# STUDIES ON THE ROLE OF PEPTIDASE AND POWDER SOYBEAN LECITHIN IN MICROPARTICLE DIETS FOR RED SEA BREAM *PAGRUS MAJOR* LARVAE

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**Abstract**: This study investigates the effects of exogenous enzyme supplementation on the digestibility of microparticle diets (MD). Powder soybean lecithin and gluten were used instead of paste soybean lecithin for evaluating its effect on the leaching rate in MD. Three kinds of MD (MD-T; MD-S; MD-U) were prepared. Based on formulation of MD-S, MD-T included powder soybean lecithin instead of paste soybean lecithin and gluten as a binder. MD-U was supplied with 0.1% of peptidase. The other ingredients were the same in the three diets. Within the first 15 minutes of submersion in water, the leaching rate for MD-T (35.5%) was lower than for MD-S (46.8%) and MD-U (45.8%). The first feeding red sea bream larvae were fed the three above mentioned MD and live food (LF) as a control. The trial lasted until 20 days after hatching. Final survival rate was highest in LF treatment (86.3%), followed by MD-T (20.7%) treatment, which was significantly higher ($P<0.05$) than the results for MD-S (13.3%) and MD-U (13.6%) treatments. Final total length (TL) of larvae fed LF was with 6.14±0.49mm significantly larger ($P<0.05$) than for those fed with MD, which reached at sizes ranged from 4.23±0.30mm (MD-S) to 4.46±0.30mm (MD-T). There were no significant differences in growth among MD treatments. Histological analysis of digestive epithelium of larvae showed thick and well-developed intestine folds at 12 days after hatching in both of LF and MD treatments. However, the intestine epithelium of larvae fed on MD appeared thin and most cell desquamation were observed at 18 days after hatching, indicating limited digestive capacity after 12 days after hatching and led to sharp decline of survival rate in MD treatments thereafter. Daily increments for protein, DNA and RNA as well as the ratio of RNA/DNA were higher in larvae fed LF compared to the respective values obtained for those fed MD. Larvae fed MD-T showed higher value of the above parameters than those fed MD-S and MD-U, suggesting larvae had a better nutritional condition in MD-T treatment than those in MD-S and MD-U treatments. The results indicated that supplementation of peptidase could not facilitate the digestion of MD for red sea bream larvae. However, using powder soybean lecithin and gluten could reduce the leaching rate from MD and consequently improved larval survival and growth.

**Key words**: *Pagrus major* larvae; Microparticle diet; Peptidase; Soybean lecithin

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Development of a MD that could match with LF for marine fish larvae has not yet been achieved, since the digestive tract of larvae is not yet fully developed and the digestive enzyme activity of the pancreas is low during the first month after hatching[1, 2]. It has been suggested that LF donates their digestive enzyme to aid larval digestion process[3—5]. However, Zambonino-Infante et al.[6] and Cahu and Zambonino-Infante[7] reported that digestive enzyme activity in sea bass *Dicentrarchus labrax* larvae fed LF and MD is similar. Kurokawa et al.[8] also showed that the exogenous enzyme derived from zooplankton in the intestine of Japanese sardine *Sardinops melanoticus* larvae less than 1%.

Although it seems that digestive enzyme activity is

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sufficient for digesting LF, there may not be enough for digestion of MD. Particulate MD contains proteins and other ingredients that are difficult for larvae to digest compared with LF[9]. Moreover, MD contains 60%—90% dry matter compared to only 10% in LF. This may lead to insufficient digestibility since it is much harder to break down dry hard particles than LF. In order to overcome this problem, several studies had been conducted to assess the effect of supplementation of dietary enzyme on rearing performance of different fish species[10—13]. Some of these reported positive results on growth and protein utilization while others did not show any effect on fish growth.

On the other hand, nutrient leaching is one of main problem in developing MD for fish larvae, low molecule weight ingredients, such as short peptide and crystal amino acid as well as water-soluble vitamins, are proved readily leached out of MD after few minutes of immersion in water[14]. If this problem cannot be solved, the most nutritive component will be lost before larvae ingested it. As a result larvae will unable to get satisfactory nutritional requirement from MD for its normal survival and growth.

