Effects of Feeding Frequency on Growth Performance, Body Composition, Digestive Enzyme Activity, Tissue Fatty Acids, and Amino Acid Content of Tiger Cuttlefish (Postprint)

Authors: Zhu Tingting, Li Chenchen, Lu You, Jin Min, Wang Xuexi, Luo Jiaxiang, Zhou Qicun

Date: 2018-12-25T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of feeding frequency on growth performance, body composition, digestive enzyme activity, tissue fatty acids, and amino acid content in tiger cuttlefish (Sepia pharaonis). One hundred eighty juvenile tiger cuttlefish with an initial body weight of (13.08±0.01) g were randomly divided into three groups (feeding frequencies of 1, 2, and 4 times per day, designated as F1, F2, and F4 feeding groups, respectively), with three replicates per group and 20 individuals per replicate. The experimental period lasted 4 weeks. The results showed that: 1) The weight gain rate, specific growth rate, and hepatosomatic index of the F2 and F4 feeding groups were significantly higher than those of the F1 feeding group ($P<0.05$), while the feed efficiency of the F1 and F4 feeding groups was significantly lower than that of the F2 feeding group ($P<0.05$). 2) The liver moisture content of the F4 feeding group was significantly lower than that of the F1 feeding group ($P<0.05$), and the liver crude lipid content was significantly higher than that of the F1 and F2 feeding groups ($P<0.05$). The muscle moisture content of the F2 and F4 feeding groups was significantly lower than that of the F1 feeding group ($P<0.05$), while the muscle crude protein and crude lipid contents were significantly higher than those of the F1 feeding group ($P<0.05$). 3) No significant differences were observed in the total amino acid, essential amino acid, and non-essential amino acid contents of muscle among all groups ($P>0.05$). The hepatic total amino acid, essential amino acid, and non-essential amino acid contents of the F1 feeding group were significantly lower than those of the F2 and F4 feeding groups ($P<0.05$), while no significant differences were observed in hepatic total amino acid and non-essential amino acid contents between the F2 and F4 feeding groups ($P>0.05$). 4) The docosahexaenoic acid (DHA) content and DHA/eicosapentaenoic acid (EPA) ratio in muscle and liver of the F1 feeding group were significantly higher than those of the F2 and F4 feeding groups ($P<0.05$), while no significant differences were observed between the F2 and F4 feeding groups ($P>0.05$). No significant differences were observed in the polyunsaturated fatty acid and n-3 highly unsaturated fatty acid contents of muscle and liver among all groups ($P>0.05$). 5) The hepatic glutathione peroxidase activity of the F1 feeding group was significantly higher than that of the F2 feeding group ($P<0.05$). The foregut -amylase, lipase, and alkaline phosphatase activities of the F1 feeding group were significantly higher than those of the F2 feeding group ($P<0.05$), while no significant differences were observed between the F2 and F4 feeding groups ($P>0.05$). Therefore, the optimal feeding frequency for juvenile tiger cuttlefish culture is twice daily.

Full Text

Effects of Feeding Frequency on Growth Performance, Body Composition, Digestive Enzyme Activities, Tissue Fatty Acid and Amino Acid Compositions of Sepia pharaonis

ZHU Tingting, LI Chenchen, LU You, JIN Min, WANG Xuexi, LUO Jiaxiang, ZHOU Qicun*

(Laboratory of Fish Nutrition, School of Marine Sciences, Ningbo University, Ningbo 315211, China)

