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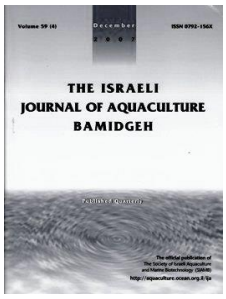

Effects of Formulated Diet Co-fed with Minced Fish on Growth, Digestion, Metabolism, and Appetite Regulation, of Mandarin Fish Hybrid (*Siniperca chuatsi* ♀× *Siniperca scherzeri* ♂)

Yan Li*, Yongqiang Li, Qinying Xu

Shanghai Fisheries Research Institute; Shanghai Fisheries Technical Extension Station, Shanghai, 200433, China

Keywords: formulated diet; co-fed; mandarin fish hybrid; appetite regulation; growth; digestion

Abstract

This study examined the effects of a formulated diet combined with minced fish at ratios of 1:1 (Diet 1), 1:3 (Diet 2) and 0:1 (Diet 3) on growth, digestion, metabolism, and appetite of juvenile mandarin fish (initial weight, 10.63 ± 0.29 g). Fish were hand fed to satiation for 194 days. Weight and growth rate of the fish in the first 97 days were negatively correlated to decreased ratio of minced fish. The weight of fish fed Diet 2 (97 days) was significantly higher than that of fish fed Diet 1. There was no significant difference in the growth of fish fed Diets 2 or 3. The activities of pepsin in the stomach, alanine transminase (ALT), and aspartate transminase (AST) in the liver, significantly increased in relation to the enhanced formulated diet ratio. The mRNA level of leptin in the liver and ghrelin in the stomach increased as the minced fish ratio decreased. The mRNA level of neuropeptide Y (NPY) in the brain increased as formulated diet ratios increased. Combined feeding strategies increased the appetite of the mandarin fish hybrid, improved digestion and metabolism of formulated diets, and improved fish growth.

***Correspondence:** Yan Li, e-mail: liyan0501@126.com. Tel: +86 021-65483715, Fax: +86 021-65483778.

Introduction

Although weaning formulated diets to mandarin fish (*Siniperca chuatsi*) is problematic, we found that after domestication, the hybrid (*Siniperca chuatsi* ♀ × *Siniperca scherzeri* ♂) accepted minced fish as easily as artificial feed (Li et al., 2015, 2017). Weaning these hybrids to artificial feed resulted in poor growth probably due to improper digestive capabilities or low attractiveness of the non-mobile particles (Li et al., 2015, 2017).

In our study, co-feeding included both minced fish and formulated feeds, where fish were gradually weaned from live prey to an artificial diet allowing gradual adaptation to the physical and biochemical characteristics of the new diets (Curnow et al., 2006; Hamza et al., 2007; Herrera et al., 2010; Ma et al., 2014). However, further study is needed on the growth performance, digestion, metabolism, and appetite of mandarin fish hybrid. The present experiment studied the effects of different ratios of formulated diets combined with minced fish on growth, digestion, and appetite-related gene expression of mandarin fish hybrid and attempted to find an appropriate method of transition.

Materials and Methods

Experimental Fish and Feeds.

The present growth trial was conducted at Shanghai Fisheries Research Institute in China. The F1 (*S. chuatsi* ♀ × *S. scherzeri* ♂) hybrids were obtained from Shanghai Sunnong Aquaculture Farm. Prior to the experiment, the juveniles were fed with dead bait and acclimated to experimental conditions for 7 days. The bait was composed mainly of a whole fish of silver carp (*Hypophthalmichthys molitrix*) with 42.4% wet weight protein and 24.0% wet weight lipid. The formulated diet was a commercial micro-particle expanded diet (Great Seven Bio-Tech Co. Ltd, Qingdao, China) which contained mainly fishmeal, krill meal, fish oil, starch, mineral mixture, a vitamin mixture, and attractant with 58.8% protein and 13.5% lipid (wet weight).

The commercial diet was ground into powder and mixed with the minced fish at the following ratios: Diet 1 = 1:1 (w: w), Diet 2 = 1:3 and Diet 3 = 0:1. It was then formed into soft dough, and then formed into spindle-like shapes. Diet 1 contained 50.6% protein and 18.7% lipid, Diet 2 contained 54.7% protein and 16.1% lipid, Diet 3 was a commercial diet with 58.8% protein and 13.5% lipid (wet weight). The experimental fish were starved for 2 days to enhance their appetite and were then fed the experimental diets. The experiment began only after the experimental diets were accepted by the fish.