In the present study, the effect of peptidase in MD on survival and growth of red sea bream *Pagrus major* larvae and powder soybean lecithin on the leaching rate of MD were investigated, meanwhile, histological analysis and measurement of protein and nucleic acid contents of red sea bream larvae were carried out for diagnosing the nutritional condition of fish larvae.

## 1 Materials and methods

**1.1 Experimental diets** The formulation and proximate compositions of experimental MDs are shown in Tab. 1. Casein hydrolysate C700 (molecular weight: 30000Da) and C800 (molecular weight: 1000—2000Da) were used as protein source, and the ratio of C700 and C800 was adjusted to 3:7. In contrast, the MD-S, MD-T included powder soybean lecithin and gluten instead of paste soybean lecithin. The MD-U was supplied with 0.1% of peptidase. The other ingredients were the same in the three diets. The manufacture methods of MD were described in our previous study[15]. S-type rotifers *Brachionus rotundiformis* (body length: 117—162μm; body width: 100—121μm) were cultured with *Nannochloropsis oculata* (Nisshin Marine Tech Co., Ltd.). These rotifers were used as LF, which were enriched with DHA Ce oil (Oriental Yeast, Co. Ltd.) prior to being fed to larvae. Two mL of enrichment material was added to 20L of the rotifers culture medium (500 rotifers/ml) at 20℃ for 16h.

**Tab.1 Formulation and proximate composition of the experimental diets (g/100g dry diet) for red sea bream larvae**

| Ingredient(%) | Diets/live food | | | |
|---|---|---|---|---|
| | MD-S | MD-T | MD-U | Rotifers |
| Peptide: C700 | 18.36 | 18.36 | 18.36 | |
| Peptide: C800 | 42.84 | 42.84 | 42.84 | |
| Fatty acid-Ca of fish oil | 25.60 | 25.60 | 25.60 | |
| Arginine | 0.40 | 0.40 | 0.40 | |
| Cystine | 0.90 | 0.90 | 0.90 | |
| Taurine | 1.40 | 1.40 | 1.40 | |
| Mineral mixture† | 2.00 | 2.00 | 2.00 | |
| *Spirulina* | 1.00 | 1.00 | 1.00 | |
| Vitamin E | 0.10 | 0.10 | 0.10 | |
| Soybean lecithin (paste) | 5.00 | — | 5.00 | |
| Soybean lecithin (powder) | — | 3.00 | — | |
| Choline chloride | 0.80 | 0.80 | 0.80 | |
| Vitamin C‡ | 0.10 | 0.10 | 0.10 | |
| Vitamin mixture§ | 1.50 | 1.50 | 1.50 | |
| Gluten | — | 2.00 | — | |
| Peptidase δ | — | — | 0.10 | |
| Proximate composition (On dry matter basis) | | | | |
| Crude protein(%) | 58.0 | 59.1 | 58.1 | 57.6 |
| Crude lipid(%) | 19.4 | 18.0 | 21.6 | 14.7 |
| Crude ash(%) | 10.3 | 10.7 | 10.4 | 11.9 |

†Ogino and Yang[16]; ‡Phospitan C; §Ogino et al[17]; δDonated by Amano Enzyme Inc, Nagoya, Japan

**1.2 Eggs and larvae** Fertilized red sea bream eggs were provided by the Owase Sea-Framing Center, located in Mie prefecture, Japan. They were transported to the National Institute of Aquaculture, Fisheries Research Agency. Fertilized eggs were incubated at 20℃ in 500L transparent cylindrical tank. Continuous aeration was provided and water flow rate was maintained at 70mL/min throughout the incubation. Period.

**1.3 Experimental design and larval rearing** Four diets were tested including live food (LF), microparticle diet T (MD-T), microparticle diet S (MD-S) and microparticle diet U (MD-U). Two thousands newly hatched larvae were randomly divided into 8 rearing tanks (100L) (4 treatments with 2 replicates). In addition, 50 larvae were stocked in 500mL vessels (duplicates) to monitor tolerance of larvae to starvation.

