Effect of winter feeding frequency on growth performance, biochemical blood parameters, oxidative stress, and appetite-related genes in Takifugu rubripes

Xiaoqiang Gao · Xinyi Wang · Xi Wang · Hongxu Li · Liang Xu · Yingying Fang · Shuquan Cao · Bin Huang · Haibin Chen · Rui Xing · Baoliang Liu

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Abstract Tiger pufferfish (Takifugu rubripes) is one of Asia’s most economically valuable aquaculture species. However, winter production of this species in North China is limited by low water temperature and unavailability of high-quality feed, resulting in high mortality and low profitability. Therefore, the aim of this study was to evaluate the effect of feeding frequency (F1: one daily meal; F2: two daily meals; F3: four daily meals; F4: continuous diurnal feeding using a belt feeder) on the growth performance, plasma biochemistry, digestive and antioxidant enzyme activities, and expression of appetite-related genes in T. rubripes (initial weight: 266.80 ± 12.32 g) cultured during winter (18.0 ± 1.0 °C) for 60 days. The results showed that fish in the F3 group had the highest final weight, weight gain rate, specific growth rate, survival rate, and best feed conversion ratio. Additionally, daily feed intake increased significantly with increasing feeding frequency. The protein efficiency and lipid efficiency ratios of fish in the F3 group were significantly higher than those of fish in the other groups. Furthermore, total cholesterol, triglycerides, and glucose levels increased with increasing feeding frequency, peaking in the F2 group and decreasing under higher feeding frequencies. The antioxidant (superoxide dismutase, catalase, glutathione, and glutathione peroxidase) and digestive (trypsin, amylase, and lipase) enzyme activities of fish in the F1 group were significantly higher than those of fish in the F3 and F4 groups. Additionally, there was a decrease in orexin expression with increasing feeding frequency. In contrast, the expression levels of tachykinin, cholecystokinin, and leptin increased with increasing feeding frequency, peaking in the F4 group. Overall, the findings of this study indicated that a feeding frequency of four meals per day was optimal for improved growth performance of pufferfish juveniles cultured during winter.

Keywords Takifugu rubripes · Feeding regime · Growth performance · Oxidative stress · Appetite-related gene
Introduction

Tiger pufferfish (Takifugu rubripes) is commercially valuable owing to its high value and limited supply and is traded in great volumes throughout China, Korea, and Japan (Miyadai et al. 2001; Tao et al. 2012). Over the years, however, wild tiger pufferfish populations have considerably declined due to high demand and habitat destruction (Jia et al. 2019). Mass culture techniques such as artificial propagation, specific nutritional and feeding strategies, and artificial ovulation induction have been adopted in northern China to protect the species and meet market demands (Ma et al. 2011; Zhao et al. 2018; Wei et al. 2019, 2020, 2021). However, winter production of tiger pufferfish in northern China is limited by low water temperature, resulting in high mortality and low profitability (Lin et al. 2017). Studies have reported decreased feeding frequency, intermittent feeding, and total cessation of feed intake under low water temperature (Rowan and Stone 1994; McNulty et al. 2000; Nanninga et al. 2011). Owing to poor performance in the wild during winter, it has been suggested that 1-year-old juveniles could be moved to offshore culture cages equipped with heaters and recirculating aquaculture systems (RAS) during winter for optimal growth performance. Lin et al. (2017) reported that seawater temperature must be maintained at approximately 20 °C to ensure optimal growth performance of tiger pufferfish, while experienced commercial tiger pufferfish farmers in Dalian have suggested that optimal growth could be supported in temperatures as low as 18 °C during winter. However, heating large volumes of cold seawater to attain either of these temperatures is neither cost-efficient nor sustainable. Some studies have shown that optimal feed management, including regulating feeding frequency, can improve the performance of this species during winter.

For instance, Kim and Lovell (1995) reported weight loss in channel catfish (Ictalurus punctatus) that were fed consistently during winter compared with those that were not fed. Similar results were observed in I. punctatus by Nanninga et al. (2011). In contrast, Burtle and Newton (1993) observed that the weight of I. punctatus can be maintained or even increased by feeding once weekly during warm winters (mean water temperature near 16.3 °C). Roy et al. (2017) reported that although bluegill (Lepomis macrochirus) and hybrid bluegill (L. cyanellus x L. macrochirus) experienced weight loss under different feeding frequencies at constant low temperatures (7–9 °C), the survival rate was high among all treatments (89–98%). Therefore, optimizing feed management (feeding rate and frequency) could be a promising research area to improve the winter production of several aquaculture species (Okomoda et al. 2019).

