of compensatory growth of *S.wangchiachii*.

1. Introduction

Compensatory growth is a phase of rapid growth that is faster than normal growth and occurs during recovery from food restriction, undernutrition or unfavorable environmental conditions [1–3]. Many studies on compensatory growth in fish have been performed; However, different results have been reported, such as overcompensation in hybrid sunfish [1], complete compensatory growth in *Labeo rohita* [4], and partial compensatory growth in *Centropomus parallelus* Poey [5]. The degree of compensation might be influenced by food quality, feeding protocol, age, sex, ontogenetic stage and sexual maturity of fish [2].

In aquaculture, the phenomenon of compensatory growth has been used as a feeding management strategy because it enhanced growth and feed efficiency, reduced production costs and maintained water quality [6,7]. However, food deprivation can cause oxidative stress when the generated reactive oxygen species (ROS) are not completely eliminated [8]. Fish are protected from ROS byantioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), etc.) and low-weight antioxidants (glutathione and vitamin E, etc.) acting in conjunction with the enzymatic defenses [9,10]. Experimental studies have demonstrated that starvation affects the activity of antioxidative enzymes and the expression of antioxidant genes in fish [11–15].

Heat shock protein 70 (HSP70) is highly conserved and involved in a wide variety of essential cellular processes in living cells as molecular chaperones, including in protein metabolism, cellular immune response and apoptosis [16,17]. HSP70, as an indicator of stress, was induced by starvation in fish. For example, starvation enhanced HSP70 levels in gilthead sea bream (*Sparus auratus*) and rainbow trout (*Oncorhynchus mykiss*) larvae [18], and the mRNA level of Hsp70 increased significantly after starvation for 21 days and suddenly decreased after starvation for 28 days in the liver of common carp(*Cyprinus carpio*). HSP70 was expressed as a protective strategy against stress caused by starvation in common carp [19].

Schizothorax wangchiachii, which belongs to Cypriniformes, Cyprinidae, Schizothorax, is a native species in China and is mainly

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distributed in the Jinsha River, Wujiang River and Ya-lung River. In recent decades, however, the population of *S.wangchiachii*has sharply declined due to habitat destruction and overfishing. Hence, some studies on artificial breeding [20] and spawning behavior [21] have been performed for species conservation. In this paper, we study whether cyclical short-term food deprivation and refeeding can elicit compensatory growth and investigate the effect of this feeding strategy on the gene expression of HSP70 (MK886829), SOD (MH223455) and GPX (MH448747) in *S.wangchiachii*.

2. Materials and methods

2.1. Fish and experimental procedure

Fingerlings of *S.wangchiachii* (average weight 0.82 g), obtained from Ludila hatchery in Dali (Yunnan, China), were acclimated to the experimental conditions for one month, and the culturing system was operated under a natural photoperiod. The water quality parameters, such as temperature (16.5–17.5 °C), pH (7.0–7.2), dissolved oxygen (> 5.0 mg/L), ammonia nitrogen and nitrite nitrogen (less than 0.1 and 0.01 mg/L, respectively), were recorded every week.

The experimental design included four treatments, each with three replicates, according to feeding restriction regimes as follows: feeding every day of the week (control), starving for one day (Monday) and feeding six days (from Tuesday to Sunday, S1F6 treatment), starving for two days (Monday and Tuesday) and feeding for five days(from Wednesday to Sunday, S2F5 treatment), starving for three days (from Monday to Wednesday) and feeding for four days (from Thursday to Sunday, S3F4 treatment). The experiment lasted for 8 weeks.

A total of 360 fish (average weight 1.20 ± 0.05 g) were randomly distributed into 12 experimental tanks (100 cm diameter, 90 cm height, 700 L capacity,30 fish per tank) in a recirculating aquaculture system. On each feeding day, commercial pelleted feed (feed no. 6610, with 33% crude protein and 6% lipid, as reported by manufacturer; Haid Group Co., Ltd. Yunnan, China) was provided twice daily (08:00 and 18:00 h) to the fish until satiation, and after 1 h, uneaten feed was siphoned, dried and weighed.

