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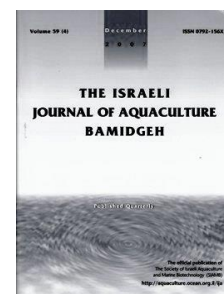
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Effect of Live, Frozen and Artificial Feeds on Digestive Enzymes, Aminotransferase, Histology of Liver and Intestine in Mandarin Fish Hybrid (*Siniperca chuatsi* ♀ × *Siniperca scherzeri* ♂)

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Abstract

To partially or entirely replace the use of live foods for mandarin fish (*Siniperca chuatsi* ♀ × *Siniperca scherzeri* ♂) with frozen or artificial feeds, a detailed understanding of their digestive physiology and the influence of these replacements is needed. The experiment was conducted in a recirculation system for 10 weeks. At termination of the feeding trial, protease activity in the alimentary tract and aspartate aminotransferase (AST) in the livers of fish fed frozen feed were significantly higher than those fed artificial feeds. Fish fed artificial feed had the highest AST and alanine aminotransferase (ALT) serum activity in serum which suggests that the liver was damaged to some extent. These results were further confirmed by histology of the liver. Fish fed the artificial feed had serious hepatocyte vacuolization and disorganization. The mucosal epithelium of intestine in fish fed frozen and artificial feed had poorly developed enterocytes with a shortening of the microvilli, decreased absorptive vacuoles and hyperplasia of the lamina propria. In addition to tissue damage there was a reduction in the ability of fish to digest frozen and artificial feed. The results of this study indicate that the mandarin fish hybrid readily feeds on frozen and artificial feeds. However, a more appropriate artificial feed for this species is needed to improve the digestive function and histology of the digestive tract.

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Introduction

The current use of live food for aquaculture is expensive and time consuming but can be alleviated by weaning fish to dry feeds. Attempts have been made to partially or completely substitute compound diets for live food (Cahu et al., 1998; Yúfera et al., 1999). Results have shown that total substitution of live food by a compound diet has produced good development, growth, and survival in European sea bass on a commercial scale (Cahu et al., 2003). However, growth in many species of fish fed artificial feeds was slower than those fed the live food (Kvåle et al., 2007; Faulk and Holt, 2009). This may be either because the nutrients in the artificial feeds failed to stimulate ingestion or the fish were unable to properly metabolize the absorbed nutrients.

Mandarin is a popular fish. It is in high demand in China but the industry has been affected by disease and inappropriate diets. Mandarin fish feed is still based on fresh fish however this is problematic as it involves a sanitary risk (live feed can be a source of pathogens or parasites), deterioration of water quality, and variability of nutritional value, as well as being impractical (Baynes and Howell, 1993). Therefore, the development of a good quality formulated diet is essential for the expansion of mandarin fish culture.

S. chuatsi and *S. scherzeri* are considered most valuable within the mandarin fish genera. *S. chuatsi* eats only live food and grows quickly. *S. scherzeri* can be easily acclimated to new environments, is relatively more resistant to diseases, but grows slowly thus a hybrid could be acclimated to eat frozen and artificial feeds. Fish fed frozen feed grow faster than those fed artificial feed (Li et al., 2014). However, knowledge about the ability of juvenile hybrid mandarin fish to adapt their digestive ability to dietary changes has not yet been investigated. The aim of this research was to analyze the activity of digestive enzymes, aminotransferase, and the histology of the digestive tract of mandarin fish fed live, frozen, and artificial feed.

Materials and Methods

Experimental Fish and Acclimation. A growth trial was conducted at the Shanghai Fisheries Research Institute in China. The F3 hybrids, *S. chuatsi* ♀ × *S. scherzeri* ♂ were obtained from the Shanghai Sunnong Aquaculture Farm. Prior to the experiment, the juveniles were fed live feed and acclimated to the experimental conditions. The live feed was mainly whole fish of silver carp (*Hypophthalmichthys molitrix*) with 16.9% wet weight protein and 9.1% wet weight lipid (Table 1).

Table 1. Proximate composition of diets used to feed the Mandarin fish during 70 days. Values are given as percentage.

Items	Moisture (%)	Ash (%)	Lipid (% wet weight)	Protein (% wet weight)
Artificial food	7.5	13.3	9.7	52.4
Bait fish	70.9	2.8	9.1	16.9

The feed was silver carp which was frozen at -20°C. The artificial feed was a commercial micro-particulated extruded diet (Qingdao Great Seven Bio-Tech Co. Ltd) which contained 52.4% wet weight protein, and 9.7% wet weight lipid.