Improving feed management is also pertinent during the grow-out phase—often the longest production stage—and represents one of the solutions for increasing the profitability of the aquaculture industry (Dias et al. 2010). Feed management includes variables such as food size, feeding rate, spatial distribution, feeding time, and feeding frequency (Xie et al. 2011; Kousoulaki et al. 2015). Among these, feeding frequency is perhaps the most important factor in maximizing feed utilization efficiency while reducing waste and lowering production costs. Therefore, it has attracted considerable attention from aquaculture research groups (Busti et al. 2020). Increasing the daily feeding frequency or apportioning daily feed rations can improve the growth rate and feed conversion ratio (Stathopoulou et al. 2021; Chen et al. 2020; Roy et al. 2017). Several studies have reported that the growth rate either decreases or is unaffected above a certain feeding frequency (Oh and Maran 2015; Küçük et al. 2013; Seo and Lee 2008). Studies attempting to optimize the daily feeding frequency of fish cultures have often focused on survival, growth, and feed efficiency; moreover, the findings of these studies are largely inconsistent (Oh et al. 2018). The effects of different feeding frequencies on the hematological parameters and oxidative status of fish have been widely investigated. Ewerton et al. (2020) reported that increasing feeding frequencies also increased the blood total protein, glucose, triglyceride, and cholesterol contents of Mugil liza, possibly due to increased intake and absorption of nutrients. Li et al. (2014) and Guo et al. (2017) also revealed that low and high feeding frequencies might cause oxidative stress of juvenile blunt snout bream Megalobrama amblycephala and Dolly Varden char Salvelinus malma, respectively. Additionally, the appetite level reflects the degree of interest, feeding frequency, and predation intensity.
of fish in bait and significantly affects the growth performance of cultured objects. Some studies reported that during fasting cycles, gut cholecystokinin (CCK) expression decreased in *Seriola quinqueradiata* and *Diplodus sargus* (Micale et al. 2012; Murashita et al. 2006) and increased in *Oncorhynchus kisutch* (Lohmus et al. 2008). Ahi et al. (2019) reported that the expression of orexigenic genes was not affected by impaired leptin signaling under normal feeding conditions; however, several orexigenic genes showed increased transcription during fasting and refeeding. Additionally, studies are yet to examine the effect of feeding frequency or time on the growth performance and physiological status of tiger pufferfish during the winter.

Measures that reduce feeding costs without negatively affecting the growth performance and quality of fish could improve the profitability and sustainability of the aquaculture industry. Therefore, the aim of this study was to evaluate the effect of feeding frequency on the growth performance, serum parameters, oxidative stress, and appetite-related genes in tiger pufferfish cultured under a relatively low temperature.

**Materials and methods**

**Culture system and feeding management**

In October 2018, *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). The fish were acclimatized for 2 weeks in the new environment before experimentation. During the acclimation period, fish were fed a commercial diet twice daily (HaiTong Group Foods, Fujian, China; size 4.0 to 5.0 mm) and maintained under a 12:12 h light:dark cycle. The daily water exchange rate was 45%, and the water conditions were maintained at 18.0 ± 0.5 °C and salinity at 28.0 ± 1.0 ppt. After acclimatization, the fish (initial weight per individual: 266.80 ± 12.32 g) were randomly assigned to 12 small cement ponds (2.0 m × 2.0 m × 1.2 m, effective depth 1.0 m) set up to have three replicate ponds per feeding regime. Each pond held 200 specimens in a water volume of 100 m³ and was equipped with a recirculating aquaculture system. The stocking density was 13.3 kg/m³ per pond.