Abstract: This experiment was conducted to investigate the effects of feeding frequency on growth performance, body composition, digestive enzyme activities, and tissue fatty acid and amino acid compositions of *Sepia pharaonis*. A total of 180 juvenile *Sepia pharaonis* with an initial body weight of (13.08±0.01) g were randomly divided into 3 groups (feeding frequencies of 1, 2, and 4 times per day, designated as F1, F2, and F4 groups, respectively), with 3 replicates per group and 20 cuttlefish per replicate. The experimental period lasted for 4 weeks. The results showed as follows: 1) The weight gain rate (WGR), specific growth rate (SGR), and hepatosomatic index (HSI) in F2 and F4 groups were significantly higher than those in F1 group ($P<0.05$), while the feed efficiency (FE) in F1 and F4 groups was significantly lower than that in F2 group ($P<0.05$). 2) The liver moisture content in F4 group was significantly lower than that in F1 group ($P<0.05$), and the liver crude lipid content was significantly higher than that in F1 and F2 groups ($P<0.05$). The muscle moisture content in F2 and F4 groups was significantly lower than that in F1 group ($P<0.05$), and the muscle crude protein and crude lipid contents were significantly higher than those in F1 group ($P<0.05$). 3) There were no significant differences in the contents of total amino acids (TAA), essential amino acids (EAA), and non-essential amino acids (NEAA) in muscle among all groups ($P>0.05$). The contents of TAA, EAA, and NEAA in liver in F1 group were significantly lower than those in F2 and F4 groups ($P<0.05$), while the contents of TAA and NEAA in liver showed no significant difference between F2 and F4 groups ($P>0.05$). 4) The docosahexaenoic acid (DHA) content and DHA/eicosapentaenoic acid (EPA) ratio in muscle and liver in F1 group were significantly higher than those in F2 and F4 groups ($P<0.05$), with no significant differences between F2 and F4 groups ($P>0.05$). There were no significant differences in the contents of polyunsaturated fatty acids (PUFA) and n-3 high unsaturated fatty acids (n-3HUFA) in muscle and liver among all groups ($P>0.05$). 5) The liver glutathione peroxidase activity in F1 group was significantly higher than that in F2 group ($P<0.05$). The activities of -amylase, lipase, and alkaline phosphatase in anterior intestine in F1 group were significantly higher than those in F2 group ($P<0.05$), with no significant differences between F2 and F4 groups ($P>0.05$). Therefore, the appropriate feeding frequency for juvenile *Sepia pharaonis* is 2 times per day.

Key words: *Sepia pharaonis*; feeding frequency; growth performance; body composition; digestive enzyme activities; tissue fatty acids; tissue amino acids

*Corresponding author, professor, E-mail: zhouqicun@nbu.edu.cn

---

Feeding strategy, comprising feeding frequency, feeding level (or feeding rate), and feeding method, constitutes an important component of intensive aquaculture management [1]. Feeding frequency is a crucial factor affecting aquaculture production; inappropriate feeding frequency can lead to slow growth rates and increased size variation among individuals [2]. Excessively high feeding frequency often reduces feed efficiency, thereby increasing production costs and deteriorating the culture environment, whereas excessively low feeding frequency results in reduced growth performance [3]. Therefore, determining appropriate feeding frequency is of great significance for fish farming.

Sepia pharaonis belongs to the phylum Mollusca, class Cephalopoda, order Decapoda, family Sepiidae, and genus *Sepia*, and is widely distributed in the South China Sea [4]. This species is characterized by large body size, delicious taste, strong disease resistance, rapid growth, and suitability for high-density culture, making it a promising economic cephalopod for aquaculture [5]. Current research on *Sepia pharaonis* has primarily focused on skin gelatin properties [6], tolerance to ecological factors [7], nutritional composition including body composition, amino acids, and fatty acids in different tissues [8], and embryonic development [9]. However, studies investigating the effects of feeding frequency on this species have not been reported. This experiment aimed to determine the optimal feeding frequency by examining its effects on growth performance, body composition, digestive enzyme activities, and tissue amino acid and fatty acid compositions of *Sepia pharaonis*, thereby providing scientific basis for rational feeding strategies, culture practices, quality control, and energy conversion during the juvenile stage.

1.1 Experimental Design

Experimental *Sepia pharaonis* were purchased from Xiangshan Laifa Farm and reared at the Ningbo Marine and Fisheries Science and Technology Innovation Base. Prior to the experiment, juvenile cuttlefish were acclimated in 300 L blue barrels for 2 weeks under indoor natural lighting with transparent plastic roofing. The water was aerated daily, and during acclimation, the cuttlefish were fed chilled shrimp twice daily (body composition and amino acid composition, fatty acid composition shown in Table 1 and Table 2 , respectively). After acclimation, the cuttlefish were fasted for 24 h, and 180 juveniles with uniform size, no surface injuries, and an initial body weight of (13.08±0.01) g were selected and randomly divided into 3 groups (feeding frequencies of 1, 2, and 4 times per day, designated as F1, F2, and F4 groups, respectively), with 3 replicates per group and 20 cuttlefish per replicate. The experimental period lasted for 4 weeks.