The weaning of the test fish began 32 days post hatch. The fish were first starved for 2 days to enhance their appetite, after which fresh dead fish were given for 2 days. The hybrid fish were then fed minced frozen silver carp fillets. Mixed diets of 50% commercial feed and 50% minced fillets helped fish to acclimate to the artificial feed. After 7 days of mixed feeding, the fish were fed with the artificial feed only.

Experimental Design. Once weaned to frozen and artificial feed, 180 fish of similar size (average weight, 37.59 ± 0.21 g) were randomly stocked into nine 250-L aquaria. The aquaria were connected to an indoor recirculation system attached to a fresh water reservoir, a head pump, a sand filter, and aerated with a blower. The different feeding groups were randomly assigned to triplicate aquaria. Fresh and frozen feed were offered to fish at a rate of 4.0–5.0% body weight daily divided into two equal feedings at 9:00 A.M. and 16:00 P.M. The commercial diet was mixed with water, made into soft dough, and fish were hand fed to apparent satiation. The feeding trial lasted for 10 weeks.

During the experimental period, the temperature was $26 \pm 2.9^{\circ}\text{C}$, and the dissolved oxygen was $7.9 \pm 0.3 \text{ mg/L}$.

Sample Collection. At the termination of the experiment, three fish per aquarium were anesthetized with (1:10000) eugenol (Shanghai Reagent Corporation, China) and 1 mL blood samples were collected from the caudal vein with a 1-mL syringe. The blood was allowed to clot in a centrifuge tube at room temperature for 2h. The clot was then removed and the residual blood cells separated from the serum by centrifugation ($836 \times g$, 10min, 4°C). The serum was then frozen at -20°C until assayed. The liver, stomach, and whole intestinal tract contents were also collected. The chyme was removed from the gut and stomach with distilled water. Each of the organs from the three fish per aquarium was pooled. The liver, stomach, and intestine samples were accurately weighed to 0.5g accuracy, then homogenized in 5 mL 0.9% ice-cold saline solution (w:v = 1:10). Following centrifugation ($3000 \times g$, 10min, 4°C), the supernatants were removed and kept at -20°C for analysis of the activity of digestive enzymes and aminotransferases.

Digestive Enzyme Activities. Total proteolytic activity in the liver and intestine was measured using the casein hydrolysis method modified by Walter (1984). Buffers used were pH 7.5 phosphate for neural protease activities in liver, and intestine, and pH 2 for pepsin. Enzyme reaction mixtures consisted of 2% (w/v) casein in pH 7.5 and pH 2 phosphate buffer (1 mL) and enzyme sample (1 mL), and were incubated for 10 min at 37°C . The reaction was stopped by adding 2 mL of 0.4 mol trichloroacetic acid. After 10 min at 37°C the samples were centrifuged at $1800 \times g$ for 10 min, and 1 mL supernatant was mixed with 0.4 mol Na_2CO_3 5 mL and 1 mL Folin reagent for 20 min at 37°C . The absorbance of the samples was recorded at 660 nm. All the samples were assayed in triplicate along with three blanks. Tyrosine was used as standard, and one unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1 μg of tyrosine per 1 min at 37°C .

Lipase activity was assayed as suggested by Borlongan (1990) with commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). 50 μL samples of the enzyme were pre-heated, mixed with 2 mL substrate buffer, and poured into a color dish. The first absorbance value (A1) was read at 30 sec after which the color solution in the centrifuge tube was held for 10 min at 37°C . The second absorbance value (A2) was read at 10 min 30 sec. The lipase activities were calculated according to the difference between the two absorbance values.

Amylase activity was determined by the starch hydrolysis method, according to the Somogy-Nelson colorimetric method, described by Robyt and Whelan (1968). The enzyme reaction mixture consisted of 2% (w/v) starch solution (0.125 mL), 0.1 M phosphate-citrate buffer, pH 7.5 (0.125 mL), and enzyme sample (0.05 mL). The reaction mixtures were incubated for 1 h at 37°C . The absorbance of samples was measured at 600 nm. All samples were assayed in triplicate. Maltose was used as the standard, and amylase activity was expressed as mmol maltose released from starch/mL/min.

The activity of digestive enzymes was expressed as enzyme activity per mg protein. The protein concentration of the supernatant solutions was determined with the Lowry et al. (1951) method, using bovine serum albumin as the standard.

