Effect of Winter Feeding Frequency on Growth Performance, Biochemical Blood Parameters, Oxidative Stress, and Appetite-Related Genes in *Takifugu Rubripes*

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Research Article

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Abstract

We evaluated the effect of winter feeding frequencies (F1: one daily meal; F2: two daily meals; F3: four daily meals; F4: continuous diurnal feeding using a belt feeder) on the growth performance, biochemical blood parameters, oxidative stress, and appetite-related genes in *Takifugu rubripes* held at a constant temperature (18.0 ± 1.0°C) for 60 days. The results showed that the final weight, weight gain rate, specific growth rate, and survival of tiger puffer in the F3 group showed the best growth performance. The total cholesterol, triglyceride, and glucose levels were significantly higher with the increased feeding frequency. We also observed the antioxidant enzymes (superoxide dismutase, catalase, glutathione, and glutathione peroxidase) and the digestive enzyme activities (trypsin, amylase, and lipase) in tiger puffers cultured in the F1 group were significantly higher than those in the F3 and F4 groups. In addition, the tiger puffers in the F1 group exhibited the highest expression of orexin and the lowest contents of glucose, tachykinin, cholecystokinin, and leptin among all the groups. In contrast, the mRNA levels of tachykinin, cholecystokinin, and leptin in the tiger puffers in the F4 group may be attributed to the negative feedback mechanism in the brain-hypothalamus-neuropeptide axis. All parameters exhibited relatively optimal levels in the F3 group. In conclusion, inappropriate feeding frequencies could have negative effects on growth and physiological indicators. The optimal feeding frequency for enhanced growth performance while maintaining a relatively good physical condition in juveniles of this species was four times a day.

1 Introduction

Tiger puffer (*Takifugu rubripes*) is the most commercially valuable and highly traded fish species in the aquaculture industry of China, Korea, and Japan due to its excellent meat quality and high nutrients (Miyadai et al., 2001; Tao et al., 2012). Unfortunately, the wild populations of tiger puffer have greatly declined in the last few decades due to high fishing pressure and habitat destruction. To protect the wild resources of this species and meet the market demands, mass culture techniques, including artificial propagation, nutritional requirements, feeding strategies, improved growth, and artificial induction of ovulation, have been established in China (Ma et al., 2011; Zhao et al., 2018; Wei et al., 2019; Wei et al., 2020; Wei et al., 2021). In recent years, the gradual expansion in the breeding of *T. rubripes* in the northern coast of China has caused the production of the cultured tiger puffer to increase rapidly and reached approximately 25,000 tons in 2018 (Department of Fisheries (DOF) 2019). Almost all the tiger puffer fish produced are sold to East Asian countries as delicacies, especially the gonad of male tiger puffer (Wang et al., 2016).

Fish farmers widely accept winter fish mortality as a major factor that decreases profitability and sustainability. Kotaro et al. (2006) reported the optimum temperature for tiger puffer growth exists within a narrow range and the weight gain of fish increases with increasing water temperature from 20°C to 25°C. In northern China, the lowest seawater temperature is below 0°C in winter, which seriously impedes the production of tiger puffer (Lin et al., 2017). Therefore, a new combined sea (offshore sea cage aquaculture system, OSCS) and land (recirculating aquaculture system, RAS) aquaculture model for tiger puffer has been developed (Jia et al., 2019). In this model, one-year-old puffer juveniles were cultured in

an OSCS from June to November and then transferred to an RAS for overwintering (Jia et al., 2020). However, in the declining water temperatures during the winter months, the tiger puffer does not feed as aggressively or at all. The decrease in food intake causes fish weight loss because the fish metabolism directly correlates with water temperature. Fontaine et al. (2007) reported that both the routine metabolic rate and the metabolic scope were significantly depressed in fishes cultured in a declining temperature. To ensure better growth and survival rates of tiger puffer, seawater needs to be heated to approximately 20 ℃ (Lin et al., 2017). However, heating large volumes of cold seawater to this temperature significantly increases operating costs. How can we reduce the heating cost and maintain fish survival? Years of breeding experience by commercial tiger puffer farmers in Dalian suggests that a water temperature of 18.0 ± 1.0 ℃ is acceptable for tiger puffer in winter. Currently, there is no standard protocol feeding strategy over winter. A vital unknown factor is the optimum feeding frequency. An insufficient feeding frequency may lead to poor growth and high mortality, especially in intensive systems (Ribeiro et al., 2012). Additionally, an increased feeding frequency can generally enhance production efficiency (Roy et al., 2017). It is well known that a reasonable feeding frequency is conducive to size individual homogeneity and optimizes feed consumption (Silva et al., 2020). At present, reports on the feeding frequency of tiger puffer during winter are virtually nil. Therefore, in the present study, the effects of feeding frequency on the growth performance, biochemical blood parameters, oxidative stress, and appetite-related genes in tiger puffer fish during wintering were examined and analyzed. This research provides a reference for establishing an appropriate feeding strategy for tiger puffer in the wintering period and provides a theoretical basis for other related breeding activities and future research.