The experimental fish were fed the same commercial feed during the experimental period using four different feeding regimes. The proximate composition of the experimental diets is presented in Table 1. The four different feeding regimes were as follows: one daily meal at 08:00 (F1); two daily meals at 08:00 and 17:00 (F2); four daily meals at 08:00, 11:00, 14:00, and 17:00 (F3); and continuous diurnal feeding using a belt feeder (F4). All groups were fed to satiation, which amounted to 1.5% of a fish’s body weight. The satiation level was assessed before the experiment was initiated, following the standardized methodology of supplying the fish with daily rations, starting with small quantities of food and gradually increasing them until almost no food remained in the ponds after each meal (Neda et al. 2019). Daily rations were equally distributed following the multiple feeding protocols, in which each meal in the F1, F2, and F3 groups lasted 45 min. The automatic belt feeder supplied feed to the different groups at the respective feeding times in equal portions (7–8 g every 5 min). The fixed ration level was adjusted every 20 days (F1-1, F2-1, F3-1, F4-1) based on the apparent satiation procedure described in Busti et al. (2020) by oversupplying the feed to one replicate in each group. The amount of feed was calculated by the average value of the four groups. Uneaten pellets were collected using a siphon, dried overnight at 105 °C, and then weighed. The mean water temperature of the circulating system was maintained at 18.0 ± 1.0 °C throughout the experiment. The water flow rate within each pond was set to 100% exchange every 3 h, where approximately 3% of the overall

| Treatments | F1  | F2  | F3  | F4  |
|------------|-----|-----|-----|-----|
| Moisture   | 4.77| 4.94| 4.91| 5.03|
| Crude protein | 54.56| 54.73| 55.06| 54.33|
| Crude lipid | 12.09| 12.22| 12.36| 12.27|
| Ash        | 8.85| 9.12| 9.22| 9.05|
| Lysine     | 2.62| 2.59| 2.61| 2.63|

F1, F2, F3, and F4 refer to groups fed for one daily meal, two daily meals, four daily meals, and continuous diurnal feeding, respectively.
RAS water volume was renewed daily. During the experimental period, each pond’s temperature, dissolved oxygen, and pH were measured twice daily (morning and evening) using a digital multi-parameter controller (DIQ/S 182XT-4, Tianjin, China). Each pond’s nitrite concentration was determined spectrophotometrically following the Griess method (Bryan and Grisham 2007). The total ammonia nitrogen content was estimated using nesslerization with NH₄Cl as the reagent (Hegazi et al. 2010). The mean water conditions were maintained as follows: dissolved oxygen at 8.05 ± 0.84 mg/L, pH at 7.5 ± 0.2, salinity: 28.0 ± 1.0 ppt, total ammonia at 0.26 ± 0.07 mg/L, and nitrite at 0.33 ± 0.04 mg/L.

Sample collection

The experimental duration was from November 1, 2020, to December 30, 2020. At the end of the experiment, the fish were fasted for 24 h and then anesthetized using tricaine methanesulfonate (MS222, Sigma, St. Louis, MO). The survival rate of the fish was determined by counting the number of surviving specimens in each pond during the final sampling, and the fish were individually weighed. Blood samples (1.5 mL) were collected from six fish per trial pond via a caudal puncture using a syringe and centrifuged at 10,000 × g for 5 min at 4 °C for plasma biochemistry analysis. Their livers, anterior intestines, and hypothalami were also sampled. Parts of the livers and sections of the anterior intestines were homogenized in ice-cold phosphate buffer (50 mM, pH 7.4) using a Teflon glass homogenizer and then centrifuged at 5000 × g for 10 min at 4 °C. The supernatants of the homogenized organ samples were then collected and stored for either liver antioxidant or intestinal digestive enzyme assays. The entire hypothalamus and the remaining liver and anterior intestine tissues were preserved in a BioSample Stabilizing Reagent (Accurate Biotechnology, Hunan, China) and stored at −80 °C for RNA extraction. Lastly, three additional fish specimens were randomly collected from each pond and stored at −80 °C for the whole-body proximate composition analysis. 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 (YSFRI-2019017).