Activity of Aminotransferases in Liver and Serum. Crude extracts of liver for assaying aminotransferases activities were determined by homogenization of frozen liver in ice-cold 0.9% saltwater. Following centrifugation ($3000 \times g$, 10 min, 4°C), activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of liver supernatants and serum were measured with commercially available kits (Jiancheng engineering Institute, Nanjing, China). Each reaction was performed at least three times. An aliquot of 100 μL refolded ALT or AST (400 $\mu\text{g/mL}$) was mixed with 500 μL substrates (alanine or aspartate and α -ketoglutarate mixture). After pre-incubation at 37°C for 30 min, 500 μL of 2, 4-dinitrophenylhydrazine was added to the mixture, which was then incubated at 37°C for 20 min. After addition of 5 mL of 400 mM NaOH into the mixture, the absorbance (OD) was measured at 510 nm within 15 min. The control was processed similarly except that the substrates and 2, 4-dinitrophenylhydrazine were added

simultaneously after the first 30 min pre-incubation. One unit of the activity was defined as the amount of enzyme that catalyzed the formation of 1 $\mu\text{mol/min}$ of alanine or aspartate, and α -ketoglutarate mixture under assay conditions.

Histology Examination. Samples for morphological examination were taken from three fish of each group. After washing, the gut and tissues from the middle part of the intestine, as well as the liver, were removed for morphologic study. All tissue samples were fixed in 10% buffered formalin. Following fixation, the samples were routinely dehydrated in graded ethanol, equilibrated in xylene and embedded in paraffin. 5 μm thick sections were cut with a rotary microtome. The sections were then stained with hematoxylin and eosin (HE) for observation under an Olympus microscope (Olympus, Tokyo, Japan). Digital micrographs were prepared with a ProgRes C14 camera (Jenoptik GmbH, Jena, Germany), and Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA) was used for image processing.

Statistical Analyses. All data were subjected to one-way analysis of variance using SPSS 16.0 for Windows. Differences among the means were tested by Tukey's multiple range tests. The level of significance chosen was $P < 0.05$.

Results

Activity of Digestive Enzymes in the Alimentary Tract. The activity of protease in the stomach, liver, and intestine of fish fed live feed were highest and significantly higher ($P < 0.05$) than those of fish fed frozen or artificial feed (Table 2).

Table 2. The activity of digestive enzymes of hybrid mandarin fish fed live, frozen or artificial feed for 70 days. Values are estimated according to the AOAC (1995) and given as Means with pooled SEM¹ (U mg/protein).

Treatments	Protease			Lipase			Amylase		
	Pepsin	Liver	Intestine	Stomach	Liver	Intestine	Stomach	Liver	Intestine
Fresh group	12.55 ^a	0.18 ^a	1.56 ^a	9.86	24.13 ^a	10.91	N	0.38 ^b	1.25
Frozen group	8.96 ^b	0.11 ^b	1.18 ^{ab}	11.84	31.08 ^a	12.42	0.20	0.61 ^a	1.19
Artificial food	5.45 ^c	0.06 ^c	0.79 ^b	10.20	13.47 ^b	10.91	0.22	0.51 ^{ab}	1.09
Pooled SEM ²	0.69	0.01	0.37	1.10	2.21	0.45		0.03	0.04
ANOVA ³									
F- value	18.239	30.635	10.420	0.301	15.176	1.031	-0.437	6.599	1.158
P- Value	< 0.001	< 0.001	0.045	0.747	< 0.001	0.384	0.647	0.004	0.328

¹Values are means of composite samples from three fish in each of three replicate groups. Means in each row with different superscripts are significantly different ($P < 0.05$).

² SEM: standard error of the mean.

³ ANOVA: one-way analysis of variance.

Fish fed artificial feed had the lowest levels of protease activity and they were significantly lower than fish fed frozen feed. No significant differences were found in the lipase activities of the stomach and the gut among fish fed different kinds of feed. However, the lipase activity of fish fed frozen feed was higher than in the other two treatments. The lipase activity in the liver of fish fed frozen feed was significantly higher than fish fed artificial feed, however there were no significant differences between the live food and frozen feed groups. The amylase activity in liver of fish fed frozen feed was significantly higher than fish fed live feed. No significant differences were found in the amylase activity in the gut among the three treatments.

Aminotransferase activity in Liver and Serum. AST activity in liver of fish fed frozen feed was significantly lower than in fish fed live feed, but was significantly higher than in fish fed artificial feed. No significant differences in the ALT activity in liver were observed among treatments. The AST and ALT activity in serum of fish fed artificial feed was significantly higher than in fish fed live feed, however, no significant differences were observed between fish fed artificial and frozen feed (Table 3).