2. Materials And Methods

2.1. Origin of fish and culture system

T. rubripes juveniles (n = 2800) were purchased from Dalian Tianzheng Industrial Corporation Limited (Liaoning, China) and transported to a greenhouse containing indoor concrete ponds (6.0 m × 6.0 m × 1.2 m) in October 2018. The fish acclimatized for approximately two weeks prior to instigating the experiment. After acclimatization, 200 fish $pool^{-1}$ (initial weight: 266.80 ± 12.32 g) were randomly assigned to 12 small cement ponds (2.0 m × 2.0 m × 1.2 m) with three replicate pools per feeding regime. Each pool was a closed recirculating aquaculture system with a total water volume of 100 m^3, and the stocking density was 13.5 kg/m^3 in each pool.

2.2 Feeding management

Tiger puffer were fed the same commercial diet (crude protein ≥ 54 %, crude fat ≥ 12 %, crude fiber ≤ 4 %, crude ash ≤ 12 %, lysine ≥ 2.5 %, calcium 3.5–9.0 %, and total phosphorus 1.5–3.0 %) during the experimental period using four different feeding regimes. The four different feeding regimes were: F1) one daily meal at 08:00; F2) two daily meals at 08:00 and 17:00; F3) four daily meals at 08:00, 11:00, 14:00, and 17:00, and F4) continuous diurnal feeding using a belt feeder. All groups were fed to the satiation level. Uneaten pellets were collected through a siphon and dried overnight at 105°C. The

uneaten pellets were then weighed and results recorded. The satiation level was assessed before beginning the experimental period following standardized methodology (Neda et al., 2019). Daily rations were equally distributed across all meals following the multiple feeding protocols. The mean water temperature in the circulating systems was maintained at 18.0 ± 1.0 ℃ throughout the experiment. The water flow rate within each pond was set at 100 % exchange every three hours, whereas approximately 3 % of overall water was renewed daily in the RAS. During the experimental period, temperature, dissolved oxygen, and pH were measured twice daily (morning and evening) using a digital multi-parameter controller (DIQ/S 182XT-4, Tianjin, China). The concentration of nitrite in each pond was determined spectrophotometrically following the Griess method (Bryan and Grisham, 2007). The total ammonia nitrogen was estimated following the nesslerization method using NH_4Cl as the standard (Hegazi et al., 2010). The mean water condition was maintained at 17.0 ± 1.0°C, dissolved oxygen at 8.05 ± 0.84 mg/L, pH at 7.5 ± 0.2, total ammonia at 0.26 ± 0.07 mg/L, and nitrite at 0.33 ± 0.04 mg/L.

2.3 Sample collection

The experimental period was 60 days from November 1 to December 30, 2018. At the end of the experiment, fishes were fasted for 24 h to empty their intestines and then anesthetized with 50 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). The fish survival rate was determined by counting the number of individuals in each pond at the final sample. All fishes were individually weighed. The growth performance was assessed using the following parameters: weight gain rate (WGR,%) = (W_t - W_o) / W_o × 100; specific growth rate (SGR,%·d^{-1}) = (lnW_t − lnW_o) / T × 100; Fulton's condition factor (K) = W_t/L_t^3 × 100; feed conversion ratio (FCR) = D_d / (W_t − W_o); and survival rate (SR, %) = N_t / N_o × 100, where W_t and W_o are the final and initial fish weights (g); L_t is the final fish length (cm); T is 60 days; D_d is total daily dry feed intake during the experimental process (g); and N_o and N_t are the final and initial fish numbers. Blood samples (1.5 mL) were collected from five fishes in each trial pond by caudal puncture using a syringe, and the serum was centrifuged at 10,000 × g for 5 min at 4°C before analysis. The fish anterior intestine and hypothalamus were sampled from each fish. Parts of the liver and anterior intestines were sampled and homogenized in an ice-cold phosphate buffer (50 mM, pH 7.4) using a Teflon glass homogenizer and centrifuged at 5000 × g for 10 min at 4°C. The supernatant was collected and stored for further analyses. The whole hypothalamus and the remaining liver and anterior intestines were removed and preserved in a BioSample Stabilizing Reagent (Accurate Biotechnology, Hunan, China) and stored at − 80°C until required for RNA extraction. This experiment strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Fishery Sciences. The procedures were approved by the Committee on Ethics of Animal Experiments of the Chinese Academy of Fishery Sciences.

2.4 Serum parameter analysis

The serum total protein (TP), total cholesterol (TC), triglyceride (TG), and glucose (Glu) was analyzed following Wu and Shang (2006) using an automatic chemistry analyzer (AU5421, OLYMPUS, Japan). The test kit was purchased from Nanjing Jiancheng Bioengineering Institute. Plasma cortisol concentration

was determined using a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) quantification kit (Mlbio, Shanghai, China).

2.5 Enzyme activity measurement

The antioxidant enzyme activity in the liver was analyzed using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. One unit of superoxide dismutase (SOD) activity was defined as the volume of enzyme required to inhibit the oxidation reaction by 50 %, expressed as units per mg protein. Catalase (CAT) activity was assayed by measuring the decrease in H_2O_2 generation rate by quantifying the absorbance at 240 nm. The glutathione (GSH) and glutathione peroxidase (GPx) contents were measured using the colorimetric method described in Gao et al. (2021).

Trypsin activity was measured by following the method of Erlanger et al. (1961), using N-α-benzoyl-dl-arginine-p-nitroanilide as a substrate. One unit of activity was defined as 1 μmol of nitroanilide released per minute. The amylase activity was quantified following the method described by Bernfeld. (1955) using a 1 % starch solution as the substrate. One unit of activity was defined as 1 μmol of maltose released per minute. The lipase activity was determined using the method described by Gjellesvik et al. (1992).