2.2. Sampling

At the beginning of the trial, the fish (starved for 24 h) were weighed and euthanized by overdose of MS-222 for body composition analysis. At the end of the trial, all fish were starved for 24 h, weighed, counted and anesthetized with MS-222. Twenty fish per tank were randomly collected, washed with distilled water and stored at −80 °C for body composition analysis. Tissues (gill, liver, spleen and kidney) were collected from 3 fish of each tank for expression analysis of SOD (EC 1.15.1.1), GPX (EC1.11.1.9) and HSP70 and kept at −80 °C until analysis.Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and were approved by the Animal Ethics Committee of the Institute of Hydroecology, Ministry of Water Resources & Chinese Academy of Sciences (approval number: IHE20160610).

2.3. Evaluation of growth performance

The various growth parameters were calculated as follows:

Specific growth rate (SGR, %/day) = [ln(final body weight)- ln(initial body weight)] × 100/experimental days;

Feeding rate (FR, %/day) = 100 × dry feed intake/[feeding days × (initial body weight + final body weight)/2];

Feed conversion efficiency (FCE, %) = [100 × (final body weight - initial bodyweight)]/dry feed intake

2.4. Body composition analysis

The biochemical composition (moisture, protein, lipid and ash content) ofthe fish body was analyzed according to standard AOAC methods (1999). Moisture content was analyzed by oven-drying at 105 °C to constant weight; crude protein content was measured by the Kjeldahl method (FOSS Tecator, Haganas, Sweden); crude lipid content was measured by the Soxhlet extraction method (Soxtec System HT6, Tecator, Haganas, Sweden); and ash content was determined by combustion in a muffle furnace at 550 °C to constant weight.

2.5. mRNA expression analysis of SOD, GPX and HSP70

Total RNA was extracted from the tissues (liver, gill, spleen and kidney) by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and treated with RNase-free DNase I (Thermo Fisher Scientific, Inc., Waltham, MA). The purity and concentration of the total RNA was measured by a NanoDrop ND-2000 UV spectrophotometer (NanoDrop Technologies, USA) with 260/230 nm absorbance ratios greater than 2.0, and the quality was checked by 0.8% agarose gel electrophoresis. cDNA was synthesized from the total RNA with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

To amplify the full-length sequences of SOD, GPX and HSP70 of *S.wangchiachii*, degenerate primers designed based on the conserved sequences of other animals's SOD, GPX and HSP70 were first used for the amplification of the middle partial fragments, respectively. The first-strand cDNA was then synthesized with the SMARTER™ RACE cDNA Ampication Kit (Clontech, USA). The full-length cDNA was obtained by using 5′/3′-RACE methods with some gene-specific primers designed based on the obtained partial sequences of SOD, GPX and HSP70. All the PCR products were ligated into thepMD18-T vector (TaKaRa, Japan) and transformed into competent *E.coli* cells. Then the positive clones were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). Finally, complete sequences were assembled using Contig Express application software.

The primers used for examination of tissue expression pattern were designed based on the complete sequence of SOD (MH223455), GPX (MH448747) and HSP70 (MK886829) and listed in Table 1. The gene expression of SOD, GPX and HSP70 was analyzed using a real-time PCR system (CFX Connect™, Bio-Rad, Inc.). The PCRs included 10 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems®, Foster City, CA), 1 μL of cDNA template,0.5 μL of each forward and reverse primer (10 μmol L^{-1}) and 8 μL of nuclease-free water. The PCR was performed as follows:95 °C for 3 min followed by 45 cycles of 95 °C for 10 s, 55–58°Cfor 20 s and 72 °C for 30 s. Each reaction was replicated three times. The housekeeping gene β-actin was used for normalization of target genes. A melting curve analysis was used to verify the accuracy of amplification. The relative expression of SOD, GPX and HSP70 was calculated according to the $2^{-\triangle\triangle Ct}$ method [22].

Table 1
Primers used for real-time PCR.

Primer name	Primer sequence(5′-3′)
SOD-F	GGAGACAACACAAACGGCTG
SOD-R	GGTCACCATTGCATCCACAA
GPX-F	TACACCCAGATGAACGAGCT
GPX-R	GAAGCCATTTCCAGGACGG
HSP70-F	CATGAACCCCACCAACACAG
HSP70-R	TCACCCTTGTAATCAACCTGGA
β-actin-F	CCTGTTCCAGCCATCCTTCT
β-actin-R	CAGCAATGCCAGGGTACATG

Table 2
Initial body weight(IBW), final body weight (FBW), specific growth rate (SGR), feeding rate (FR) and feed conversion efficiency (FCE) of *Schizothorax wangchiachii* subjected to feed restriction regimes for eight weeks.

