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Research Article

Study on formulated diet for large yellow croaker (*Larimichthys crocea*) larvae at the early feeding stage

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Abstract

Most hatcheries in China rely on live prey for successful rearing of fish and shrimp larvae. Besides the high costs, live prey with pathogenic bacteria also increases the risk of contracting foodborne diseases. More than 3.5 billion large yellow croaker (*Larimichthys crocea*) larvae (total length over 4-centimeters) were raised in 2016. However, no formulated diet is available for replacing live feed in early stages of this croaker so far. In the present study, a new microdiet (Yq) is investigated for rearing croaker larvae at early stages (15 days after hatching). A commercial import diet (Cd) and live prey (Lp) were fed as controls. About 20,000 larvae per tank were hatched from Ningde Fufa Fishery Company in May 4th, 2017. Quality parameters, such as growth performance, survival and three digestive enzyme activities were measured. The survival rate of Yq (58.5%), Cd (51.5%) and Lp (69.6%) exhibited no significant difference (p>0.05). Furthermore, the cost of Yq was only about 23.3% of the cost of Lp. Specific growth rate in Lp was about 8 times higher (p<0.05) than that in two other microdiet treatments. Tryptic activity was slightly higher than other enzymes' activities, revealing that trypsin plays an important role in the degradation process of large yellow croaker larvae. In the Lp group, tryptic activity was 4 times higher than that of the other two groups. In addition, the activity of three diets on α-amylase and lipase activities were not significantly different (p>0.05). The high survival rate and low cost of Yq showed that it is suitable in rearing *Larimichthys crocea* larvae at the early stages.

Keywords: Larvae, Large yellow croaker, Formulated diet, Survival, Digestive enzyme

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Introduction

Larval stage is one of the critical developmental stages of fish. In this stage fish is passing from endogenous to exogenous feeding (Yúfera and Darias, 2007). Traditionally, early stages of larvae generally depend on live prey. Live prey is always with high levels of pathogenic bacteria, poor hygienic conditions and high costs. However, no formulated diet is available for replacing live feed of large yellow croaker larvae at the early stages (Cahu and Infante, 2001). Although feed delivery method is reported to be important, proper nutritional supply for fish larvae and juveniles is essential for fish growth and survival (Tanaka *et al.*, 2008; Ma *et al.*, 2015). Specific growth rate (SGR) and survival data are reported to be powerful tools for understanding the effects of both live and manufactured diets on first-feeding fish larvae. Some researchers reported formulated diets are completely replaced by live feeds from the onset of exogenous feeding in larvae of *Penaeus japonicus*, *Cyprinus carpio* and *Sparus aurata* (Teshima *et al.*, 1983; Escaffre and Kaushik, 1995; Robin and Vincent, 2003). Wang *et al.* (2005) found live feeds were healthier for *Plelteobagrus fulvidraco* larvae while larvae fed test diets showed higher SGR.

Large yellow croaker has been widely cultured since 1990s, mainly in floating cages; in terms of the estimated national marine fish culture production at species level the croaker is currently at the first rank with 165,496 tons in 2016 (Bureau of Fisheries, 2017). However, high mortality during the larval stage has severely deferred the production efficiency and profitability of large yellow croaker hatchery. Most hatcheries relied on live feeds such as rotifers, brine shrimp and copepods (Mai *et al.*, 2005; Yu *et al.*, 2012). Also, live feeds supply is usually required beyond metamorphosis until larvae are weaned onto a compound inert diet (Alves *et al.*, 2006). Therefore, it is necessary to formulate diets for supporting the growth of large yellow croaker which can substitute live preys.

Studies showed that the transition from endogenous to exogenous nutrition is crucial moments in marine fish. In large yellow croaker transition occurs during 5 to 8 days after hatching (DAH) (Mai *et al.*, 2005; Yu *et al.*, 2012). Although the development of digestive systems and nutritional requirements of large yellow croaker larva are well studied, few researches are focused on the nutritional information about early stage of *Larimichthys crocea* larvae (Mai *et al.*, 2005; Ai *et al.*, 2008). In this study, we tested the effects of a new formulated diet (Yq) on survival, growth and enzyme activities in early stages larvae of *Larimichthys crocea*, which was compared with the effects of a commercial import diet (Cd) and live prey (Lp).