Growth parameter calculations

Growth parameters were calculated using previously described formulas (Kim et al. 2010; Oh and Maran 2015):

Weight growth rate (WGR, %) = 100 × [(W₀f − W₀i)/W₀i],

Specific growth rate (SGR, % · day⁻¹) = 100 × [(ln W₀f − ln W₀i)/T],

Feed conversion ratio (FCR) = D/(W₀f − W₀i + W₀d),

Daily feed intake (DFI, g/g) = 100 × D/[(W₀f + W₀i + W₀d)/2 × T],

Protein efficiency ratio (PER, %) = 100 × (W₀f × P₀f − W₀i × P₀i + W₀d × P₀d)/D × P₅

Lipid efficiency ratio (LER, %) = 100 × (W₀f × L₀f − W₀i × L₀i + W₀d × L₀d)/D × L₅

Fulton’s condition factor (K) = W₀f/L₀f³ × 100.
Survival rate (SR, %) = \( N_t / N_o \times 100 \),
where \( W_{af} \) and \( W_{ai} \) are the final and initial average fish weights (g), respectively; \( W_{tf}, W_{ti}, \) and \( W_{td} \) are the total final, initial, and dead fish weights (g), respectively; \( L_{af} \) is the final average fish length (cm); \( T \) is 60 days; \( D \) is the total amount of the consumed feed during the experimental process (g); \( P_i \) (%) and \( P_f \) (%) are the protein contents of the whole fish at the beginning and at the end of the experiment, respectively, and \( P_d \) (%) is the protein content of the feed; \( L_i \) (%) and \( L_f \) (%) are the lipid contents of the whole fish at the beginning and end of the experiment, and \( L_d \) (%) is the lipid content of the feed; and \( N_o \) and \( N_t \) are the initial and final fish numbers, respectively.

Serum parameter analysis

The serum total protein (TP), total cholesterol (TC), triglyceride (TG), and glucose (Glu) were analyzed using an automatic chemistry analyzer (AU5421, Olympus, Japan), following procedures described by Wu and Shang (2006). The test kit was purchased from the Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Plasma cortisol concentration was determined using a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) quantification kit (Mlbio, Shanghai, China).

Liver antioxidant enzyme activity

The antioxidant enzyme activity of 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 amount of enzyme required to inhibit the oxidation reaction by 50%, expressed as unit per milligram of protein. Catalase (CAT) activity was determined by measuring the decrease in \( \text{H}_2\text{O}_2 \) 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).

Measurement of intestinal enzyme activity

Trypsin activity was determined following the method of Erlanger et al. (1961), using N-\( \alpha \)-benzoyl-dl-arginine-p-nitroanilide as a substrate. One unit of activity was defined as 1 \( \mu \)mol of nitroanilide released per minute. Amylase activity was quantified following the method described by Bernfeld (1955), using 1% starch solution as the substrate, and one unit of activity was defined as 1 \( \mu \)mol of maltose released per minute. Lipase activity was determined using the method described by Gjellesvik et al. (1992).

Proximate compositions of fish and diet

The proximate compositions of the experimental fish and diet were determined using the standard methodology of AOAC (2000). Moisture content was determined by oven drying the samples at 105 °C for 24 h until a constant weight was attained. Ash content was determined by incinerating the sample in a muffle furnace at 550 °C for 5 h. Crude protein content was determined using the Kjeldahl method (N×6.25). Crude fat content was determined using the ether extraction method. All analyses were performed in triplicate.

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 total RNA mixture was treated with RNase-free DNase I (Promega Madison, Hunan, China) and purified to remove genomic DNA. The quality of the extracted RNA was determined by measuring its absorbance at 260 and 280 nm using the GeneQuant 1300 (GE Healthcare Bioscience, Piscataway, NJ). The integrity of the extracted RNA was tested by electrophoresis on a 2.0% agarose gel. Total RNA (1 \( \mu \)g) was then treated with DNase I, and first-strand cDNA was synthesized using an AMV enzyme (Promega, Hunan, China). Conditions for the reverse transcription (RT) reaction were 46 °C for 48 min and 72 °C for 8 min.

The expression of leptin in the liver, tachykinin in the anterior intestine, and orexin and cholecystokinin (CCK) in the hypothalamus were analyzed. The primer sequences used for measuring orexin,
tachykinins, CCK, and leptin mRNA levels are listed in Table 2. PCR amplification for appetite-related genes was performed using a GoTaq Flexi DNA polymerase (Promega, Hunan, China). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green Premix Pro Taq (AG11702, Accurate Biotechnology Co., Ltd., Hunan, China) and an ABI 7300 System (PerkinElmer Applied Biosystems, USA). The real-time PCR (20 μL) reaction contained 10 μL GoTaq® qPCR Master Mix, 2 μL of cDNA, and 0.8 μL of each primer. Ultrapure water was added to make up the final reaction volume. The real-time PCR conditions were as follows: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s, and 60 °C for 20 s. All samples were amplified in triplicate, and the mean value of these triplicate measurements was used to calculate the mRNA expression. The housekeeping gene β-actin was used as an internal standard. Relative gene expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Prior to statistical analysis, the data’s normality and homogeneity of variance were tested using the Kolmogorov–Smirnov and Levine’s tests, respectively. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test, using SPSS 22.0 software. All data are presented as mean ± SD. Means were considered significantly different at $p < 0.05$.