Experimental Design.

180 fish of similar size (av. weight, 10.63 ± 0.29 g) were randomly stocked into nine 250L aquaria that were connected to an indoor recirculation system annexed to a fresh water reservoir, high-head pump, sand filter, and an aeration system. Three different mixed diets were randomly assigned to triplicate aquaria. Fish were hand-fed to apparent satiation at 9:00 and 16:00. The feeding trial lasted for 194 days. The fish were weighed after 97 days. During the experimental period, the temperature was 27.1 ± 2.0°C, and the dissolved oxygen was about 7.6 mg/L.

Sampling.

After 194 days, six fish per aquarium were anesthetized with Eugenol (1:10000) (Shanghai Reagent Corporation, China) and their liver, stomach, and whole intestinal tract contents were collected; the chyme was removed from the gut and stomach with distilled water. Each section of the organs of six fish per aquarium was pooled. The liver, stomach, and intestine samples were accurately weighed, then homogenized in 0.9% ice-cold saline solution (w: v = 1:10). Following centrifugation (3000 × *g*, 10min, 4^0C), the supernatants were removed and kept at -20^0C for analysis of digestive and metabolic enzyme activity. The liver, stomach, and brain samples of another three fish were collected and stored at -80^0C for RNA extraction.

Digestive enzyme activities.

Total protease activity in the gastrointestinal tract was measured with the casein hydrolysis method, modified by Walter (1984). pH 7.5 phosphate buffers were used for the analysis of neural protease activity in intestine, and pH 2 phosphate buffers were used for the analysis of pepsin in the stomach. 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 with commercial kits as suggested by Borlongan (1990) (Jiancheng Bioengineering Institute,

Nanjing, China). 50 uL enzyme samples were mixed with 2 mL pre-heating substrate buffer in the color dish. The first absorbance values (A1) were read at 30 sec, the second absorbance values (A2) were read after 10 min 30 sec at 37°C. Lipase activity was calculated according to the difference between the two absorbance values. Amylase activity was determined by the starch hydrolysis method (Robyt and Whelan, 1968). Maltose was used as the standard, and amylase activity was expressed as mmol maltose released from starch as mL^{-1}. The digestive enzymes activity was as expressed as mg/protein. Protein concentration of the supernatant solutions was determined with the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Metabolic enzyme activities.

Pyruvate kinase (PK) (EC 2.7.1.40) activities were measured in the liver homogenate with PK assay kits (Nanjing Jiancheng Bioengineering Institute, China). One unit of enzyme activity was defined as 1μmol phosphoenolpyruvate (PEP) converted to pyruvic acid per minute per gram tissue protein at 37℃ pH 7.6. Lipoprotein lipase (LPL) activity in the liver was also analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). One unit of LPL activity was defined as 1mmol of free fatty acid released per hour per gram of liver protein. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in the liver were measured with commercially available kits (Jiancheng Engineering Institute, Nanjing, China). Each reaction was performed in triplicate. One unit of the activity was defined as the amount of enzyme that catalyzed 1 μmol/min of alanine or aspartate, and α-ketoglutarate mixture.

Real-time PCR analysis.

Total RNA was extracted from the liver, stomach, and brain using the TRIzol method. Agarose gel electrophoresis at 1% and spectrophotometric analysis (A260:A280 nm ratio) were used to assess RNA quality and quantity. Subsequently, cDNA synthesis was performed at 70℃ for 5min, 42℃ for 60min, and 95℃ for 5min. Quantitative real-time PCR (qPCR), specific primers for the target genes were designed. The primer sequences are shown in Table 1. Quantitative real-time PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa Biotechnology (Dalian) Co., Ltd.).