Variables	Treatments			
	Control	S1F6	S2F5	S3F4
IBW(g/fish)	1.19 ± 0.02	1.20 ± 0.09	1.18 ± 0.02	1.20 ± 0.08
FBW(g/fish)	3.27 ± 0.31	3.06 ± 0.15	3.01 ± 0.08	3.17 ± 0.15
FR (%/day)	2.93 ± 0.06^{a}	3.53 ± 0.09^{ab}	4.13 ± 0.37^{bc}	4.96 ± 0.36^{c}
SGR(%/day)	1.78 ± 0.13	1.67 ± 0.08	1.67 ± 0.02	1.72 ± 0.10
FCE(%)	56.10 ± 3.33	51.48 ± 2.03	53.79 ± 4.75	56.73 ± 5.14

Values are the means ± SEs. Values in the same row with different superscripts are significantly different ($P < 0.05$).

2.6. Statistical analysis

All data were analyzed with SPSS 17.0 (SPSS Inc., USA) and are presented as the mean ± standard error of three replicates. Homogeneity of variance was tested with Levene's test and normality with the Shapiro-Wilk test. One-way analysis of variance was performed at a significance level of 0.05 following the confirmation of normality and homogeneity of variance. Duncan's multiple-comparison test was used to analyze the significance of differences between treatments. For data that were not normally distributed or exhibited no homogeneity of variance, the Kruskal-Wallis H-test and all pairwise comparisons were used to determine the significance of differences among treatments. $P < 0.05$ was considered to indicate significant difference.

3. Results

3.1. 1 Growth performance

Final body weight (FBW), SGR, FR and FCE values are shown in Table 2. No significant differences in FBW, SGR and FCE were found among the treatments ($P > 0.05$). The FR significantly increased with extended periods of food deprivation and peaked in the S3F4 group ($P < 0.05$).

3.2. Body composition

The whole-body composition exhibited different changes in *S.wangchiachii* under varying degrees of food restriction (Table 3). No obvious change was observed in the whole-body crude protein content among all the treatments ($P > 0.05$). Compared with the control group, the moisture content of fish subjected to feed restriction regimes (S1F6, S2F5 and S3F4) was high($P < 0.05$), and the lipid content of fish in S1F6, S2F5 and S3F4 was lower than that of the control ($P < 0.05$). However, no significant difference was observed in moisture content or lipid content among these three treatments ($P > 0.05$). The ash content of S1F6 was lower than that of the control ($P < 0.05$), and there was no significant difference compared with S2F5 and S3F4 ($P > 0.05$).

Table 3
Body composition of *Schizothorax wangchiachii* after eight cycles of food deprivation and refeeding.

Treatments	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Control	68.81 ± 0.45^{a}	14.55 ± 0.10	14.52 ± 0.36^{a}	1.93 ± 0.01^{a}
S1F6	70.93 ± 0.92^{b}	14.15 ± 0.23	13.03 ± 0.69^{b}	1.74 ± 0.06^{b}
S2 F5	71.22 ± 0.63^{b}	14.43 ± 0.27	12.17 ± 0.41^{b}	1.84 ± 0.05^{ab}
S3 F4	71.83 ± 0.40^{b}	14.50 ± 0.29	11.69 ± 0.25^{b}	1.85 ± 0.03^{ab}

Data are presented as the means ± SEs. Values with different superscripts in a column differ significantly ($P < 0.05$).

3.3. Expression levels of HSP70 in tissues

In the present study, the expression of HSP70 in tissues was examined at the mRNA level (Fig. 1). The expression levels of HSP70 in the gill, liver and spleen were not significantly affected by different feed restriction regimes ($P > 0.05$). In the kidney, the expression levels of HSP70 were significantly downregulated in S1F6 and S2F5 compared to the control ($P < 0.05$), and no significant differences were observed between the control and S3F4($P > 0.05$).

3.4. Gene expression of SOD and GPX in tissues

The expression levels of SOD and GPX in tissues were examined at the mRNA level (Figs. 2 and 3). The expression levels of SOD and GPX in the gill, liver, spleen and kidney were not significantly affected by different feed restriction regimes ($P > 0.05$).