Table 3. The activities of aminotransferase of mandarin fish hybrid fed live, frozen and artificial feed during 70 days. Values are estimated according to the AOAC (1995) and given as Means with pooled SEM¹.

Groups	Liver (U mg/protein)		Serum(U/L)	
	AST ²	ALT ³	AST	ALT
Fresh bait fish	115.76 ^a	2060.9	15.92 ^b	6.69 ^b
Frozen bait fish	97.34 ^b	1801.0	25.87 ^{ab}	11.10 ^a
Artificial feed	72.02 ^c	2417.3	34.10 ^a	12.58 ^a
Pooled SEM ⁴	5.08	141.02	2.52	0.84
ANOVA ⁵				
F -value	19.751	2.313	7.793	26.983
P -Value	< 0.001	0.131	0.003	< 0.001

¹Values are means of composite samples from three fish in each of three replicate groups. Means in each row with different superscripts are significantly different. ($P < 0.05$).

²AST: aspartate aminotransferase.

³ALT: alanine aminotransferase.

⁴SEM: standard error of mean.

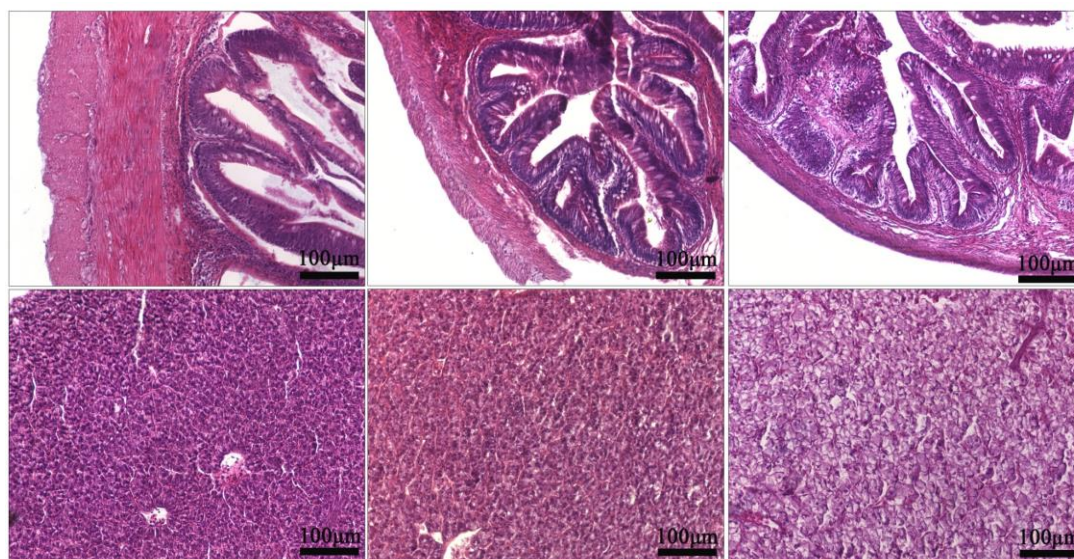
⁵ANOVA: one-way analysis of variance.

The Histology of Intestine and Liver. The intestinal tissue of fish fed live feed was normal. The mucosal epithelium had well-developed enterocytes with prominent microvilli. Goblet cells were regularly distributed among the mucosal epithelium (Fig. 1). Fish fed frozen (Fig 2) and artificial feed (Fig 3) exhibited pronounced pathological changes of the intestinal epithelium. The mucosal epithelium had poorly developed enterocytes with shortening of microvilli and decreasing absorptive vacuoles, and hyperplasia of the lamina propria was also observed. An apparent change in the circular muscle layer is shown in the longitudinal muscles of fish fed different feed. The tunica muscularis of fish fed frozen bait fish was thicker than fish fed artificial feed and thinner than fish fed fresh bait fish.

Fig. 1. The Intestine of fish fed fresh bait fish (× 20).

Fig. 2. The intestine of fish frozen bait fish (× 20).

Fig. 3. The intestine of fish fed artificial food (× 20).



a : microvilli ; b: goblet cells; c: tunica muscularis; d: lamina propria; e: hepatocyte vacuolization

Fig. 4. The liver of fish fed fresh bait fish (× 20).

Fig. 5. The liver of fish fed frozen bait fish (× 20).

Fig. 6. The liver of fish fed artificial food (× 20).