2.6 RNA extraction, cDNA synthesis, and real-time PCR

The total RNA was extracted from the liver, anterior intestine, and hypothalamus using commercial assay kits (RNAiso Reagent kit and Evo M-MLV RT Kit II with gDNA Eraser, AG11711; Accurate Biotechnology Co., Ltd, Hunan, China) following the manufacturer's instructions. The quality of the extracted RNA was determined by measuring its absorbance at 260 and 280 nm using GeneQuant 1300 (GE Healthcare Bioscience, Piscataway, NJ). The integrity of the extracted RNA was tested by electrophoresis on a 2.0 % agarose gel. The primer sequences for used for measuring orexinB, tachykinins, cholecystokinin, and leptin mRNA levels are listed in Table 1. The real-time PCR (20 μL) was amplified using SYBR Green Premix Pro Taq (AG11702, Accurate Biotechnology Co., Ltd., Hunan, China), containing 10 μL GoTaq®qPCR Master mix, 2 μL of cDNA, 0.8 μL of each primer with ultrapure water added to reach the final total reaction volume. The real-time PCR conditions were as follows: 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 20 s. Relative gene expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All samples were amplified in triplicate.

Table 1
Primers used in the gene expression analysis using qRT-PCR

Genes	Primer sequence (5'–3')	Size (bp)
OrexinB	F:TGACCGGAGATGCGGCCGCTGG	182
	R:CTGGGCAAACGCAAGGAAGAAGGG	
Tachykinins	F:CCCAGTACATTCTATGGCGTTCGG	228
	R:GTGACAACGATGTAAATCGT	
Cholecystokinin	F:GATATCAAGCAAGCAGGAAAGG	198
	R:TAGAGAAACCTTAGCTCTGACAGC	
Leptin	F:TACCCCGAGGTTCCTGCTGAT	174
	R:CTTGGGTCTATTATGGACACCC	
β-actin	F: CAGGGAGAAGATGACCCAGA	125
	R: CATCACCAGAGTCCATGACG	

2.7 Statistical analysis

Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test using SPSS 22.0 software. All data are presented as mean ± SD. In all statistical tests, statistically significant difference was set at $P < 0.05$.

3. Results

3.1. Performance measures

After 60 days of culture, tiger puffer in the F1 group exhibited the lowest final weight, WGR, SGR, survival, and the highest FCR (Table 2). In contrast, the F3 group showed the highest levels for the same growth performance and feed conversion ratio parameters. There were no differences between the feeding regimes for K ($P > 0.05$).

Table 2
Various feeding parameters in tiger puffers fed on different feeding regimens

Parameter	F1	F2	F3	F4
Initial weight (g)	264.95 ± 6.38	266.12 ± 10.50	268.14 ± 9.53	267.51 ± 9.19
Final weight (g)	340.35 ± 7.07^{a}	369.68 ± 11.12^{b}	417.37 ± 13.07^{c}	391.02 ± 12.29^{bc}
WGR (%)	28.46 ± 0.43^{a}	38.39 ± 4.28^{b}	55.67 ± 1.40^{c}	46.31 ± 7.5^{bc}
SGR (% d^{-1})	0.42 ± 0.01^{a}	0.54 ± 0.05^{b}	0.74 ± 0.02^{c}	0.63 ± 0.09^{bc}
FCR(%)	2.49 ± 0.06^{a}	1.92 ± 0.04^{b}	1.78 ± 0.02^{c}	2.48 ± 0.05^{a}
K	3.53 ± 0.27	3.68 ± 0.18	3.67 ± 0.09	3.73 ± 0.15
Survival (%)	73.18 ± 2.33^{a}	84.67 ± 3.04^{b}	90.58 ± 2.22^{b}	87.24 ± 3.17^{b}

3.2. Biochemical characteristics of blood

The TP content in juveniles from the F1 treatment group was significantly lower than in juveniles from the other groups ($P < 0.05$). There was no significant difference in the TP content among juveniles from the F2, F3, and F4 groups ($P > 0.05$). The Glu, TC, and TG contents in juveniles in the F1 group were significantly lower than in juveniles in the other groups ($P < 0.05$). There was a marked increase in the Glu, TC, and TG contents in juveniles in the F2 group compared to juveniles in the F3 and F4 group ($P < 0.05$), and there was no significant difference in these parameters between F3 and F4 groups ($P > 0.05$) (Fig. 1).

3.3. Antioxidant enzymes activities

Feeding frequency significantly decreased the antioxidant enzymes examined (Fig. 2). All antioxidant enzyme activities showed similar trends. The results showed that the SOD, CAT, GSH, and GPx activities in tiger puffers in the F1 group were significantly higher than in tiger puffers in the F3 and F4 groups ($P < 0.05$). However, the enzyme levels in the F2 group were not statistically different from the other groups ($P > 0.05$), except for GSH.

3.4. Digestive enzyme activities

The digestive enzyme activities followed the same trends as the feed efficiency results. The activities of amylase, trypsin, and lipase in the F1 group were the highest among all the groups and were significantly higher than those in the fishes in the F3 and F4 treatment groups ($P < 0.05$). However, the levels of amylase and lipase in the F2 group were not statistically different from the other groups ($P > 0.05$).