Table 1 Body composition (wet weight basis) and amino acid composition (DM basis) of chilled shrimp

Table 2 Fatty acid composition of chilled shrimp (percentage of total fatty acids)

The three groups of *Sepia pharaonis* were randomly distributed among 9 barrels, with 20 cuttlefish per barrel. The different feeding frequencies and schedules during the experiment were as follows: F1 group, fed once daily (09:00); F2 group, fed twice daily (09:00, 18:00); F4 group, fed four times daily (09:00, 12:00, 15:00, and 18:00). Each feeding was conducted to satiation. Frozen shrimp were thawed before feeding, surface moisture was removed and the shrimp were weighed. After 1 h of feeding, residual feed was siphoned out, surface moisture was removed and weighed. Feed intake was calculated as the weight of feed offered minus residual feed. Water exchange was performed daily at 08:00 using a micro-flow system, with 30% water exchanged. The culture period lasted for 4 weeks, during which water temperature was maintained at 29.0–34.9 °C, dissolved oxygen concentration was above 6.0 mg/L, and salinity ranged from 23.0‰ to 27.3‰.

1.3 Sample Collection

At the end of the feeding trial, the cuttlefish were fasted for 24 h, anesthetized with 0.15 mol/L magnesium chloride (MgCl) [10], weighed and counted to calculate weight gain rate (WGR), survival rate (SR), and specific growth rate (SGR). In each culture barrel, 3 cuttlefish were randomly selected, weighed individually, and measured for mantle length. The liver was dissected and weighed to calculate condition factor (CF) and hepatosomatic index (HSI). Ten cuttlefish were dissected on ice packs; the anterior intestine and liver were collected in 2.0 mL centrifuge tubes for determination of digestive enzyme activities and amino acid and fatty acid contents. Abdominal muscle was stripped and placed in 4 mL centrifuge tubes for determination of muscle composition, amino acids, and fatty acids. All samples were stored at -80 °C.

1.4 Analytical Methods

Routine nutrient contents in muscle and liver were determined according to AOAC (1995) [11] methods. Moisture content was determined by oven drying at 105 °C to constant weight. Crude protein content was measured using a protein analyzer (Leco FB-528) based on the principle of thermal conductivity absorption. Crude lipid content was determined using a lipid analyzer (SX360) based on the Soxhlet extraction principle. Crude ash content was measured by muffle furnace incineration at 550 °C.

Fatty acid analysis: Liver and muscle samples were freeze-dried for 48 h. Lipids were extracted using hydrochloric acid-methanol solution and potassium hydroxide-methanol solution for pretreatment, then sent to the Testing Center of Ningbo Institute of Materials Technology & Engineering, Chinese Academy of Sciences. Fatty acid methyl esters were analyzed using a gas chromatography-mass spectrometer (GCMS-QP2010 Plus, SHIMADZU, Japan), and the relative percentages of various fatty acids were calculated using area normalization.

Amino acid analysis: Liver and muscle samples were hydrolyzed with 6 mol/L HCl in a sand bath for 24 h, then diluted to 50 mL in a volumetric flask. One milliliter of the solution was rotary evaporated, and the residue was dissolved in 0.02 mol/L HCl for analysis using an amino acid analyzer (L-8900).

Intestinal digestive enzyme and liver antioxidant enzyme activity analysis: Activities of intestinal trypsin, -amylase (AMS), lipase (LPS), creatine kinase (CK), -glutamyl transferase (-GT), alkaline phosphatase (AKP), and Na , K -ATPase, as well as liver malondialdehyde (MDA) content and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, were all determined using assay kits from Nanjing Jiancheng Bioengineering Institute.

1.5 Calculation Formulas

Survival rate (%) = 100 × Nt/N
Weight gain rate (%) = 100 × (Wt − W)/W
Specific growth rate (%/d) = 100 × (ln Wt − ln W)/t
Feed intake (FI, g) = amount fed − residual feed
Feed efficiency (FE, %) = 100 × (Wf + Wd − Wi)/F
Condition factor (g/cm^3) = 100 × W/L^3
Hepatosomatic index (%) = 100 × Wh/W

Where: Nt = number of cuttlefish at experiment end; N = initial number of cuttlefish; Wt = final average weight of cuttlefish (g); W = initial average weight of cuttlefish (g); t = experimental duration (days); Wf = total final weight of cuttlefish (g); Wd = total weight of dead cuttlefish (g); Wi = total initial weight of cuttlefish (g); F = feed intake (g); W = body weight of cuttlefish (g); L = mantle length of cuttlefish (cm); Wh = liver weight (g).

1.6 Data Processing and Analysis

Data were initially processed using Excel 2007 and then subjected to statistical analysis using SPSS 16.0 software. One-way analysis of variance (ANOVA) was performed on the data. When significant differences were detected, Turkey' s test was used for multiple comparisons. $P<0.05$ was considered statistically significant, and results were expressed as mean ± standard error.