4. Discussion

In this study, our results showed that eight cycles of food deprivation and refeeding elicited full compensatory growth in *S.wangchiachii*. Here, full compensatory growth was triggered as a result of alternating feeding, as evidenced by the similar FBWs of deprived fish and fish fed daily after eight cycles of food deprivation and refeeding. The compensatory growth response observed here is similar to other reports in fish, such as juvenile *Lophiosilurus alexandri* [3], blackhead seabream (*Acanthopagrus schlegelii schlegelii*) [23], and channel catfish (*Ictalurus punctatus*) [24]. The exact mechanisms of compensatory growth include an increase in feed intake, an improvement in feed utilization or a decrease in metabolic costs [2]. For *S.wangchiachii*, the feed restriction regimes significantly influenced the FR; the longer the restriction time was, the higher the FR, indicating that hyperphagia was the main mechanism of compensatory growth in *S. wangchiachii.* Some studies have reported the effects of food deprivation on gene expression related to the regulation of food intake. In grass carp, zebrafish and common carp, the authors concluded that the anorectic genes play a more important role in the well-fed fish than the feed-restricted fish [25–27].

Fish utilize stored nutrients to sustain life when deprived of food, and metabolism is mainly based on lipids and proteins [28]. For *S.wangchiachii*, the protein content in the bodies of fish in different treatment groups was not affected by the feed restriction regimes, but the lipid content of fish in S1F6, S2F5 and S3F4 was lower than that of fish in the control group and decreased with extension of the period of feed restriction, which indicated that lipids were depleted as an energy source during feed deprivation. The longer the restriction time was, the lower the body lipid content in *S. wangchiachii*. Similar reports were observed in *C.carpio* var. Jian [29], sea bream (*S.aurata*) [30], Atlantic salmon (*Salmo salar L.*) [31], Atlantic halibut (*Hippoglossus hippoglossus*) [32]. According to Gong et al. [25], after grass crap were subjected to food restriction, the body lipid content decreased via an increase in fatty tissue decomposition and inhibition of lipid synthesis.

Under feed restriction conditions, HSP70 is a molecular chaperone involved in protein synthesis and catabolism as well as the mobilization of other nutrients. In European sea bass (*Dicentrarchus labrax*) and *L.rohita* fingerlings, HSP70 levels were upregulated by feed restriction regimes in liver and muscle [33,34], which indicated that HSP70 expression has the potential to be used as an indicator of nutritional stress and health status in fish. However, food deprivation did not affect HSP70 expression in the gills of juvenile Atlantic salmon [35] or in the muscles of channel catfish [36]. In this study, the expression level of HSP70 varied among tissues and feed restriction regimes. HSP70 expression in the gill, liver and spleen was not influenced by feed restriction regimes; perhaps the degree of feed restriction was not severe enough to induce the heat shock response in these tissues. In contrast,