Materials and methods

Rearing system and feed ingredients

Fertilized *Larimichthys crocea* eggs were collected within 12h from Fufa Fishery Company limited, Ningde, Fujian, China. Zygotes were then randomly stocked into nine 2,000L conical tanks with light blue walls at 17.5 eggs/L (mean weight 1.19 ±

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0.13 mg) and hatched within 3 days (May 4th 2017). The seawater (24.8 ppt) was filtered through three sand filters before use and the total ammonia was kept below 0.08 mg/L. Four airstones were evenly placed in each tank to maintain the dissolved oxygen concentration and provide sufficient water current for the eggs and larvae to keep them within the water column. The seawater exchange began 4 days after hatching at a rate of 20-30% per day to make sure temperature stayed above 22.0°C. Fluorescent lights (about 500 Lux at the water surface) were suspended at the center of each rearing tank for a constant photoperiod (LD 10h: 14h) during the experiment. The bottom of the pool was cleaned by siphoning from 5 DAH (1d after feeding). Concentration of *Chlorella* sp. was maintained at 2-5×10^6 cells/mL from 4 to 15 DAH (5 days after yolk sac disappear in Lp).

Artificial feed

At the beginning of the trial, about 20,000 fish were placed into each tank (stocking density: 10 individuals/L). Experiment was conducted with larvae at 3 DAH (mean initial weight 0.71±0.14 mg). Three treatments in triplicate were randomly assigned to larval rearing tanks including two formulated diet treatments (Yq-experimental diet, Cd-commercial import diet) and live prey treatment (Lp). Larvae in Lp group was fed mainly on rotifers (*Brachionus plicatilis*) at 09:00 and 15:00 to maintain a target density (15-20 ind/mL) from 4 to 6 DAH, *Artemia* nauplii (15-20 ind/mL) from 7 to 8 DAH and copepod from 9 to 15 DAH. From 4 to 15 DAH, other two groups of larvae were fed with Yq and Cd seven times a day (06:00, 09:00, 12:00, 15:00, 18:00, 21:00 and 00:00). The experimental diet was formulated according to the patent, was made by Yuequn Feed Company, Jieyang, Guangdong, China (Hong, 2014) (Chinese invention patent, ZL 201410300888.0; inventor: Yuequn Hong). Cd was purchased from Japan which is used to feed ornamental fish and mariculture fish species. The particle size ranged from 60 to 130 μm for Yq and 150 to 300 μm for Cd. Compositions of the two formulated feeds, Yq and Cd, are listed in Table 1. Feeding levels were decided on a dry weight basis at 15% and 20% of larval wet weight, corresponding to 2 and 3 g/tank time (dry weight), respectively.

Table 1: Feed formulation and proximate composition of Yq (experimental microdiet) and Cd (commercial import diet).

Diet	Protein (%)	Lipid (%)	Fiber (%)	Ash (%)	Calcium (%)	Phosphorus (%)	Moisture (%)	Lysine (%)
Yq	52.0	8.0	3.0	16.0	2.0	1.5	10.0	3.0
Cd	52.0	8.0	5.0	17.0	2.0	1.5	10.0	--

Data provided by manufacturers.

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Sampling

Growth rate was monitored by randomly sampling ten larvae from each tank at 15:30 from 3 to 15 DAH. The samples were captured and measured using light microscope (Olympus BX51 microscope, Tokyo, Japan). Thirty larvae were weighed immediately after briefly dried on a filter paper at 3, 6, 9 and 15 DAH. Equations used to calculate data in this study are as follow: SGR= $((\ln W_t - \ln W_0)/t) \times 100\%$; and K= $(W/L^3) \times 100\%$, where K is Fulton's condition factor, W is wet weight, t is days, L is total length. Fish in each of the triplicate aquaria were collected and survival number was counted with random sampling at fifteen different places of each tanks. Numbers of larvae counted in the entire fifteen samples were totaled and the sum was divided by the total number of sample and multiplied with the total volume of water in the tank.