2.1 Effects of Feeding Frequency on Growth Performance and Morphological Indices of *Sepia pharaonis*

The effects of feeding frequency on growth performance and morphological indices of *Sepia pharaonis* are presented in Table 3 . The results showed that survival rate and condition factor did not differ significantly among groups ($P>0.05$). Feed intake increased significantly with increasing feeding frequency ($P<0.05$). Feed efficiency in F4 group was significantly higher than that in F1 and F4 groups ($P<0.05$), with no significant difference between F1 and F4 groups ($P>0.05$). Weight gain rate, specific growth rate, and hepatosomatic index in F2 and F4 groups were significantly higher than those in F1 group ($P<0.05$), while no significant differences were observed between F2 and F4 groups ($P>0.05$).

Table 3 Effects of feeding frequency on growth performance and morphology indexes of *Sepia pharaonis*

2.2 Effects of Feeding Frequency on Liver and Muscle Composition of *Sepia pharaonis*

The effects of feeding frequency on liver and muscle composition of *Sepia pharaonis* are shown in Table 4 . The results indicated that liver crude protein content did not differ significantly among groups ($P>0.05$). Liver moisture content in F4 group was significantly lower than that in F1 group ($P<0.05$). Liver crude lipid content in F4 group was significantly higher than that in F1 and F2 groups ($P<0.05$), with F2 group being significantly higher than F1 group ($P<0.05$). Muscle moisture content in F2 and F4 groups was significantly lower than that in F1 group ($P<0.05$), while muscle crude protein and crude lipid contents were significantly higher than those in F1 group ($P<0.05$). However, no significant differences were observed between F2 and F4 groups ($P>0.05$).

Table 4 Effects of feeding frequency on liver and muscle composition of *Sepia pharaonis* (wet weight basis)

2.3 Effects of Feeding Frequency on Tissue Amino Acid Composition of *Sepia pharaonis*

The effects of feeding frequency on muscle amino acid composition of *Sepia pharaonis* are presented in Table 5 . The results demonstrated that the contents of essential amino acids threonine (Thr), leucine (Leu), and lysine (Lys) in muscle of F2 group were significantly higher than those in F1 group (P<0.05), with no significant differences between F4 group and either F1 or F2 groups (P>0.05). The contents of non-essential amino acids aspartic acid (Asp), serine (Ser), glutamic acid (Glu), and alanine (Ala) in muscle of F2 group were significantly higher than those in F1 group (P<0.05), while F4 group showed no significant differences compared with F1 and F2 groups (P>0.05).

Table 5 Effects of feeding frequency on muscle amino acid composition of *Sepia pharaonis* (DM basis)

The effects of feeding frequency on liver amino acid composition of *Sepia pharaonis* are shown in Table 6 . The results revealed that the contents of Thr, valine (Val), methionine (Met), isoleucine (Ile), Leu, phenylalanine (Phe), Lys, histidine (His), arginine (Arg), Asp, Ser, Glu, glycine (Gly), Ala, cysteine (Cys), tyrosine (Tyr), total amino acids, essential amino acids, and non-essential amino acids in liver of F1 group were significantly higher than those in F2 and F4 groups (P<0.05). Among these, the contents of Val, Met, His, Arg, Asp, Glu, Gly, Ala, Cys, total amino acids, and non-essential amino acids in liver showed no significant differences between F2 and F4 groups (P>0.05).

Table 6 Effects of feeding frequency on liver amino acid composition of *Sepia pharaonis* (DM basis)

2.4 Effects of Feeding Frequency on Tissue Fatty Acid Composition of *Sepia pharaonis*

The effects of feeding frequency on muscle fatty acid composition of *Sepia pharaonis* are presented in Table 7 . The results indicated that EPA, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and n-3 high unsaturated fatty acids (n-3HUFA) contents in muscle did not differ significantly among groups (P>0.05). The DHA content, n-6 high unsaturated fatty acids (n-6HUFA) content, and DHA/EPA ratio in muscle of F1 group were significantly higher than those in F2 and F4 groups (P<0.05), with no significant differences between F2 and F4 groups (P>0.05). The n-3/n-6 ratio in F2 group was significantly higher than that in F1 group (P<0.05), but showed no significant difference with F4 group (P>0.05).

Table 7 Effects of feeding frequency on muscle fatty acid composition of *Sepia pharaonis* (percentage of total fatty acids)

The effects of feeding frequency on liver fatty acid composition of *Sepia pharaonis* are shown in Table 8 . The results demonstrated that EPA, MUFA contents, and n-3/n-6 ratio in liver of F2 and F4 groups were significantly higher than those in F1 group (P<0.05), while no significant differences were observed between F2 and F4 groups (P>0.05). The DHA content, SFA content, and DHA/EPA ratio in liver of F1 group were significantly higher than those in F2 and F4 groups (P<0.05), with no significant differences between F2 and F4 groups (P>0.05).