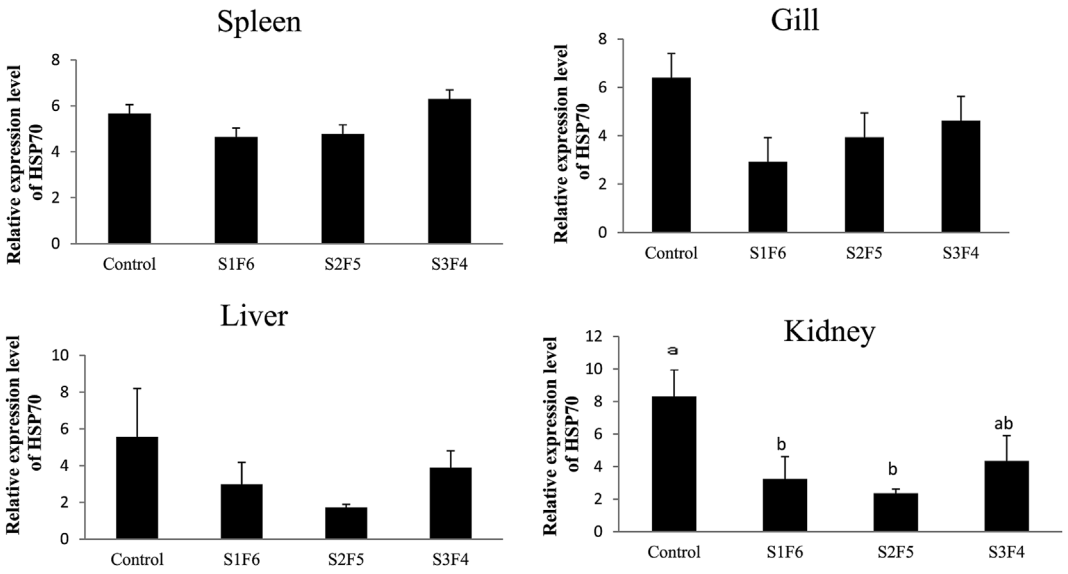

Fig. 1. The effect of cyclical short-term food deprivation and refeeding on the relative expression levels of the HSP70 gene in the gill, spleen, liver and kidney of *Schizothorax wangchiachii.* Values are the means ± SDs (n = 3 tanks, three pooled fish per tank). Bars assigned with different superscripts are significantly different ($P < 0.05$).

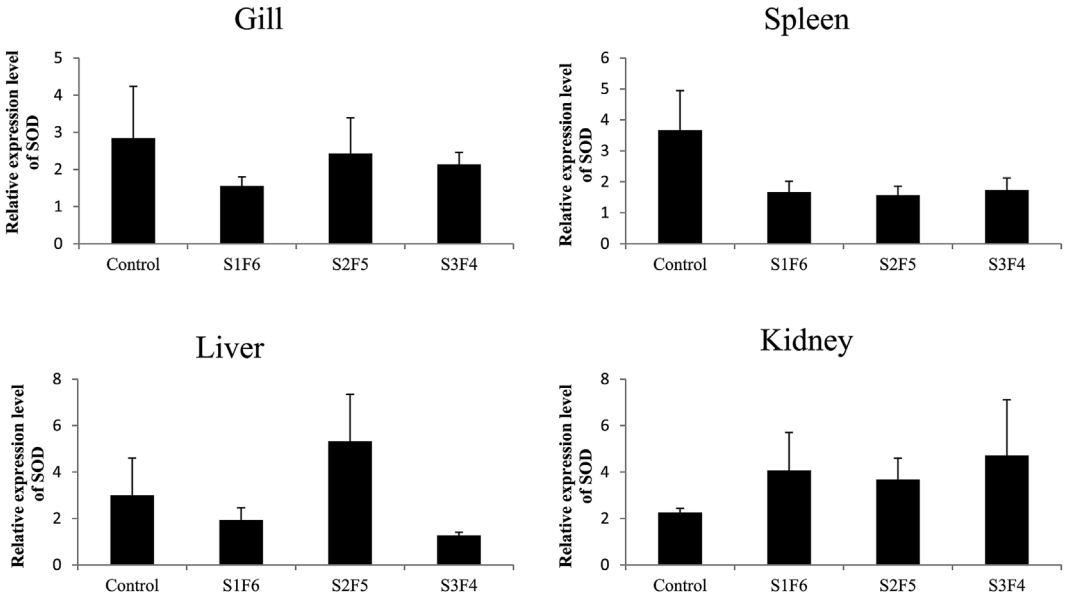

Fig. 2. The effect of cyclicalshort-term food deprivation and refeeding on the relative expression levels of the SOD gene in the gill, spleen, liver and kidney of *Schizothorax wangchiachii.* Values are the means ± SDs (n = 3 tanks, three pooled fish per tank). Vertical bars assigned with different superscripts are significantly different ($P < 0.05$).

the kidney was susceptible to the adopted feed restriction regimes, and HSP70 levels were significantly downregulated in S1F6 and S2F5 compared to the control. Similar results were reported in white sturgeon larvae(*Acipenser transmontanus*), wherein suboptimal feeding for two weeks reduced HSP70 levels in the liver [37], and starvation for 72 h reduced HSP70 levels in white sturgeon larvae due to, but not limited to, the low metabolic rate and the disruption of protein metabolism during starvation [38]. There were discrepancies in HSP70 expression among different experiments, which may be related to differences in species, developmental stages, experimental conditions, duration of fasting and refeeding, target tissues, etc.