3.5. Appetite-related gene expression

The mRNA levels of the appetite-related genes displayed different expression patterns under the different feeding frequencies. There was a considerable decrease in orexin expression with an increasing feeding

frequency from F1 to F4 ($P < 0.05$; Fig. 4A). Conversely, the orexin mRNA levels in the tiger puffers were not significantly different between the F3 and F4 groups ($P > 0.05$). The mRNA levels of tachykinin, CCK, and leptin in the tiger puffers in the F1 group were the lowest among all the groups and significantly lower than those in the fishes in the F3 and F4 groups ($P < 0.05$; Fig. 4B, C, and D). In contrast, the mRNA levels of tachykinin, CCK, and leptin in the F4 group tiger puffers were the highest among all the groups ($P < 0.05$).

4 Discussion

Water temperature significantly influences fish growth. The growth performance of tiger puffer fish decreases with water temperature. Puffer fish require transferring to indoor breeding ponds for overwintering. The study aimed to determine how to maintain an acceptable growth rate through an optimal feeding frequency in an overwintering environment. One of the most important criteria for aquaculture success is feeding efficiency. Previous research shows the higher the feeding frequency, the better the fish growth performance (Azzaydi et al., 2000; Gilannejad et al., 2019). In the present study, the final weight, WGR, SGR, and survival of the tiger puffer significantly increased with increasing feeding frequency from F1 to F3, and then decreased in the F4 group. We speculate that only providing the correct volume of food is not optimal for fish growth, as demonstrated by the low frequency feeding regime groups (F1 and F2). Conversely, the F3 group (4 meals d^{-1}) allowed the tiger puffers more opportunities to obtain enough food and ensured a relatively high growth rate. In addition, excessive feeding times (F4 group) could lead to a lower growth rate. The FCR data further illustrated the problem, and the FCR of the tiger puffers in the F4 group was significantly higher than the FCR of tiger puffers in the F2 and F3 groups and was not significantly different from that of the F1 group. The results revealed that a higher feeding frequency could cause food to move through the digestive tract more quickly, resulting in a decrease in the feed utilization rate. These results are consistent with those previously reported for *Limanda ferruginea*, *Pseudosciaena crocea*, and *Mugil liza* (Dwyer et al., 2002; Xie et al., 2011; Ewerton et al., 2020). Tiger puffer is a fierce fish with a serious cannibal phenomenon. In the present study, an inadequate supply of food cause the tiger puffers in the F1 and F2 groups to display higher cannibal rates, reducing the survival rates in the F1 and F2 groups due to cannibalization. The results suggest an appropriate feeding frequency (F3, 4 meals d^{-1}) is more conducive to tiger puffer survival and growth.

Hematological parameters are reliable indicators of the pathology and physiological status of fish (Barcellos et al., 2004). Jahanbakhshi et al. (2013) identified total plasma proteins as the main component involved in diet metabolism and are important in detecting alterations in protein metabolism. Silva et al. (2020) reported a significant decrease in the total blood protein levels in *mugil liza* when fish were fed only once a day. Chagas et al. (2005) also reported that fish fed once daily contained lower concentrations of total plasma protein. Additionally, our results support previous studies, a decrease in TP levels may be attributed to malnutrition caused by a chronic lack of food intake. In the present study, Glu, TG, and TC levels were significantly higher with an increased feeding frequency. We presume that a high feeding frequency may lead to an excessive accumulation of dietary carbohydrates and lipid classes

in tiger puffers, consequently causing a substantial increase in blood glucose and lipids. In addition, the decrease in Glu, TG, and TC levels when fed only once daily (F1 group) is likely to reflect the decreased concentration of blood glucose and lipid metabolites in starved fish (Mccue, 2010).

Fishes are often challenged by various stressors, including nutritional stress originating from environmental changes. Under stress, fish exhibit antioxidant defense mechanisms to protect against the effects of ROS by enhancing the levels of antioxidant enzymes. Vijayan and Moon (1992) reported that fasted rainbow trout are more sensitive to handling stress. Previous studies identified an enhanced production of ROS coupled with a limited capacity to scavenge ROS is observed in fish under suboptimal and/or adverse conditions (Farombi et al., 2007; Dabas et al., 2012). Li et al. (2014) also reported that low feeding frequencies could cause oxidative stress in juvenile *Megalobrama amblycephala*. In the present study, we observed that SOD, CAT, GSH, and GPx activities in the tiger puffers in the F1 group were significantly higher than in the tiger puffers in the F3 and F4 groups. This result indicates that low feeding frequency (one daily meal, F1 group) produced oxidative stress in tiger puffers. Various assumptions may explain these findings. Due to a shortage of food under the F1 conditions, the tiger puffers are prone to attack each other, which may cause oxidative stress. This suggestion was supported by the reduction in the survival rate in this group. In addition, Davis and Gaylord (2011) reported the stress response was less severe in fasted sunshine bass than in fed fish. Ruane et al. (2002) reported that lower ration levels in common carp (*Cyprinus carpio*) lowered stress levels compared to fish fed high ration levels. This is likely to be a result of interspecific differences in physiological responses to stressors.