Table 1. Real-time PCR primer sequences

Name	*Primer*	*Sequence*	*Size*
Ghrelin	Forward	5'- TTGCTGGTCTTCCTGTTGT -3'	162bp
	Reverse	5'- GTGGTTGTCCTCAGTGGGT -3'	
NPY[1]	Forward	5`- TACCTCTATGAGCCGCCTTGT -3'	150bp
	Reverse	5`- GCCCTCCCGTACTTTACTTTT -3'	
Leptin	Forward	5`- AGTTGACTACAGCCAGAGGA -3'	187bp
	Reverse	5`- AATAGCCAGTTTGGAAGAGC -3'	
IGF-I	Forward	5`- ATTGTGGACGAGTGCTGCTT -3'	202bp
	Reverse	5`- TTGTCTTGTCTGGCTGCTGT -3'	

[1]NPY: neuropeptide Y.

Data analysis.

Specific growth and survival rates were calculated for each treatment as follows:

SGR1 = $(\ln W_m - \ln W_i \times 100) / t$ SGR2 = $(\ln W_f - \ln W_m \times 100) / t$

Survival rate (%) = Nf / Ni ×100

Where W_m = mean weight for 97 days

W_f = mean final weight for 194 days W_i = mean initial weight

t = the experimental days

N_f = the final number of fish

N_i = the initial number of fish

Results are presented as mean ± stand error (S.E.). All data were subjected to one-way analysis of variance. Differences between the treatment means were determined using Turkey's multiple-range test at a $P < 0.05$ level of significance. Statistical analyses were done using the statistical/scientific software 16.0 (SPSS 16.0).

Results

Growth performance.

The growth of mandarin fish hybrid appears in Table 2. The weight of the fish during the first 97 days showed decreasing trends in relation to the decreased ratio of minced fish; the trend of growth rate was the same. During the next 97 days the average the weight of the fish fed Diet 1 was significantly lower than those fed Diet 2 ($P < 0.05$) and their growth rate subsequently decreased. No significant difference was observed between fish fed Diets 2 and 3 ($P > 0.05$). In contrast, the growth rate of fish fed Diet 2 was greater than the other treatment groups. Gene expression of IGF-I in the liver increased with an increase of the formulated diet ratio and thereafter declined. Survival rate of the fish over 194 days ranged from 78.3% to 81.7% and was independent of experimental diets.

Table 2. The growth performance of fish fed diets for 97 days and 194 days

Groups	*MW (g)*[2]	*SGR1*[3]*(%)*	*FW(g)*[4]	*SGR2*[5] *(%)*	*SR (%)*[6]	*IGF-I*[7]
Diet 1	65.5±2.8^{a}	1.87±0.08^{ab}	142.8±3.0^{b}	0.93±0.07^{b}	80.0±5.8	1.00±0.00^{b}
Diet 2	53.7±4.3^{ab}	1.65±0.04^{b}	160.6±2.8^{a}	1.35±0.04^{a}	81.7±8.8	1.65±0.50^{a}
Diet 3	48.2±1.6^{b}	1.61±0.04^{b}	149.7±3.5^{ab}	1.31±0.13^{a}	78.3±12.0	1.13±0.10^{b}
One-way ANOVA analysis						
F value	7.943	6.176	5.297	7.141	9.038	27.562
P value	0.021	0.035	0.047	0.026	0.006	0.001

[1]Values are presented as means ± S.E.M. Values in the same column with the same superscripts are not significantly different determined using Turkey' test ($P > 0.05$).
[2]MW, the average weight of mandarin fish hybrid fed diets for 97 days.
[3] SGR1, the special growth rate between fish fed diets from 1 day to 97 days.
[4] FW, the final weight of mandarin fish hybrid fed diets for 194 days.
[5] SGR2, the special growth rate between fish fed diets from 97 days to 194 days.
[6]SR, the survival rate of mandarin fish fed diets for 194 days.
[7]IGF-I, the insulin-like growth factors in liver at the end of experiment.

Digestive enzyme activity. At the end of experiment, we found that the activity of protease in the stomach significantly increased in relation to the increase of formulated ratio in the diet. No significant differences were observed between fish fed Diets 2 and 3 (Table 3). Protease activity in the intestine significantly increased with the increase of formulated diet ratio and thereafter significantly declined. Amylase activity in the stomach and intestine significantly decreased in relation to the increase of formulated diet ratio. Lipase activity in the stomach significantly decreased in relation to the increase of formulated diet ratio, but in the intestines, it increased significantly and thereafter decreased significantly.