Diet leaching rates

Leaching rates were evaluated for both Yq and Cd. Known amount of each dried diet was added in a beaker containing 50 mL of seawater for 4, 8 and 16 hours and mixed occasionally. The beaker contents were subsequently filtrated, dried over night at 65°C and weighted to the nearest 0.0001 g (BSA124S, Sartorius Corp., Edgewood, NY, USA). Lost part of the diet was calculated by subtraction of the remaining dry matter from the initial weight.

Enzyme activities

At 15 DAH, the croaker larvae from all 9 tanks were frozen overnight in liquid nitrogen and then transferred to -80°C for further enzyme analysis. The whole body of larvae were weighted and homogenized in 1.5 mL tubes, respectively. Lipase (LPS) activity was assayed following Verduin *et al.* (1973) and α-amylase (AMS) activity was assayed according to the method of Somogyi (1960). Trypsin (TRY) activity was assayed according to the methods of Iwamori *et al*. (1997) using a trypsin kit purchased from Nanjing Jiancheng Biochemical Corporation (Nanjing, China). According to the manufacturer' protocol, the samples were centrifuged and the supernatants were then used to detect LPS, AMS and TRY activities, as well as soluble protein content. LPS activity units were expressed in U/g protein, whereas AMS and TRY activities were expressed in U/mg protein. Bovine serum albumin (BSA, sigma) was used as standard when detecting protein content of *Larimichthys crocea* larvae.

Statistical analysis

Data on growth, survival and enzyme activities levels among dietary treatments were tested for significant differences ($p<0.05$) using one-way ANOVA followed by Tukey's post-hoc test. The data were calculated as mean ± SD and statistical analysis was performed using GraphPad Prism 5.0 and Microsoft Excel.

Results

Growth and survival

Among the three treatments (Yq, Cd and Lp), total length and height of larvae were the same before feeding (3 DAH) and showed similar trends during the

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experiment. Since 6 DAH, both total length and height in Lp group was significantly higher (p<0.05) than that in two microdiet treatments (Cd and Yq). When weaning trial was conducted at 15 DAH, the total length in Lp reached 6.81±0.52 mm, while croaker larvae in Yq and Cd were 4.41±0.23 mm and 4.81±0.36 mm, respectively. The level of wet weight in Lp increased sharply since 6 DAH while slowly increased in Yq and Cd (Fig. 1). The total length and wet weight in Yq was similar with that in Cd.

SGRs of croaker larvae in Lp were 26.94-48.72% per day which was about 8 times higher than that in Yq and Cd from 7 to 15 DAH (p<0.05, Table 2). While SGRs in Yq were lower than that in Cd, which were only 8.37±2.08% and 5.52±0.15%, respectively. Compared to Cd (51.5±6.6%) and Lp (69.6±6.0%), the survival rate of Yq (58.5±15.3%) showed no significant difference.

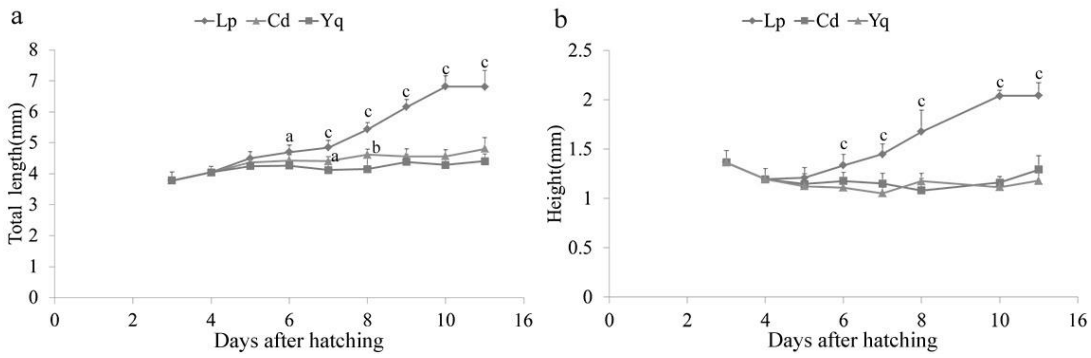

Figure 1: Total length (a) and height (b) of large yellow croaker larvae (n=10) at different weaning times. Values are mean, different superscripts are significantly different (p<0.05), error bars show standard deviation.