| Genes             | Primer sequence (5’–3’)                  | Size (bp) |
|-------------------|-----------------------------------------|-----------|
| OrexinB           | F: TGACCCGGAGATGCGCGCCGCTGG             | 182       |
|                   | R: CTGGGCAAACGCAAGGAAGAAGGG             |           |
| Tachykinins       | F: CCCAGTACATTCTATGGCGTTCCG             | 228       |
|                   | R: GTGACACAGTGATAATCGT                  |           |
| Cholecystokinin   | F: GATATCAAGCAAGCAGAAAGG               | 198       |
|                   | R: TAGAGAAACCTTAGCTGACAGC              |           |
| Leptin            | F: TACCCCGAGGTTCTGCTGAT                | 174       |
|                   | R: CTTGGGTCTATTTAGGGACACC              |           |
| β-actin           | F: CAGGGAGAAAGATGACCCAGA              | 125       |
|                   | R: CATCACAGAGTCCATGACG                 |           |

Results

Performance measures

Tiger pufferfish in the F1 group exhibited the lowest final weight, WGR, SGR, and survival rate, and the highest FCR, followed by those in the F2 group (Table 3). In contrast, the F3 group demonstrated the highest values for the parameters and the lowest FCR, followed by those in the F4 group. DFI increased significantly with increasing feeding frequency ($p < 0.05$); however, no significant differences were observed between the F1 and F2 groups ($p > 0.05$). Additionally, fish in the F3 group had significantly higher ($p < 0.05$) PER and LER values than those in the other groups, with the lowest value observed in the F4 group. However, feeding frequency did not significantly affect ($p > 0.05$) the $K$ values of the fish.

Biochemical characteristics of blood

The TP content of fish in the F1 group was significantly lower ($p < 0.05$) than those of fish in the other groups; however, there was no significant difference ($p > 0.05$) among the F2, F3, and F4 groups. Similarly, the Glu, TC, and TG contents of fish in the F1 group were significantly lower ($p < 0.05$) than those of fish in the other groups. Additionally, the Glu, TC, and TG contents of fish in the F2 group were significantly higher ($p < 0.05$) than those of juveniles in the F3 and F4 groups; however, no significant difference ($p > 0.05$) was observed between the F3 and F4 groups (Fig. 1).
Table 3 Growth performance of juvenile tiger puffer reared under different feeding frequencies over 60 days

| 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    |
| DFI (g/g)         | 2.30 ± 0.10^a    | 2.31 ± 0.2^a     | 2.93 ± 0.07^b    | 3.52 ± 0.42^c    |
| PER (%)           | 26.37 ± 0.49^a   | 28.27 ± 0.59^b   | 31.36 ± 0.57^c   | 24.41 ± 0.59^d   |
| LER (%)           | 14.59 ± 0.29^a   | 16.78 ± 0.49^b   | 19.53 ± 0.42^c   | 12.25 ± 0.20^d   |
| K                 | 3.53 ± 0.27      | 3.68 ± 0.18      | 3.67 ± 0.09      | 3.73 ± 0.15      |
| Survival (%)      | 73.17 ± 2.57^a   | 84.50 ± 3.04^b   | 90.50 ± 2.18^b   | 87.33 ± 3.25^b   |

WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; DFI, daily feed intake (g/g); PER, protein efficiency ratio (%); LER, lipid efficiency ratio (%); K, Fulton’s condition factor. F1, F2, F3, and F4 refer to groups fed for one daily meal, two daily meals, four daily meals, and continuous diurnal feeding, respectively. Different superscript letters in each row indicate significant differences between values (p < 0.05).