The digestive enzyme activities are a considerable factor in the digestion and absorption of food. Proteases, lipases, and amylases are crucial digestive enzymes involved in the digestion of proteins, lipids, and carbohydrates, respectively (Suzer et al., 2008). Our findings showed that digestive enzyme activities in juvenile tiger puffers were influenced by feeding frequency. The activities of amylase, trypsin, and lipase in fishes in the F1 group were the highest among all the groups and significantly higher than the fishes in the F3 and F4 groups. We speculate the contradiction between poor growth at the low feeding frequency and the high enzymatic activity could be caused by food shortages, suggesting the fish may improve their utilization of feed ingredients by increasing enzyme activities. These results are consistent with those previously reported for Asian sea bass, *Lates calcarifer*, and Brazilian sardine, *Sardinella brasiliensis* (Harpaz et al., 2005; Baloi et al., 2017). In addition, Silva et al. (2019) reported that mullet juveniles provided higher feeding frequencies had lower enzymatic activities, suggesting an excess in nutrients may reduce enzymatic activity to avoid unnecessary expenditure. In the present study, we did not observe a similar phenomenon in the F4 groups (continuous diurnal feeding), which may be related to the digestive physiology and metabolic functions in different fish species.

Neuropeptides originating from the hypothalamus regulate food intake by stimulating or inhibiting appetite. These appetite-regulating factors can be divided into orexigenic factors (orexin) and anorexigenic factors (tachykinin, CCK, and leptin). Most of these factors are short-term and regulated by the central nervous system or the peripheral regulatory system of the fish. Previous studies showed that orexin is a kind of neuropeptide secreted from specific hypothalamic neurons that regulate feeding,

foraging, reward, sleep, and arousal in fish and mammals (Sakurai, 2007 and 2014; Qiu et al., 2018; Yang et al., 2020). Tachykinin is a neuropeptide that inhibits appetite and participates in immune, respiratory, and digestive activities (Jensen et al., 1987). CCK is a synthetic peptide hormone produced by neuroendocrine I cells of GIT in the presence of food in the gut, which can be influenced by food deprivation and feeding (Chaudhri et al., 2008; MacDonald and Volkoff, 2009). Leptin is secreted by the pancreas of animals and automatically regulates energy and metabolism in fishes. Karteris et al. (2005) reported that starvation increases the expression of prepro-orexin/hypocretin mRNAs and the orexin/hypocretin protein. In addition, leptin levels can be upregulated after a period of fasting in fish species, such as rainbow trout (Kling et al., 2009), fine flounder (Fuentes et al., 2012), and Atlantic salmon (Trombley et al., 2012). Previous studies show CCK slows gastric emptying and suppresses food intake (Olsson et al., 1999; Volkoff et al., 2005). Volkoff et al. (2006) also found that leptin initiates the action of the anorexigenic factor CCK and inhibits the action of the orexigenic peptide orexin. In the present study, the tiger puffers in the F1 group exhibited the highest expression of orexin and the lowest content of glucose, tachykinin, CCK, and leptin. This result indicates the orexigenic factors were activated in the starved state. In contrast, the mRNA levels of tachykinin, CCK, and leptin in the tiger puffers in the F4 group were the highest, suggesting increases in the anorexigenic levels in the F4 group may be attributed to the negative feedback mechanism in the brain-hypothalamus-neuropeptides axis. These results further illustrate that varying ingestion or excessive feeding can induce changes in the mRNA expression of appetite-related genes, suggesting that nutritional status modulates actions in the nervous system.

5. Conclusion

This study investigated the effects of different feeding frequencies (1/2/4 meals d^{-1} and continuous feeding with an automatic feeding machine) on growth performance, biochemical characteristics of blood, oxidative stress, and appetite-related genes in tiger puffer fish during wintering in RAS. Our results indicate that inappropriate feeding strategies (too low or too high feeding frequencies) could reduce the growth performance, reduce the feed conversion ratio, and alter blood physiology and the expression of appetite-related genes. Additionally, we identified that growth and physiological indicators in the F3 group (4 meals d^{-1}) showed a consistent positive trend. Our results suggest feeding 4 meals d^{-1} is the optimal feeding frequency for tiger puffer juveniles when overwintering at 18.0 ± 1.0°C.

Declarations

6. Funding

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7. Conflicts of interest

All authors have no any potential sources of conflict of interest.

8. Ethics approval/declarations

All animals used in the research were treated humanely according to the guidelines of the university committees, with due consideration to the alleviation of distress and discomfort.

9. Consent for publication

All authors have read the manuscript and agree that the work is ready for submission to the journal and they accept the responsibility for the manuscript's content. The study is performed in accordance with guidelines of Fish Physiology and Biochemistry.

10. Data Availability Statement

The data that support the findings of this study are openly available at https://figshare.com/s/9c6a82240eaf655aaeb8, DOI: 10.6084/m9.figshare.14955105.

11.Code availability

Not applicable

12. Authors' contributions

In this paper, Xiao-Qiang Gao mainly designed experiment and wrote the manuscript. Xi Wang, Xinyi Wang and Shu-Quan Cao finished biochemical parameters analysis experiment and collect partly data. Hong Xu Li, Liang Xu and Ying-Ying Fang mainly undertaken blood, tissues collection and fish management. Bin Huang provided partly funding support and idea. Chen Hai-Bin and Xing Rui were mainly responsible for technical guidance for aquaculture during the experiment. Bao Liang Liu mainly revised the manuscript. All authors joined the analysis and interpretation of data and approved the final version of the manuscript.