Each tank was supplied with sand-filtered seawater from the Gokasho Bay, Mie Prefecture, Japan. The water flow rates were adjusted to 1—2 turns-over per day in LF treatment and 2—6 turns-over per day in MD treatments during the feeding experiments, respectively. Water temperature was maintained at 20—20.5℃ using a controlled heating system. Rearing tanks were illuminated by fluorescent light sources to provide 12L: 12D photoperiod with light intensity between 250—400 lx at the water surface. Mild aeration was supplied via one air stone in each tank. MD treatments were fed daily one time per hour between 08:00 and 17:00 hours by hand, increasing size of particles were fed to the larvae as they grew. Daily ration was 1.5—2.0g per tank. Larvae in LF treatment were offered rotifers *Brachionus rotundiformis* twice daily at 09:00 and 14:00 hours, Rotifers were maintained at a density of 5—10 ind. /mL in the rearing tank. During the experimental period, rearing tanks were cleaned and siphoned daily and dead larvae were removed and counted. In order to collect enough fish sample for analyses of protein and nucleic acid, a parallel feeding trail with a 500L tank stocked with 10000 larvae was use for every MD treatment. The rearing conditions and methods were kept identical to those in the 100L tanks. The feeding trial was terminated at 20 days after hatching.

**1.4 Sampling** Thirty larvae per tanks (100L) were sampled at day 1, 5, 10, 15, and 20 in order to monitor growth. Histological samples were taken at 12 days after hatching and 18 days after hatching, respectively. They were immersed in Bouin's fixative for 24h and then preserved in 70% ethanol for later histological analysis. At the end of the feeding trial, larval survival in each tank was determined by counting remaining individuals, and the total length of 30 larvae from each tank was measured. Remaining fish were washed thoroughly with tap water and allowed to remain on tissue paper to drain off excess water. They were then immediately frozen and stored at −80℃ until analysis.

Larvae reared in 500L tanks were sampled at day 3, 7, 10, 13, 16, 20 for nucleic acid analysis, three hundreds larvae were sampled, rinsed with distilled water, and pipetted into vinyl bags, and immediately frozen at −80℃ for later analysis.

**1.5 Analytical methods** The leaching rates of soluble nitrogen from MD were measured according to the method described in our previous study[18]. For histological analysis, larval samples were embedded in paraffin and sagittal sections were cut from the blocks. The sections were stained with Mayer's hematoxylin-eosin. A modification of the Schmidt-Thannhauser-Schneider method was used for nucleic acid analysis as described by Nakano[19]. DNA and RNA contents are both expressed as μg / fish. Total protein content was determined using a Bio-Rad protein kit (Bio-Rad, Tokyo, Japan). Results are expressed as μg of protein per fish.

The daily growth rate for nucleic acids and protein were calculated using the following formula[20]:

$$\text{Daily growth rate } (\%/\text{day}) = [10^{\log W_n + t - \log W_n / t} - 1] \times 100$$

Where, $W_n$ is the nucleic acids and protein content at time $n$ and $t$ is the interval in days.

**1.6 Statistical analysis** Growth and survival rate data were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests to determine differences among means. All the statistical analysis was performed with the software SPSS 10.0 for Windows.

## 2 Results

### 2.1 Leaching rates from MD

Leaching rates from MD, were presented as the loss of water-soluble nitrogen, and the results are shown in Fig.1. The initial soluble nitrogen concentration of MD-

T, MD-S and MD-U were 9.0%, 8.7% and 8.8%, respectively, on which the percentages leaching are based. The leaching rate from MD-T was lower than from both MD-S and MD-U, which both behaved similarly within 15 minutes, whereas, the leaching rate from MD-S increased thereafter when compared with MD-U.

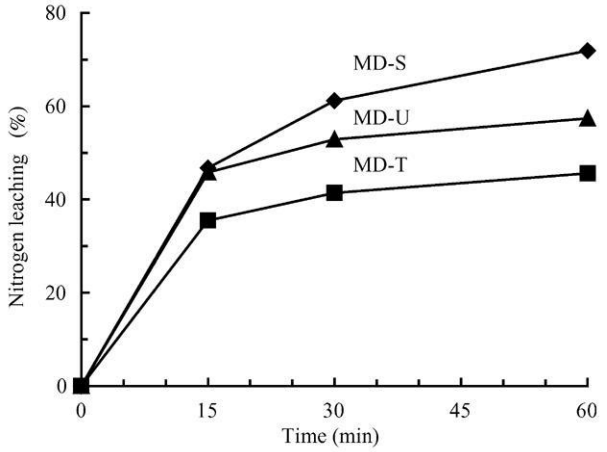


Fig. 1 Leaching rates of nitrogen from microparticle diets. MD-S, microparticle diet S; MD-T, microparticle diet T; MD-U, microparticle diet U. Values are means of duplicate experiment, n=2