Table 2: Specific growth rate (SGR), condition factor (CF) and survival of larval large yellow croaker fed on Yq, Cd and Lp (live prey), respectively.

Diet	SGR (%) from 3 to 7 DAH	SGR (%) from 7 to 15 DAH	CF (%)	Survival (%)
Yq	0.084±0.021	0.055±0.001	1.03±0.23	58.5±15.3
Cd	0.157±0.015[a]	0.063±0.006	1.19±0.26	51.5±6.6
Lp	0.269±0.030[b]	0.487±0.089[b]	1.49±0.33[b]	69.6±6.0

Values are mean ± SD, different superscript letters within a column were significantly different (p<0.05).

Digestive enzyme activity

TRY activity was relatively high between different enzyme's activities, revealing that trypsin plays a more important role in degradation process of large yellow croaker larvae at the early stage. In Lp group, tryptic activity was four times higher than that in the other two groups. Figure 2 shows that both AMS and LPS activity of large yellow croaker larvae were not significant (p>0.05) effected by different diets (Yq and Cd). The analysis showed that LPS activity was relatively

lower than the activity of AMS and TRY in all three groups.

Diets

Yq and Cd were similar in proximate composition but differed in terms of fiber (3.0% for Yq and 5.0% for Cd). Besides, lysine was additionally added to Yq. The leaching loss was significantly different at 16 hours in Yq (10.3±1.1%) and Cd (9.4±0.5%) in sea water.

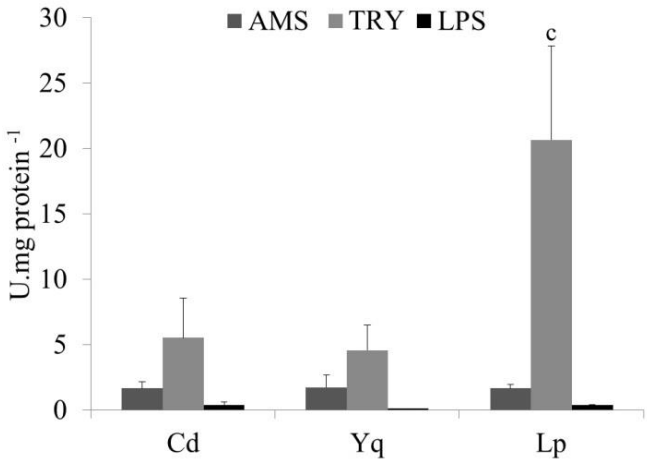

Figure 2: Effect of different diets on lipase (LPS, U/g protein), α-amylase (AMS, U/mg protein) and trypsin (TRY, U/mg protein) activity in large yellow croaker larvae after feeding 15 days. Different letters above bars in same enzyme represent significant difference (*p*<0.05).

Discussion

In most cultured species, larval fishes ingest food primarily by swallowing the particle identified as food directly by the sensory organs. Mouth gape and oesophagus diameters are defining factors in food-particle preferences during development (Yúfera and Darias, 2007; Russo *et al.*, 2009). In this study, the size of experimental diet was smaller than the mouth gape of croaker. Previous studies suggested that feeding frequency could influence growth and development of fish larvae (Dada *et al.*, 2002; Xie *et al.*, 2011). Here, the feeding frequency was set as 7 times per day according to previous findings to maintain the prey density. Although ingestion rate of large yellow croaker larvae was not measured, visual observation showed that ingestion of larvae in both two microdiet treatments showed no difference with live prey. Rapid leaching of low molecular weight and water-soluble molecules is thought to be a major problem for delivering balanced nutrients by microdiet. Those two factors might lead to low growth and survival rate for early weaning (Langdon, 2003; Muguet *et al.*, 2011).