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Figures

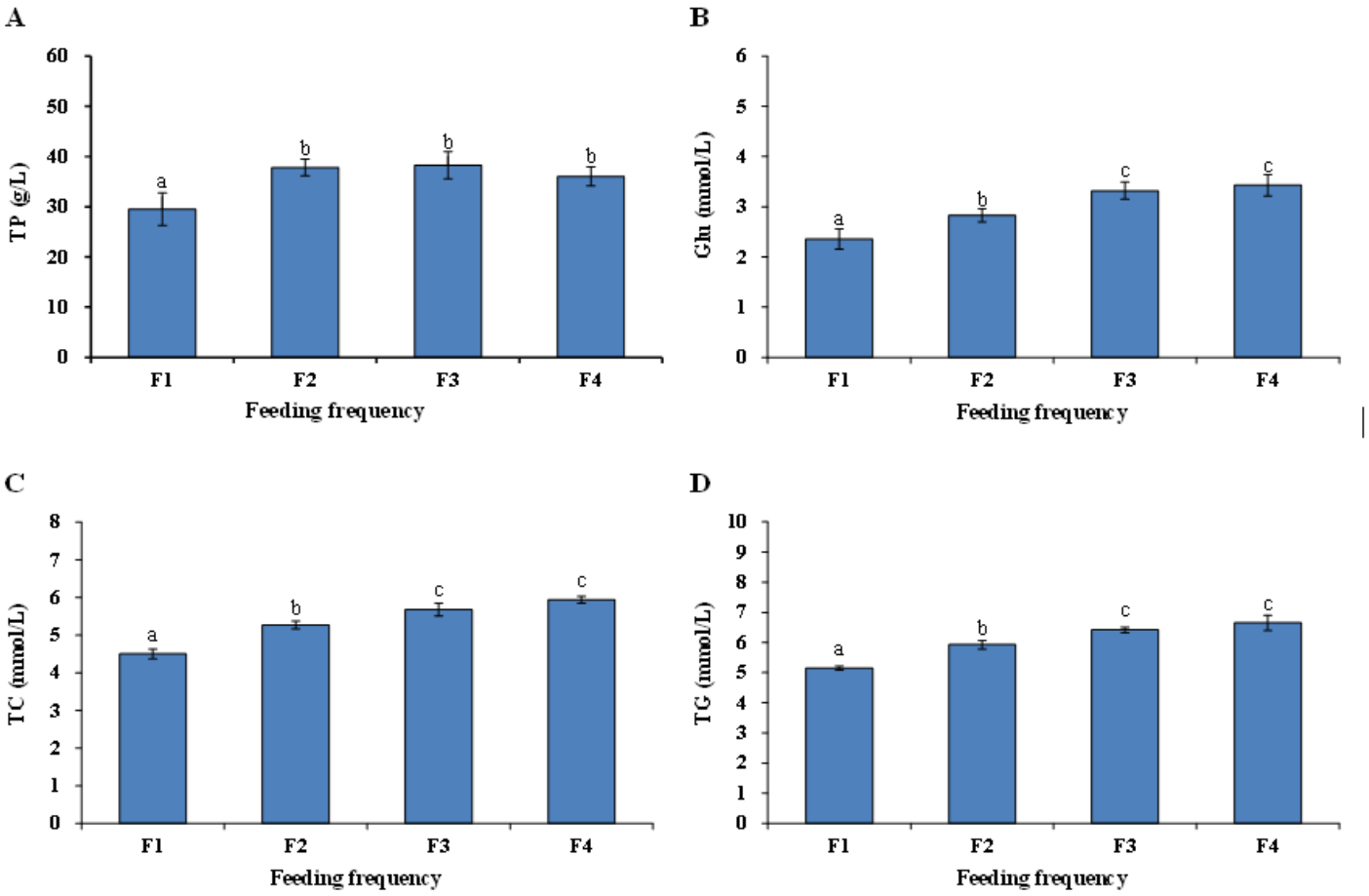

Figure 1

Biochemical blood parameters of Tiger puffer juveniles with different feeding frequencies for 60 days. The values are expressed as the mean ± S.D. The significant difference between groups at $P < 0.05$ is showed by different letter.