Fig. 1 Biochemical blood parameters of juvenile tiger puffer with different feeding frequencies for 60 days. These parameters include total protein (TP, A), glucose (Glu, B), total cholesterol (TC, C), and triglycerides (TG, D). F1, F2, F3, and F4 refer to groups fed for one daily meal, two daily meals, four daily meals, and continuous diurnal feeding, respectively. The values are expressed as the mean ± S.D. The significant difference between groups at p < 0.05 is showed by different letters.
Liver antioxidant enzyme activity

There was a significant decrease in the activities of antioxidant enzymes in the fish’s liver with increasing feeding frequency (Fig. 2), with the enzymes showing similar trends in all groups. Specifically, SOD, CAT, GSH, and GPx activities were significantly higher ($p < 0.05$) in the F1 group compared with the F3 and F4 groups. Additionally, GSH activity was significantly higher in the F2 group compared with the F3 and F4 groups; however, there were no significant differences ($p > 0.05$) in the activities of the other enzymes among the F2, F3, and F4 groups.

Digestive enzyme activity

Fish in the F1 group had the highest amylase, trypsin, and lipase activities, which were significantly higher ($p < 0.05$) than those of fish in the F3 and F4 groups. However, the amylase and lipase activities of fish in the F2 group were not significantly different ($p > 0.05$) from those of fish in the other groups (Fig. 3).

Appetite-related gene expression

The experimental fish’s mRNA expression levels of appetite-related genes varied under different feeding frequencies. Specifically, there was a significant decrease ($p < 0.05$) in orexin expression in the hypothalamus with increasing feeding frequency (Fig. 4A). However, there was no significant difference ($p > 0.05$) in orexin mRNA levels between the F3 and F4 groups. Additionally, the mRNA expression levels of tachykinin (anterior intestine), CCK (hypothalamus), and leptin (liver) of fish in the F1 group were significantly lower ($p < 0.05$) than those of fish in the F3 and F4 groups (Fig. 4B, C, and D), indicating that the

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**Fig. 2** Antioxidant enzyme activity of juvenile tiger puffer with different feeding frequencies for 60 days. These parameters include superoxide dismutase (SOD, A), catalase (CAT, B), glutathione (GSH, C), and glutathione peroxidase (GPx, D). F1, F2, F3, and F4 refer to groups fed for one daily meal, two daily meals, four daily meals, and continuous diurnal feeding, respectively. The values are expressed as the mean ± S.D. The significant difference between groups at $p < 0.05$ is showed by different letters.
expression levels increased with increasing feeding frequency. Overall, the highest mRNA levels ($p < 0.05$) of tachykinin, CCK, and leptin were observed in fish in the F4 group.

**Discussion**

The aim of this study was to determine the optimal feeding frequency for optimal growth and performance of tiger pufferfish during winter. Previous research has shown that feeding frequency is directly proportional to the growth performance of fish (Azzaydi et al. 2000; Gilannejad et al. 2019). In the present study, there was a significant increase in the final weight, WGR, SGR, PER, LER, and survival rate of tiger pufferfish with increasing feeding frequency from groups F1 to F3, followed by a decrease in the F4 group, indicating a quadratic pattern. Among the feeding frequencies examined, four meals per day supported optimal growth of juvenile tiger pufferfish.

Inappropriate feeding strategies (feeding frequencies that are too low or high) have been shown to negatively affect the growth performance and survival of fish (Lee et al. 2016; Ribeiro et al. 2012). The findings of the present study showed that a feeding frequency of one meal per day was insufficient to meet the fish’s nutritional requirement, resulting in malnutrition (Ewerton et al. 2020), as evidenced by the poor growth performance of fish in the F1 group. Moreover, excessive feeding (F4 group, continuous diurnal feeding) resulted in lower values of SGR, PER, and LER, and higher values of FCR and DFI. Busti et al. (2020) reported that a high feeding frequency (three times/day) had no considerable effect on overall performances and feed efficiency of gilthead seabream (*Sparus aurata*). Moreover, an increase in the number of daily meals can cause rapid dumping of food in the digestive tract, reducing the time needed to digest and absorb the feed efficiently, which may eventually lead to poorer feed conversion and nutrient efficiency ratio (Riche et al. 2004; Booth et al. 2008; Costa-Bomfim.
et al. 2014). These results were 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). Furthermore, inappropriate feeding frequencies can cause cannibalism in fish species, as observed in *Clarias gariepinus* (Kaiser et al. 1995), *Channa striatus* (Muntaziana et al. 2017), and *Limanda ferruginea* (Dwyer et al. 2002). In the present study, an inadequate supply of feed caused higher rates of cannibalism in the F1 and F2 groups. In contrast, excess supply of feed in the F4 group resulted in fierce competition for food intake, which increased biting between conspecifics. This contributed to the higher mortality rate in the F1 and F4 groups.