SOD and GPX are some of the important antioxidant enzymes in the fish antioxidant defense system [13]. Gene expression of these antioxidant enzymes is considered to be an accurate estimate of fish antioxidant capacity when interference of biochemical origin is not involved [15]. In large yellow croaker (*Pseudosciaena crocea*), the gene expression levels of SOD and GPX were upregulated in the right lobe of the liver after 4 days of starvation, indicating that the antioxidant defense system was activated by starvation [13]. In this study, the adopted feed restriction regimes did not significantly affect SOD and GPX expression levels in the examined tissues between the control and other treatments. Fish immune systems are affected by nutritional status, and fish immunity is often reduced under poor nutritional conditions, leaving the fish susceptible to pathogens and parasites [39–41]. In *S.wangchiachii*, although starvation was a stressor, refeeding alleviated the negative effects of stress caused by starvation to some extent, which was why both SOD and GPX showed no differences in expression in the examined tissues among treatments. Similarly, a recent study in *L.rohita* fingerlings also demonstrated that the expression of SOD and CAT genes in the liver and gills was significantly upregulated after

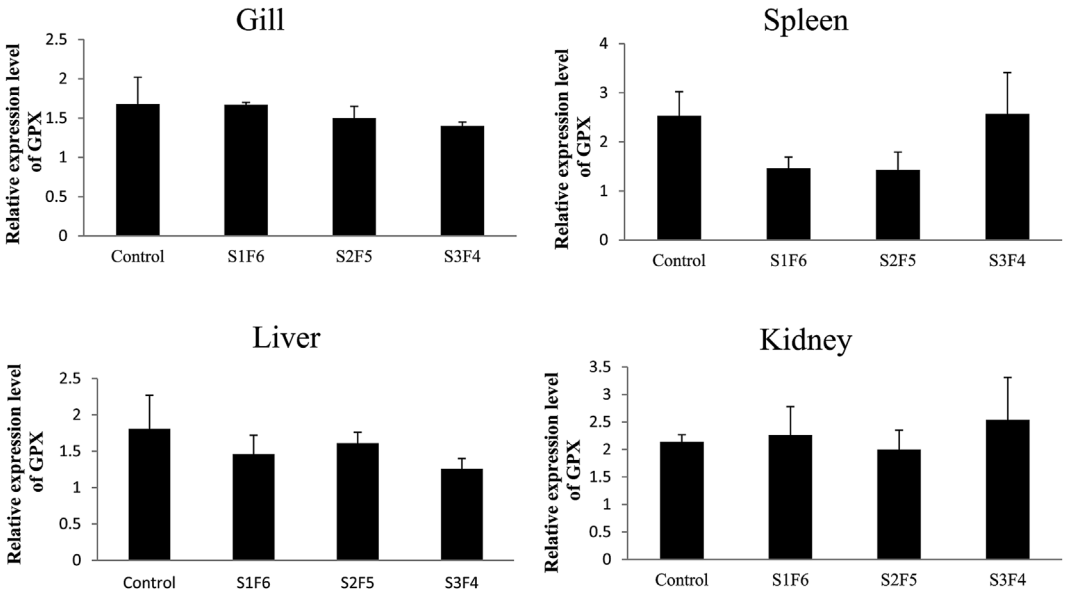

Fig. 3. The effect of cyclical short-term food deprivation and refeeding on the relative expression levels of the GPX gene in the gill, spleen, liver and kidney of *Schizothorax wangchiachii.* Values are the means ± SDs (n = 3 tanks, three pooled fish per tank). Vertical bars assigned with different superscripts are significantly different ($P < 0.05$).

starvation for 7 days, downregulated after refeeding for 3 days, and returned to basal values after refeeding for 8 days [42]. In addition, the immune response is an energy-demanding process that affects many physiological pathways, including protein and lipid metabolism [43]. These results, together with the results of the body composition analysis, may indirectly explain why the lipid content (energy reserves) of fish subjected to feed restriction regimes was lower than that of the control, possibly because lipids are involved in the immune response.

5. Conclusion

In conclusion, full compensatory growth was observed in *S.wangchiachii* subjected to eight cycles of food deprivation and refeeding. Hyperphagia was the main mechanism of compensatory growth in *S. wangchiachii.* The expression levels of SOD and GPX in the examined tissues were not affected by different feed restriction regimes. Regarding the expression levels of HSP70, no significant differences were observed in the gill, liver and spleen, but in the kidney, the expression levels of HSP70 were significantly downregulated in S1F6 and S2F5 compared to the control. The present findings are meaningful for future detailed research on the underlying mechanisms of the relationship between feed restriction and immune response in fishes.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

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