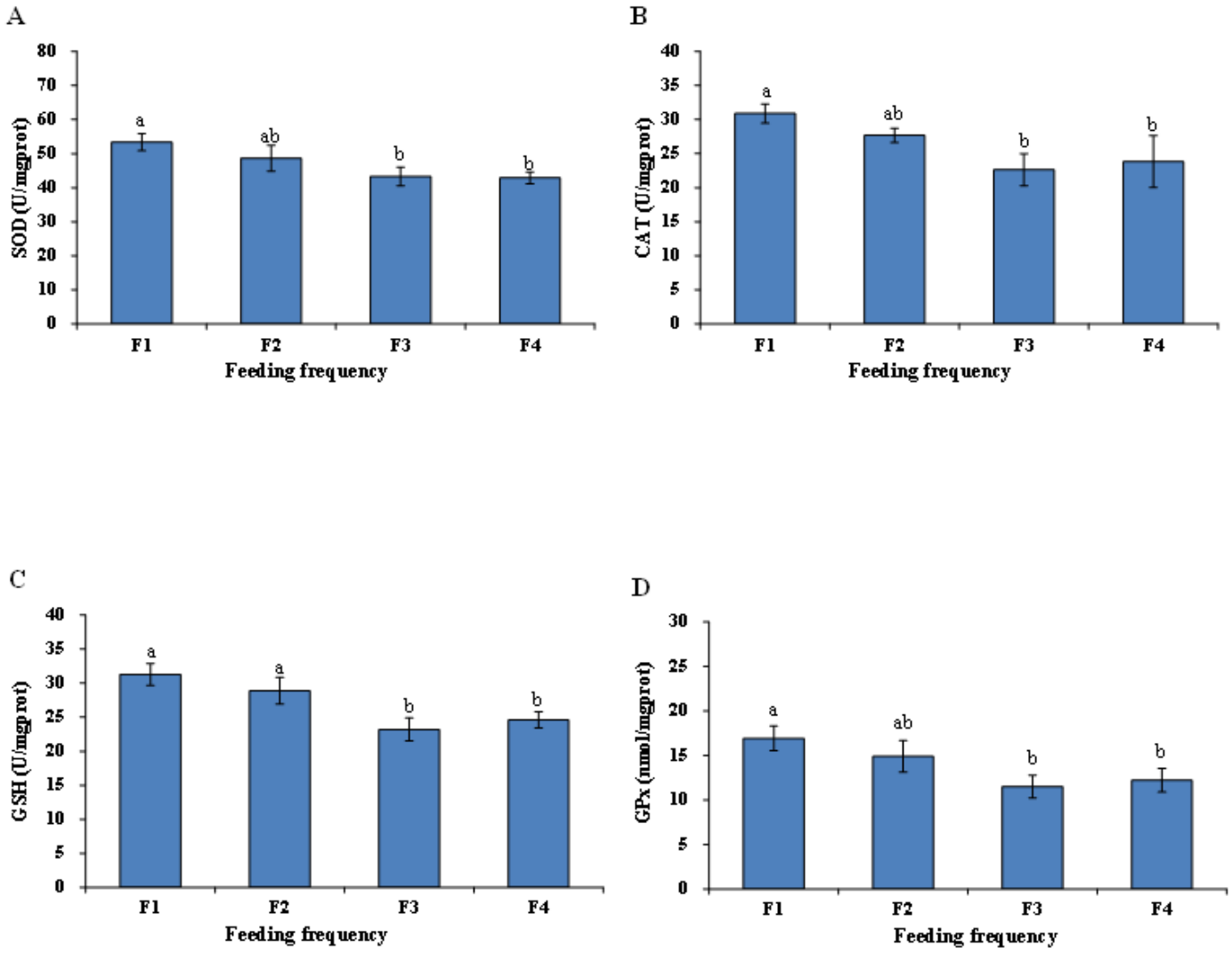

Figure 2

Antioxidant enzymes activity of Tiger puffer juveniles with different feeding frequencies for 60 days. The values are expressed as the mean ± S.D. The significant difference between groups at $P < 0.05$ is showed by different letter.