Hematological parameters are reliable indicators of the physiological status of fish and their pathology (Barcellos et al. 2004). Jahanbakhshi et al. (2013) identified total plasma proteins as the main component involved in dietary metabolism and to be important in detecting altered protein metabolism. Silva et al. (2020) reported that *M. liza* fed only once a day displayed a significant decrease in total blood protein levels, similar to Chagas et al. (2005) and this study. This indicates that the decrease in total blood protein levels in the F1 group individuals may be attributed to malnutrition caused by a severe lack of feeding. Generally, there was an increase in plasma Glu, TG, and TC levels with increasing feeding frequency. This result indicated that a high feeding frequency might lead to excessive accumulation of dietary carbohydrates and lipids in tiger pufferfish, resulting in a substantial increase in blood glucose and lipids. Moreover, Al-Khafaji et al. (2017) observed significantly lower plasma cholesterol, TG, and Glu levels in jade perch (*Scortum barcoo*) fed once daily compared with those fed thrice daily. Similarly, the present study’s findings showed the lowest Glu, TG, and TC levels in the fish specimens fed only once daily.
daily (F1 group). This indicates that the tiger pufferfish mobilized more energy during starvation to maintain normal metabolic levels (Mccue 2010).

Fish species are often challenged by various stressors, including nutritional stress originating from environmental changes. For example, Vijayan and Moon (1992) reported that fasted rainbow trout (*Oncorhynchus mykiss*) are more sensitive to stress. Under stressful conditions, fish exhibit antioxidant defense mechanisms to protect against reactive oxygen species (ROS) by augmenting the levels of antioxidant enzymes. Enhanced production of ROS and limited ROS scavenging capacity in fish under suboptimal or adverse conditions could lead to death (Farombi et al. 2007; Dabas et al. 2012). An example is the study by Li et al. (2014) that reported that low feeding frequencies could cause oxidative stress in juvenile *Megalobrama amblycephala*. In this study, we observed significantly higher SOD, CAT, GSH, and GPx activities in the F1 group than those in the F3 and F4 groups. This result indicated that a low feeding frequency (one daily meal, F1 group) could induce oxidative stress in tiger pufferfish. Moreover, the tendency of tiger pufferfish to attack each other may also result in oxidative stress. These findings corroborate those of Qin and Fast (1996), who observed exacerbated aggression and cannibalistic behaviors among *C. striatus* juveniles; however, Davis and Gaylord (2011) reported that the stress response in the fasted sunshine bass was less severe than that in the well-fed fish. Additionally, Ruane et al. (2002) reported that supplying a low amount of feed to common carp (*Cyprinus carpio*) reduced stress levels compared with those in the fish specimens fed a larger ration. These differences in the experimental results could be attributed to interspecific differences in physiological responses to stress.

Digestive enzyme activities are important factors in the digestion and absorption of food. Proteases, lipases, and amylases are crucial enzymes in the digestion of proteins, lipids, and carbohydrates, respectively (Suze et al. 2008). Contradictory to the lower growth rates in the F1 group, the amylase, trypsin, and lipase activities were highest in the F1 group juvenile specimens compared with those of the F3 and F4 group specimens. We speculated that this contradiction could be caused by the food shortages, as the fish may have improved their feed utilization by increasing enzyme activities while experiencing food scarcity. These results were consistent with previous findings in Asian sea bass (*Lates calcarifer*) and Brazilian sardines (*Sardinella brasiliensis*) (Harpaz et al. 2005; Baloi et al. 2017). Furthermore, Silva et al. (2020) observed low enzymatic activities in mullet juveniles under high feeding frequency, indicating that nutrients in excess induce a reduction in enzymatic activity to avoid unnecessary energy expenditure. However, we did not observe a similar phenomenon in the F4 group (continuous diurnal feeding) in our investigations, which may be due to the differences in digestive physiology and metabolic functions.