Table 8 Effects of feeding frequency on liver fatty acid composition of *Sepia pharaonis* (percentage of total fatty acids)

2.5 Effects of Feeding Frequency on Liver Antioxidant Capacity and Intestinal Digestive Enzyme Activities of *Sepia pharaonis*

The effects of feeding frequency on liver antioxidant capacity and intestinal digestive enzyme activities of *Sepia pharaonis* are presented in Table 9 . The results showed that liver superoxide dismutase activity and malondialdehyde content did not differ significantly among groups (P>0.05). Liver glutathione peroxidase activity in F1 group was significantly higher than that in F2 group (P<0.05), but showed no significant difference with F4 group (P>0.05).

The activities of trypsin, creatine kinase, -glutamyl transferase, and Na , K -ATPase in anterior intestine did not differ significantly among groups (P>0.05). The activities of -amylase, lipase, and alkaline phosphatase in anterior intestine of F1 group were significantly higher than those in F2 group (P<0.05), with no significant differences between F2 and F4 groups (P>0.05).

Table 9 Effects of feeding frequency on liver antioxidant ability and intestinal digestive enzyme activities of *Sepia pharaonis*

3.1 Effects of Feeding Frequency on Growth Performance of *Sepia pharaonis*

Within an appropriate range of feeding frequency, the growth rate of most fish species increases with feeding frequency [12]. Lin et al. [13] reported that the specific growth rate and weight gain rate of *Megalobrama amblycephala* were significantly higher when fed 5 times daily compared with 1 or 2 times daily. Wang et al. [14] also found that the specific growth rate of *Pelteobagrus vachelli* richardson increased significantly with increasing feeding frequency. For juvenile *Sebastes schlegeli*, the specific growth rate was highest when fed 4 times daily, being 2.25%-15.43% higher than groups fed 1, 2, or 3 times daily [1]. Similarly, this experiment found that when fed twice daily (F2 group), *Sepia pharaonis* exhibited significantly higher weight gain rate and specific growth rate than F1 group. This may be because at appropriate higher feeding frequencies, cuttlefish had more opportunities to approach or achieve satiety daily, obtaining more nutrients and energy for weight gain [15], consequently improving feed efficiency. Similar results were reported in *Megalobrama amblycephala* [13], indicating that moderately increasing feeding frequency benefits fish growth. In this study, feed efficiency in F2 group was significantly higher than in F4 group, possibly because excessively high feeding frequency led to increased energy expenditure for feeding activities, reducing energy available for growth. Additionally, frequent activity behaviors in aquatic animals accelerate energy consumption, which is detrimental to growth and increases feed costs [16]. Furthermore, as cuttlefish are stomach-bearing animals capable of holding substantial food quantities, repeated multiple feedings may cause reflexive rapid movement of digestive tract contents, resulting in undigested food being expelled and reducing feed conversion efficiency, thereby affecting growth.

3.2 Effects of Feeding Frequency on Liver and Muscle Composition, Morphological Indices, and Tissue Amino Acid Composition of *Sepia pharaonis*

Some studies have found that feeding frequency had no significant effect on hepatosomatic index of *Oreochromis niloticus* [17], whereas the hepatosomatic index of *Pelteobagrus vachelli* decreased with increasing feeding frequency [18]. Other reports have indicated that increasing feeding frequency elevates feed intake, and excess energy beyond that required for normal growth is converted to and stored as fat, consequently increasing body lipid content and correspondingly decreasing moisture content [19]. Dong et al. [20] reported that in *Ictalurus punctatus*, higher feeding frequency provided abundant food, reducing energy expenditure on food competition and cannibalism, allowing large amounts of dietary fat and protein to be stored in the liver. Conversely, at lower feeding frequencies, more energy was utilized for competing for food and avoiding cannibalism, leading to catabolism of most protein nutrients. In this study, with increasing feeding frequency, crude lipid contents in muscle and liver of *Sepia pharaonis* increased significantly while moisture content decreased significantly. The elevated lipid content also led to an increasing trend in hepatosomatic index, consistent with the aforementioned reports. This may be attributed to reduced feeding activity with increased feeding frequency [21], causing excess dietary nutrients to be converted to and stored as fat in the liver [22]. The decreasing liver amino acid content with increasing feeding frequency, along with the highest liver crude protein content in F2 group (though not significantly different from other groups), further supports this hypothesis. These findings are similar to those reported in *Larimichthys crocea* [23] and *Acanthopagrus schlegeli* [24]. Changes in muscle crude protein and crude lipid contents were consistent, both increasing significantly with feeding frequency, which aligns with the trends of numerous essential and non-essential amino acids in muscle. This suggests that excess energy consumed by *Sepia pharaonis* is stored as fat and protein in muscle, indicating that muscle is an important energy storage tissue. However, the trends of Thr, Leu, Lys, Asp, Ser, Glu, and Ala contents in muscle were opposite to those in liver with increasing feeding frequency, representing the result of amino acid transfer among body tissues. We speculate this may also contribute to the reduced liver crude protein content. The essential amino acids/total amino acids (EAA/TAA) ratio did not differ significantly among feeding frequency groups but was greater than the ideal protein pattern recommended by FAO/WHO: EAA/TAA 40% [25]. Cuttlefish fed chilled shrimp exhibited balanced amino acid profiles with complete varieties, representing high-quality aquatic protein.