Liver samples from fish fed fresh feed showed normal histology (Fig. 4). The hepatocytes were regular and compact. The hepatocyte vacuolization and disorganization were present in samples from fish fed both frozen (Fig. 5) and artificial feed (Fig. 6); however,

it was much more pronounced in fish fed artificial feed. The lost of tissue structure, an evident pyknosis (nuclear destruction) and some necrosis and hemorrhagic tissue were also apparent in the liver of fish fed artificial feed.

Discussion

Research has been conducted to find artificial feeds for full or partial replacement of live feed in juvenile hybrid mandarin fish. Although significant improvements have been reported, artificial feeds have not matched the results obtained with live feed (Li et al., 2014). Many studies have shown that the relatively low level of enzyme activity was one of the reasons for limited success of diets and the poor growth of fish fed solely on artificial diets (Verreth et al., 1992; Day et al., 1997). The present study also revealed that the digestive enzymes activity, especially the protease of fish fed artificial feed, was significantly lower than in fish fed live and frozen feed. The decreased protease activity indicated that degradation of protein compounds and the rate of digestion and absorption of essential amino acids decreased (Chong et al., 2002), and retarded the growth of fish. This may be because the live feed organisms consumed by fish "donated" their digestive enzymes, either by autolysis or as zymogens that activate the endogenous digestive enzymes in fish (Pedersen and Hjelmeland, 1988; Kolkovaski, 2001). However, these substances are frequently omitted in formulated diets (Kolkovaski, 2001), and artificial feeds sometimes contain proteins and other ingredients that are difficult for fish to digest (Lindner et al., 1995), as well as other anti-nutritional factors. In addition, observations of feeding showed that when juveniles were offered fresh and frozen bait fish, they fed by 'visually locking on' to the feed and striking to consume it. However, when fed artificial diets, they carefully scrutinised the feed before striking. Once the feed fell to the bottom of the tank it was ignored by the juvenile mandarin fish.

ALT and AST are the most important aminotransferases in fish livers (Cowey and Walton, 1989; Fynn-Aikins et al., 1995), and are considered valuable tools to evaluate the response of fish to diet modifications (Dean et al., 1986). In this study, the AST activity was significantly higher in fish fed live and frozen feed compared to artificial feed, indicating that fish responded to the bait fish positively. The ALT and AST activity is also closely related to amino acids metabolism in fish. The transaminase activity increased with the increase of amino acid metabolism (Deng et al., 2009, Cheng et al., 2010). Hybrid Mandarin fish fed artificial feed had the lowest AST activity. This suggests that protein metabolism was insufficient and did not increase amino acid levels in the liver, which in turn activates this enzyme (Trenzado et al., 2006). Aminotransferases in serum are indicative of the damage of organs and in particular of liver cells (Kumar et al., 2010). When liver cells are damaged, aminotransferases, including ALT and AST leak into the blood (Racicot et al., 1975). Significantly higher ALT and AST levels in serum were found in hybrid mandarin fish fed the artificial feed. These results suggest that fish liver could have been damaged to some extent and have affected the process of protein metabolism. These results are in agreement with the histology of the liver of fish fed artificial feed which caused serious hepatocyte vacuolization and disorganization. This may indicate that hybrid mandarin fish cannot totally adapt to artificial feed.

The histology of animals is important in understanding pathological alterations in response to nutritional sources (Hu et al., 2013). In this experiment, the liver of fish fed artificial feed exhibited pathological changes. This result was also obtained with seabass (*Dicentrarchus labrax*), which showed hepatic disturbances induced by artificial feed (Mosconi-bac, 1987). Similarly the metabolism of fat and fatty acid was also apparently disturbed.

Goblet cells are associated with the immune system by producing mucus which acts as a lubricant in the alimentary tract and provides protection against chemical and mechanical damage. The reduced amount of goblet cells in the intestine of fish fed frozen and artificial feed suggests a decrease in the defense mechanism of the fish. In addition, the intestine of fish fed frozen and artificial feed exhibited pronounced pathological changes which negatively affected the digestion and absorption of feed. In this study, the tunica muscularis in the gut of fish fed live feed was thicker than in fish fed frozen and

artificial feed. Concurrent with tissue degradation is a reduction in the ability of the gut to process feeds.

Conclusion

Although our research suggested that the most appropriate feed for hybrid mandarin fish is fresh bait fish, we found that hybrid mandarin fish can feed on frozen and artificial feed after acclimation. The main reason why fish could not grow faster was that frozen and artificial feed decreased digestive enzymes activity and caused pathological changes of the alimentary canal.

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