Neuropeptides originating from the hypothalamus regulate feed intake by stimulating or inhibiting appetite. These appetite-regulating factors can be divided into orexigenic (orexin) and anorexigenic factors (tachykinin, CCK, and leptin). Most of these factors are short-term and are regulated by the central nervous system or the peripheral regulatory system of the fish. Previous studies showed that orexin is a neuropeptide secreted from specific hypothalamic neurons that regulate feeding, foraging, reward, sleep, and arousal in fish and mammals (Sakurai 2007, 2014; Yang et al. 2020). Tachykinin is a neuropeptide that inhibits appetite and participates in immune, respiratory, and digestive activities (Jensen et al. 1987). Similar to tachykinin, CCK is an anorexigenic factor, a synthetic peptide hormone produced by neuroendocrine I cells in the gastrointestinal tract in the presence of food, indicating that it can be influenced by food deprivation and feeding (Chaudhari et al. 2008; MacDonald and Volkoff 2009). Leptin is secreted by the pancreas of animals and regulates energy and metabolism. Karteris et al. (2005) reported that starvation could increase the expression of prepro-orexin/hypocretin mRNAs and raise orexin/hypocretin protein levels. Additionally, there is an upregulation of leptin receptors after a period of fasting in certain fish species, such as rainbow trout (Kling et al. 2009), fine flounder (Fuentes et al. 2012), and Atlantic salmon (Trombley et al. 2012). Other studies have shown that CCK can suppress gastric emptying and feed intake (Olsson et al. 1999; Volkoff et al. 2005). Volkoff (2006) also reported that leptin initiates the action of the anorexigenic factor CCK and inhibits the action of orexin. In this study, tiger pufferfish specimens in the F1 group exhibited the highest expression of orexin; the lowest expression of tachykinin, CCK, and leptin;
and the lowest glucose content. This result indicated that a starved state activates the orexigenic factors.

In contrast, the mRNA levels of tachykinin, CCK, and leptin were highest in the F4 group specimens, which may be attributed to the negative feedback mechanism in the brain-hypothalamus-neuropeptides axis. These results further illustrated that varying ingestion rates or excessive feeding could induce changes in the mRNA expression profiles of appetite-related genes, suggesting that nutritional status modulates actions in the nervous system of tiger pufferfish.

Conclusion

This study investigated the effect of different feeding frequencies (one, two, or four meals/day or continuous feeding with an automatic feeding machine) on the growth performance, blood serum parameters, oxidative stress, and appetite-related genes in tiger pufferfish during winter in RAS. Our results indicated that inappropriate feeding strategies (feeding frequencies that are too low or high) could reduce the growth performance and feed conversion ratio and alter blood physiology and the expression of appetite-related genes. Overall, the findings of this study indicated that feeding four meals per day was the optimal feeding frequency for tiger puffer juveniles during winter (18.0 ± 1.0 °C).

Author contribution Xian-Qiang Gao designed the experiments and wrote the manuscript. Xi Wang, Xinyi Wang, and Shu-Quan Cao completed the biochemical parameters analysis experiment and assisted in data collection. Hong Xu Li, Liang Xu, and Ying-Ying Fang primarily undertook blood and tissue collection and fish management. Bin Huang provided funding support and the project idea. Chen Hai-Bin and Xing Rui were responsible for aquaculture technical guidance during the experiment. Bao Liang Liu primarily revised the manuscript. All the authors contributed in the analysis and interpretation of data and approved the final version of the manuscript.

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Data availability The data that support the findings of this study are openly available at https://figshare.com/s/9e6a82240eaf655aaeb8, https://doi.org/10.6084/m9.figshare.14955105.

Code availability Not applicable.

Declarations

Ethics approval All animals used in this research were treated humanely following the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Fishery Sciences, considering alleviation of distress and discomfort. The procedures used in this research were approved by the Committee on Ethics of Animal Experiments of the Chinese Academy of Fishery Sciences (permit no. YSFRI-2019017).

Consent for publication All the 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 was performed in accordance with the guidelines of Fish Physiology and Biochemistry.

Conflict of interest The authors declare no competing interests.

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