3.3 Effects of Feeding Frequency on Tissue Fatty Acid Composition of *Sepia pharaonis*

Marine fish cannot synthesize sufficient EPA and DHA to meet their growth requirements and must obtain these from dietary sources [26]. Deficiency in EPA and DHA adversely affects visual system development and pigmentation, reduces predation ability in larvae, impairs neuroendocrine system development, and increases mortality [27]. Arachidonic acid (ARA, C20:4n-6), as one of the high unsaturated fatty acids, also influences normal development in some marine fish [28]. In this study, with feeding frequency increasing from once to twice daily, C20:4n-6 contents in both muscle and liver of cuttlefish decreased significantly, with no significant differences between F2 and F4 groups. Although EPA content in muscle showed no significant differences with increasing feeding frequency, an overall increasing trend was observed, while DHA content and DHA/EPA ratio in muscle decreased significantly. These changes were consistent with EPA and DHA contents in liver, indicating a positive correlation in fatty acid content and composition between muscle and liver, which may be related to fat storage and selective retention of fatty acids. The n-3/n-6 ratio in fish is an important indicator for evaluating product quality, with WHO/FAO recommending a minimum level of 0.1-0.2 [29]. In this experiment with *Sepia pharaonis*, the n-3/n-6 ratio of chilled shrimp and cuttlefish muscle ranged from 9.01 to 13.54, substantially exceeding the WHO/FAO recommendation, indicating that chilled shrimp as feed can satisfy the fatty acid requirements of *Sepia pharaonis*.

3.4 Effects of Feeding Frequency on Liver Antioxidant Capacity and Intestinal Digestive Enzyme Activities of *Sepia pharaonis*

The liver is a vital metabolic organ and a major site of free radical production. Free radicals can cause fatty acid oxidation [30]. Malondialdehyde, as the end product of lipid peroxidation, exhibits cytotoxicity [31]. In this study, liver malondialdehyde content in *Sepia pharaonis* showed an increasing trend with elevated feeding frequency, indicating that high-frequency feeding induces hepatic oxidative stress. This is consistent with findings in *Megalobrama amblycephala*, where liver malondialdehyde content peaked when fed 6 times daily [32]. In fish research, superoxide dismutase and glutathione peroxidase are important indicators for evaluating cellular antioxidant capacity [33]. In this experiment, F4 group exhibited the highest liver superoxide dismutase activity, which may alleviate oxidative stress induced by high-frequency feeding. Glutathione peroxidase activity in F2 group was significantly lower than in F1 group, while F4 group showed no significant differences with either F1 or F2 groups. However, current research on the precise mechanisms of feeding frequency effects on oxidative stress in *Sepia pharaonis* remains limited and requires further investigation.

Digestive enzyme activity is considered an indicator of fish ability to absorb and digest food [34]. In this study, the activities of three digestive enzymes (-amylase, lipase, and trypsin) showed a trend of initially decreasing then increasing with feeding frequency. The activities of -amylase and lipase in anterior intestine of F1 group were significantly higher than other groups, while trypsin activity showed no significant differences among groups. These findings are similar to those reported in *Hippocampus erectus* [35], *Oplegnathus fasciatus* [3], and *Oreochromis niloticus* × *O. affreus* [36]. This may be because at lower feeding frequencies, insufficient food intake necessitates increased intestinal digestive enzyme activity to ensure adequate absorption of nutrients to meet growth energy requirements. When food intake increases, cuttlefish can meet growth energy demands with lower digestive enzyme activity, further confirming that cuttlefish in F2 group had higher feed utilization efficiency compared with F4 group. Recent studies have found that intestinal creatine kinase, -glutamyl transferase, alkaline phosphatase, and Na , K -ATPase show similar trends to digestive enzymes [37], suggesting enhanced nutrient absorption capacity in fish under optimal feeding frequencies.