Table 3. The digestive enzyme activities of fish fed diets for 194 days (U/mgprot)[1]

Groups	*Protease*		*Amylase*		*Lipase*	
	Stomach	*Intestine*	*Stomach*	*Intestine*	*Stomach*	*Intestine*
Diet 1	16.28 ± 0.07^{b}	1.69 ± 0.30^{b}	0.42 ± 0.00^{a}	0.28 ± 0.01^{a}	72.74 ± 2.78^{a}	31.52±1.93^{b}
Diet 2	19.94 ± 0.38^{a}	5.13 ± 0.18^{a}	0.34 ± 0.00^{b}	0.18 ± 0.00^{b}	56.27 ± 4.49^{ab}	52.71±3.16^{a}
Diet 3	20.37 ± 0.19^{a}	0.47 ± 0.05^{c}	0.23 ± 0.00^{c}	0.18 ± 0.00^{b}	38.97 ± 11.37^{b}	32.97±2.61^{b}
One-way ANOVA analysis						
F value	80.843	140.302	854.124	127.437	6.064	20.482
P value	< 0.001	< 0.001	< 0.001	< 0.001	0.007	0.002

[1]Values are presented as means ± S.E.M. Values in the same column with the same superscripts are not significantly different determined using Turkey' test ($P > 0.05$).

Metabolic enzyme activity.

At the end of experiment, PK activity in liver showed an increase with the increase of formulated diets ratio and thereafter significantly declined (Table 4). LPL activity in the liver significantly decreased with the decrease of minced fish ratio. ALT and AST activity in liver significantly increased with the decrease of minced fish ratio.

Table 4. The activities of metabolic enzyme of fish fed diets for 193 days[1]

Groups	*PK*[2] *(U/gprot*[1]*)*	*LPL*[2] *(U/mgprot)*	*ALT*[2] *(U/gprot)*	*AST*[2] *(U/gprot)*
Diet 1	166.1 ± 13.2[ab]	3.18 ± 0.21[a]	70.02 ± 0.82[b]	42.91 ± 2.09[b]
Diet 2	200.8 ± 11.6[a]	0.71 ± 0.00[b]	86.71 ± 1.34[a]	45.34 ± 2.78[b]
Diet 3	150.1 ± 10.5[b]	1.03 ± 0.03[b]	87.57 ± 2.06[a]	54.95 ± 2.81[a]
One-way ANOVA analysis				
F value	4.561	45.417	43.669	6.082
P value	0.019	< 0.001	< 0.001	0.012

[1]Values are presented as means ± S.E.M. Values in the same column with the same superscripts are not significantly different determined using Turkey' test ($P > 0.05$).
[2]PK, pyruvate kinase; LPL, lipoprotein lipase; ALT, alanine transminase; AST, aspartate transminase.

Appetite related gene expression.

The mRNA level of leptin in the liver and ghrelin in stomach increased with the decrease of minced fish ratio and thereafter declined (Table 5). However, the mRNA level of NPY in the brain increased with the increase of minced fish ratio.

Table 5. The mRNA levels of appetite related gene expression in fish fed different feeds for 193 days[1]

Groups	*Leptin in liver*	*NPY*[2] *in brain*	*Ghrelin in stomach*
Diet 1	1.00 ± 0.00[b]	40.47 ± 19.27	1.00 ± 0.00
Diet 2	2.05 ± 0.08[a]	50.97 ± 9.13	4.52 ± 2.32
Diet 3	1.30 ± 0.01[b]	89.64 ± 14.69	1.30 ± 0.35
F value	54.298	3.001	2.079
P value	< 0.001	0.125	0.206

[1]Values are presented as means ± S.E.M. Values in the same column with the same superscripts are not significantly different determined using Turkey' test ($P > 0.05$).
[2]NPY: Neuropeptide Y.