Larval stage is a critical phase in fish. In this stage fish is passing from endogenous feeding to exogenous feeding. Since then, different nutritions, such as dietary lipid, vitamin and protein, affecting the growth and survival rate of large yellow croaker larvae has been well studied (Ai *et al.*, 2006; Yu *et al.*, 2012). However, few studies were focused on feeding starter diet for early stages larvae of large yellow croaker without live food

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(Wang *et al.*, 2009). Our study showed that the formulated diet Yq was treated as starter diet of croaker larvae and had comparable growth and SGR with Cd, but lower than Lp. And the survival rate did not show significant difference among treatments while Yq had higher survival rate than Cd. More importantly, the price of Yq (about 7 yuan/day tank) was much lower than live prey (about 30 yuan/day tank), which was only about 23.3% of live prey. After the experiment, large yellow croaker larvae were transferred to three cement pools for 7 weeks rearing. The final survival rate of Yq was about 30% which showed no significant difference with live prey. Interestingly, a previous study found that formulated diets for initial feeding of *Plelteobagrus fulvidraco* larvae showed better growth, but higher mortality and higher size hierarchy (Wang *et al.*, 2005). The difference might be due to a better feeding regime and suitable size of diet, also lysine was added extra in Yq diet. As crystalline lysine could be utilized efficiently by large yellow croaker larvae and was reported to affect the growth and survival of this fish (Zhang *et al.*, 2008).

One of the main challenges when formulated diet is used as initial feed for early stages of marine fish larvae is their weak ability to digest microdiets when compared to digest live preys. It was generally accepted that trypsin is the most important proteolytic enzyme in early stages of marine fish larvae (Rønnestad *et al.*, 2013). Tryptic activity was relatively high compared with total alkaline proteolytic activity in laboratory-reared herring (*Clupea harengus*) and turbot (*Psetta maxima*) larvae (Ueberschär, 1993). The same results were observed in the present study and proved that trypsin might be the most important proteolytic enzyme in large yellow croaker larvae at early stages. Phospholipid was nutritionally the most important lipid class in fish larvae. The dietary phospholipid requirement is high during initial feeding of marine fishes (Tocher *et al.*, 2008). The analysis showed that lipase activity was lower than other two digestive enzymes. As was reported, it was difficult to assess the ability of marine fish larvae to digest lipids. Lipase activity has been detected from initial feeding in several species, where it either diminished or remained stable during early larval stage (Izquierdo and Henderson, 1998; Murray *et al.*, 2006; Darias *et al.*, 2007). Ai *et al.* (2008) found suitable concentrations of diary lipid (172-177g/kg) in large yellow croaker larvae based on survival and growth data. Otherwise, α-amylase as a key enzyme for digestion of complex carbohydrates has been determined in larva of several fish species (Cara *et al.*, 2003; Gisbert *et al.*, 2009). The α-amylase maintained a high level after initial feeding that tended to decrease during the following weeks (Lauff and Hofer, 1984; Ma *et al.*, 2005; Alvarez-González *et al.*, 2008). Related researches demonstrated that exogenous enzymes from live prey were of minor significance for the overall digestive capacity of marine fish larvae (Hjelmeland *et al.*, 1988; Ueberschär, 1995). All enzyme activities we tested were considered as endogenously.

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Results of the present study indicated clearly that survival rate of the test diet (Yq) showed no difference with Cd and Lp in large yellow croaker larvae at early stages. However, the total length, height and wet weight of two microdiet treatments were significantly lower when comparing with Lp under the present experimental conditions. Tryptic activity was relatively high between different enzyme activities and was significantly higher in Lp which indicated that Lp was easier to digest for large yellow croaker larvae. Although the present nutritional composition of Yq still had place to improve, it was suitable for large yellow croaker larvae at the early stages. Otherwise, cofeeding of live prey with formulated diet has been proved to be an effective protocol to enhance fish survival during weaning in fish larvae. Therefore, more researches on the larval nutritional requirements are needed to be conducted and the appropriate nutritional composition of this diet for larva needs to be developed.

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