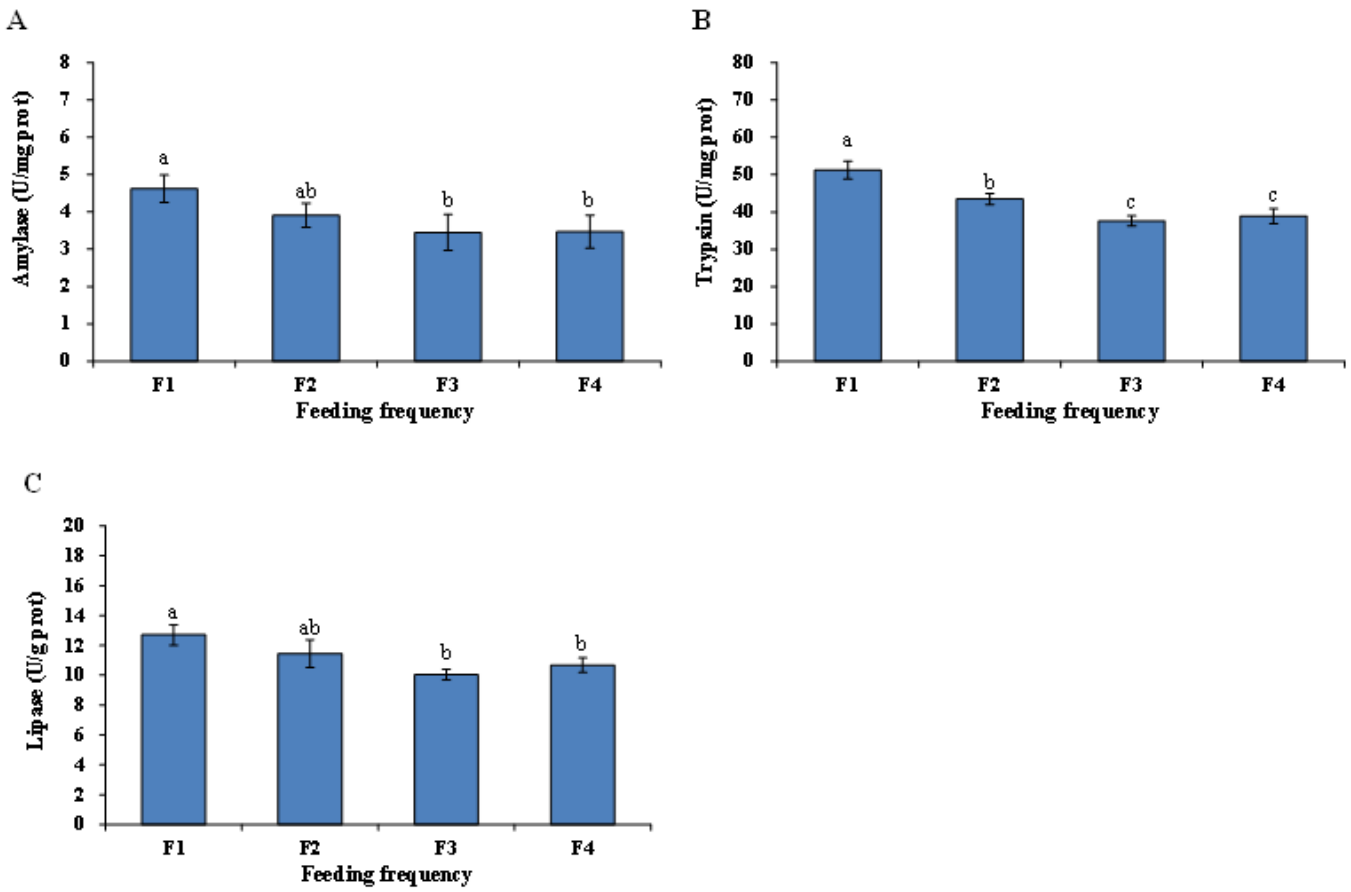

Figure 3

Specific activities of amylase, trypsin and lipase of Tiger puffer juveniles with different feeding frequencies for 60 days. The values are expressed as the mean ± S.D. The significant difference between groups at $P < 0.05$ is showed by different letter.

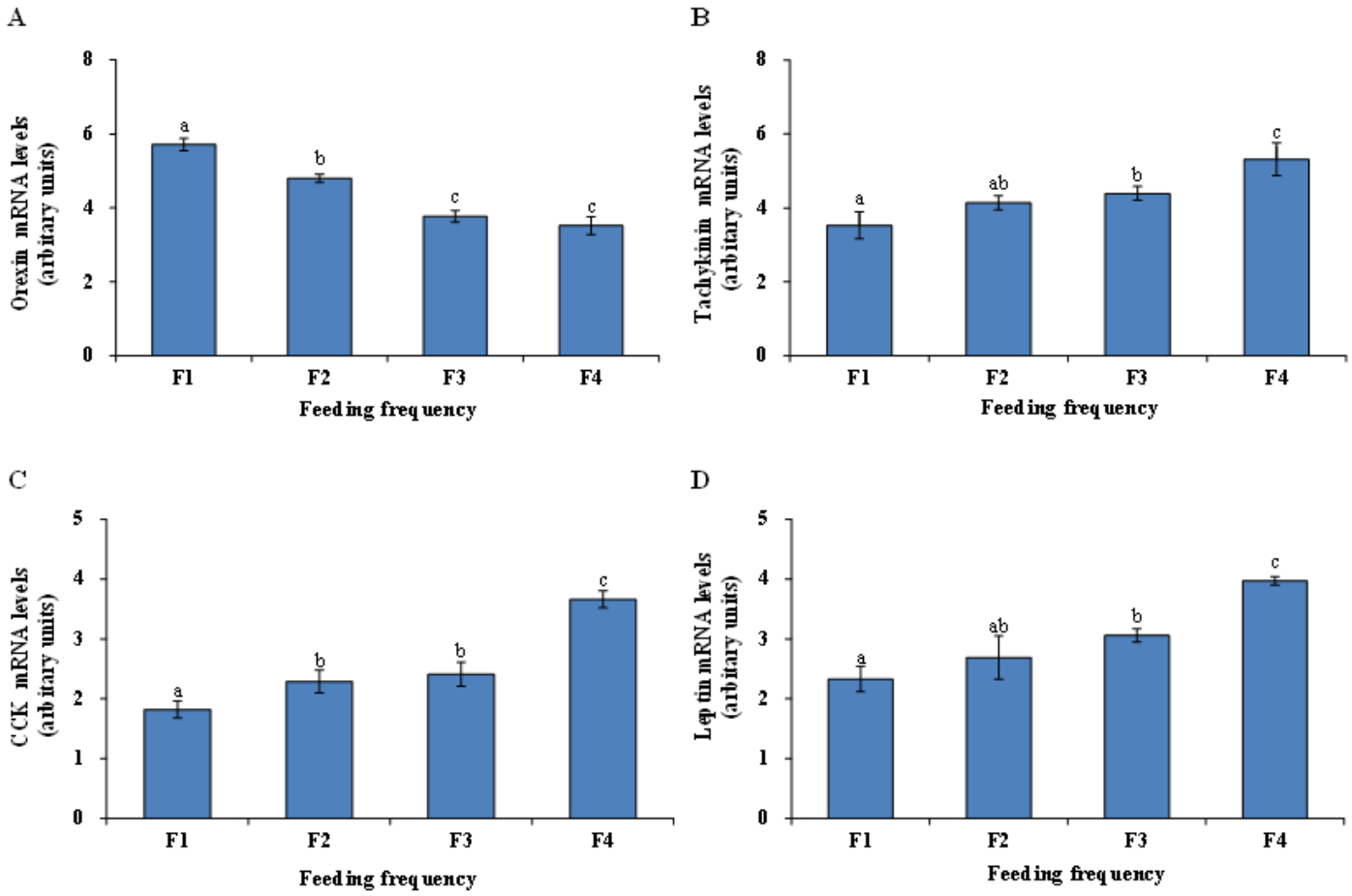

Figure 4

Relative expression levels of Orexin, Tachykinin, CCK and Leptin of Tiger puffer juveniles with different feeding frequencies for 60 days. The values are expressed as the mean ± S.D. The significant difference between groups at $P < 0.05$ is showed by different letter.