Discussion

This study found that the growth of fish in the first 97 days showed decreasing trends in relation to the decreased ratio of minced fish. At the end of 97 days, the growth of fish showed increasing trends in relation to the decreased ratio of minced fish. Results indicated that growth of mandarin fish hybrid improved, and co-feeding increased the acceptability of artificial diets when live feeds are gradually withdrawn. Improved growth when live feeds were combined with microdiets has also been reported for cobia *Rachycenton canadum* (Nhu et al., 2010), loach *Misgurnus anguillicaudatus* (Wang et al., 2009), tongue sole *Cynoglossus semilaevis* (Chang et al., 2006) and Senegalese sole *Solea senegalensis* (Engrola et al., 2009). It was reported that live feed given together with a formulated diet could enhance the efficiency of the formulated diet by promoting the assimilation and deposition of dietary nutrients in the fish body (Kolkovski et al., 1997). Growth of juvenile mandarin fish hybrids was enhanced in the early co-feeding treatments as the palatability and digestibility of minced fish was better compared to artificial diets. The nutritional requirements of mandarin fish hybrid are age-dependent. Formulated diets have higher protein and lower lipids compared to minced fish and balance the nutritional composition of bait fish especially with respect to amino acids which are not easily modified in bait fish (Rønnestad et al., 1999). Therefore, mixed diets with higher formulated diet ratios resulted in significant improvement of growth performance at the end of experiment, and the gene expression of IGF-I in the liver paralleled fish growth.

In this experiment, protease activity of mandarin fish hybrid fed Diet 2 was significantly higher than in the fish fed Diets 1 or 3. This increase in protease activity may explain why growth of fish fed Diet 2 was better (Caruso et al., 1993; Chong et al., 2002). The digestive system of younger fish may lack the specific capacity to cope with any specific diet, but as the fish grow, there may be physiological adaptations to particular diets. In this experiment, the digestive enzyme activity levels were more

moderate. Co-feeding may have helped mandarin fish hybrids accept the formulated diets and improve their digestive capacity. They were able to ingest and digest the diets more easily.

Dietary carbohydrate seems to be the main factor involved in PK activity regulation observed in fish (Enes et al., 2010). In this experiment, higher content of carbohydrate in formulated diet improved feed adhesiveness and helped reduce soluble loss of feed in the water. The PK activity in liver of mandarin fish hybrid increased with the increase of formulated diet ratio. PK activity in grass carp *Ctenopharyngodon idella* increased with increasing dietary carbohydrate supplementation (Yuan et al. (2013). The inhibited activity of PK in fish fed formulated diets may occur because carnivorous mandarin fish hybrids have a low capacity for carbohydrate regulation. LPL is a key enzyme in lipoprotein metabolism, and LPL activity can increase with increasing dietary lipid levels (Li et al., 2013). In this experiment, Diet 1 contained 18.7% lipid, Diet 2 contained 16.1% lipid and Diet 3 contained 13.5%lipid, therefore, LPL activities decreased with the decrease of minced fish ratio. ALT and AST are the most important aminotransferases in fish livers (Cowey and Walton, 1989; Fynn-Aikins et al., 1995). Moreover, AST or ALT activity is closely related to amino acid metabolism in fish; the transaminase activity was enhanced with the increase of amino acid metabolism (Cheng et al., 2010; Deng et al., 2009; Feng et al., 2012; Luo et al., 2012). In this study, ALT and AST activity significantly increased with the increase of formulated diet ratio. This suggests that higher protein feed could enhance amino acid metabolism of mandarin fish hybrid after fish adapted to the artificial diets. It can also explain why the growth of fish increased with the increase of formulated diet ratio.

In mammals, leptin is synthesized and secreted predominantly by adipose tissue. It plays a major role in the regulation of body fat mass by decreasing food intake and inducing weight loss (Doyon et al., 2001). Ghrelin can stimulate food intake and the release of growth hormone from the pituitary gland in goldfish (Matsuda et al., 2011; Peng et al., 1993). In the present study, the mRNA levels of leptin in liver and ghrelin in stomach increased with the decrease of minced fish ratio and thereafter declined. Discrepancies in the expression of orexigenic and anorexigenic peptides among fish species may result from species-specific and tissue-specific differences in the expressional response to food (Babaei et al., 2017). NPY is considered the most potent orexigenic peptide in the mammalian brain (Woods et al., 1998). The upregulation of NPY was observed in the brain of mandarin fish hybrid fed diets with increased formulated diet ratio. The results indicated that after adapting to artificial diets the appetite of mandarin fish hybrid increased therefore explaining the rapid growth of the fish.

Conclusion

Co-feeding strategies could shorten the weaning time by improving the palatability and digestibility of formulated diets and the appetite of mandarin fish hybrid. The result of this experiment further confirmed that suitable formulated diets could be used in the future for commercially farmed mandarin fish hybrid.

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