4 Conclusion

Considering growth performance, feed utilization, liver and muscle composition, liver antioxidant enzyme activities, intestinal digestive enzyme activities, and tissue fatty acid and amino acid compositions, the appropriate feeding frequency for juvenile *Sepia pharaonis* under the conditions of this experiment is 2 times per day.

References:

[1] SVEIER H, LIED E. The effect of feeding regime on growth, feed utilisation and weight dispersion large Atlantic salmon (*Salmo salar*) reared seawater[J]. *Aquaculture*, 1998, 165(3/4): 333-345.

[2] JOBLING M. Effect of feeding frequency on food intake and growth of Arctic charr, *Salvelinus alpinus* L[J]. *Journal of Fish Biology*, 1983, 23(2): 177-185.

[3] 宋国, 彭士明, 孙鹏, 等. 饥饿与再投喂及投喂频率对条石鲷幼鱼生长和消化酶活力的影响 [J]. 中国水产科学, 2011, 18(6): 1269-1277.

[4] 黄梓荣. 南海北部陆架区头足类的种类组成和资源密度分布 [J]. 南方水产科学, 2008, 4(5): 1-7.

[5] MINTON J W, WALSH L S, LEE P G, et al. First multi-generation culture of the tropical cuttlefish *Sepia pharaonis* Ehrenberg, 1831[J]. *Aquaculture International*, 2001, 9(5): 379-392.

[6] HOQUE M S, BENJAKUL S, PRODPRAN T. Properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin incorporated cinnamon, clove anise extracts[J]. *Food Hydrocolloids*, 2011, 25(5): 1085-1097.

[7] 戴远棠, 谢晓晖, 黄国光, 等. 虎斑乌贼幼体对盐度及 pH 值耐受力的研究 [J]. 河北渔业, 2012(10): 8-11, 16.

[8] 陈道海, 文菁, 赵玉燕, 等. 野生与人工养殖的虎斑乌贼肌肉营养成分比较 [J]. 食品科学, 2014, 35(7): 217-222.

[9] 陈道海, 王雁, 梁汉青, 等. 虎斑乌贼 (*Sepia pharaonis*) 胚胎发育及孵化历期观察 [J]. 海洋与湖沼, 2012, 43(2): 394-400.

[10] MESSENGER J B, NIXON M, RYAN K P. Magnesium chloride as an anaesthetic for cephalopods[J]. *Comparative Biochemistry Physiology C: Comparative Pharmacology*, 1985, 82(1): 203-205.

[11] BELLARDI S, BIANCHINI M L, DOMENIS L, et al. Effect of feeding schedule and feeding rate on size and number of adipocytes in rainbow trout *Oncorhynchus mykiss*[J]. *Journal of the World Aquaculture Society*, 1995, 26(1): 80-83.

[12] 孙丽慧, 王际英, 丁立云, 等. 投喂频率对星斑川鲽幼鱼生长和体组成影响的初步研究 [J]. 上海海洋大学学报, 2010, 19(2): 190-195.

[13] 林艳, 缪凌鸿, 戈贤平, 等. 投喂频率对团头鲂幼鱼生长性能、肌肉品质和血浆生化指标的影响 [J]. 动物营养学报, 2015, 27(9): 2749-2756.

[14] 王武, 周锡勋, 马旭洲, 等. 投喂频率对瓦氏黄颡鱼幼鱼生长及蛋白酶活力的影响 [J]. 上海水产大学学报, 2007, 16(3): 224-229.

[15] TUCKER B J, BOOTH M A, ALLAN G L, et al. Effects of photoperiod and feeding frequency performance newly weaned Australian snapper *Pagrus auratus*[J]. *Aquaculture*, 2006, 258(1/2/3/4): 514-520.

[16] LEE S M, PHAM M A. Effects of feeding frequency and feed type on the growth, feed utilization composition juvenile olive flounder, *Paralichthys olivaceus*[J]. *Aquaculture research*, 2010, 41(9): e166-e171.

[17] 孙晓锋, 冯健, 陈江虹, 等. 投喂频率对尼罗系吉富罗非鱼幼鱼胃排空、生长性能和体组成的影响 [J]. 水产学报, 2011, 35(11): 1677-1683.

[18] 方巍. 黄颡鱼摄食和投喂策略的研究 [D]. 硕士学位论文. 武汉: 华中农业大学, 2010.

[19] CHO S H, LIM Y S, LEE J H, et al. Effects of feeding rate and feeding frequency on survival, growth, and body composition of Ayu post-larvae *Plecoglossus altivelis*[J]. *Journal of the World Aquaculture Society*, 2003, 34(1): 85-91.

[20] 董桂芳, 胡振雄, 黄峰, 等. 投喂频率对斑点叉尾鮰幼鱼生长、饲料利用和鱼体组成的影响 [J]. 渔业现代化, 2012, 39(2): 48-53.

[21] DWYER K S, BROWN J A, PARRISH C, et al. Feeding frequency affects consumption, feeding pattern and growth of juvenile yellowtail flounder (*Limanda ferruginea*)[J]. *Aquaculture*, 2002, 213(1/2/3/4): 279-292.

[22] 覃希. 投喂频率和投喂水平对吉富罗非鱼幼鱼生长性能和生理机能的影响 [D]. 硕士学位论文. 南宁: 广西大学, 2014.

[23] 孙瑞健, 徐玮, 米海峰, 等. 饲料脂肪水平和投喂频率对大黄鱼生长、体组成及脂肪沉积的影响 [J]. 水产学报, 2015, 39(3): 401-409.

[24] 楼宝, 毛国民, 骆季安, 等. 饲喂频率对黑鲷生长及体生化成分的影响 [J]. 海洋水产研究, 2006, 27(6): 19-24.

[25] Joint FAO/WHO Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, World Health Organization. *Codex alimentarius: general requirements (food hygiene)*[M]. [s.l.]: Food & Agriculture Organization, 2001.

[26] WATANABE T, KITAJIMA C, FUJITA S. Nutritional values of live organisms used in Japan for mass propagation of fish: a review[J]. *Aquaculture*, 1983, 34(1/2): 115-143.

[27] BELL M V, BATTY R S, DICK J R, et al. Dietary deficiency of docosahexaenoic acid impairs vision light intensities juvenile herring (*Clupea harengus* L.)[J]. *Lipids*, 1995, 30(5): 443-449.

[28] SARGENT J R, MCEVOY L A, BELL J G. Requirements, presentation and sources of polyunsaturated fatty acids marine larval feeds[J]. *Aquaculture*, 1997, 155(1/2/3/4): 117-127.

[29] 何志刚, 刘文革, 伍远安, 等. 饲料脂肪水平对芙蓉鲤鲫形体指标、组织脂肪含量与脂肪酸组成的影响 [J]. 饲料研究, 2016(6): 36-41.

[30] FLOOD L P, CARVAN M J, JAEGER L, et al. Reduction in hepatic microsomal P-450 and related catalytic activity in farm-raised red drum[J]. *Journal of Aquatic Animal Health*, 1996, 8(1): 13-21.

[31] BANERJEE B D, SETH V, BHATTACHARYA A, et al. Biochemical effects of some pesticides lipid peroxidation free-radical scavengers[J]. *Toxicology Letters*, 1999, 107(1/2/3): 33-47.

[32] LI X F, TIAN H Y, ZHANG D D, et al. Feeding frequency affects stress, innate immunity and disease resistance of juvenile blunt snout bream *Megalobrama amblycephala*[J]. *Fish & Shellfish Immunology*, 2014, 38(1): 80-87.

[33] JIN Y, TIAN L X, ZENG S L, et al. Dietary lipid requirement on nonspecific immune responses juvenile grass (*Ctenopharyngodon idella*)[J]. *Fish & Shellfish Immunology*, 2013, 34(5): 1202-1208.

[34] 尹飞, 彭士明, 孙鹏, 等. 低盐胁迫对银鲳幼鱼肠道消化酶活力的影响 [J]. 海洋渔业, 2010, 32(2): 160-165.

[35] 席寅峰, 张东, 施兆鸿. 投喂频率对雌雄分化后灰海马生长发育、饵料转换效率及消化酶活力的影响 [J]. 海洋渔业, 2013, 35(1): 77-85.

[36] 强俊, 王辉, 李瑞伟, 等. 饲喂频率对奥尼罗非鱼仔稚鱼生长、体成分和消化酶活力的影响 [J]. 广东海洋大学学报, 2009, 29(4): 79-83.

[37] TIAN H Y, ZHANG D D, LI X F, et al. Optimum feeding frequency of juvenile blunt snout bream *Megalobrama amblycephala*[J]. *Aquaculture*, 2015,

437: 60-66.