## 2.2 Survival and growth

Changes in survival and growth are shown in Figs. 2 and 3, respectively. Larvae fed MD showed relatively good survival rate ranging from 71.4% to 76.5% at 10 days after hatching, although survival was considerable lower when compared with the series fed LF (91.5%). Most of the mortalities in MD treatments occurred from 12 days after hatching onward to the end of the experiment. Final survival rate was highest in the LF treatment. In MD treatments, survival rate was significantly higher ($P<0.05$) in larvae fed MD-T (20.7%) than those fed MD-S (13.3%) and MD-U (13.6%). There were no significant differences in survival rate between MD-S and MD-U treatments. Unfed (starved) larvae died completely by 8 days after hatching.

No significant differences in growth were detected among all treatments until 5 days after hatching. The growth of larvae fed LF showed an overall increase from 5 days after hatching and reached 6.14±0.49mm of total length at the end of the experiment. The growth of larvae fed MDs was very low and only attained final mean TL ranged from 4.23±0.30mm to 4.46±0.30mm. There were no significant differences in the growth rates among MD treatments until the end of the experiment.

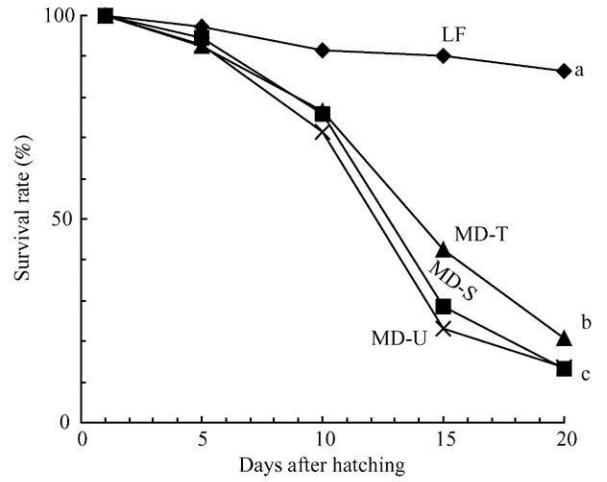


Fig. 2 Survival rate of larval red sea bream fed LF and MD. LF, live food; MD-S, microparticle diet S; MD-T, microparticle diet T; MD-U, microparticle diet U. Initial number of larvae per tank were two thousands. Results are means of duplicate experiments. Letters denote significant differences between the diets ($P<0.05$)

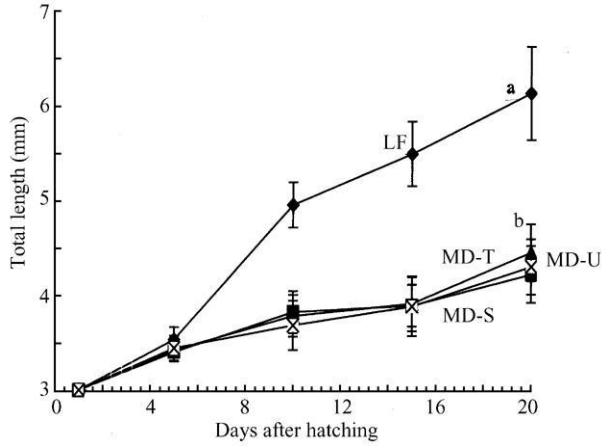


Fig. 3 Growth of larval red sea bream fed LF and MD. Abbreviations were shown in Fig. 2. Values are means±S.D. from two replicates, n=2. The bars represent standard deviations. Letters denote significant differences between the diets ($P<0.05$)

## 2.3 Histological observation

The development of the digestive epithelium of larvae, fed with LF and MDs at the first feeding, is shown in Fig. 4. Microscopic observation of samples taken from histological sections indicated that larvae fed MDs showed thick and well-developed intestine folds at 12 days after hatching similar to those seen in LF-fed larvae. However, the intestine folds of larvae fed MDs appeared somewhat deteriorated

or started to vanish towards 18 days after hatching, indicating the limited digestive capacity of this epithelium thereafter. In contrast, larvae fed LF showed excellent quality of the digestive tissue at 18 days after hatching.

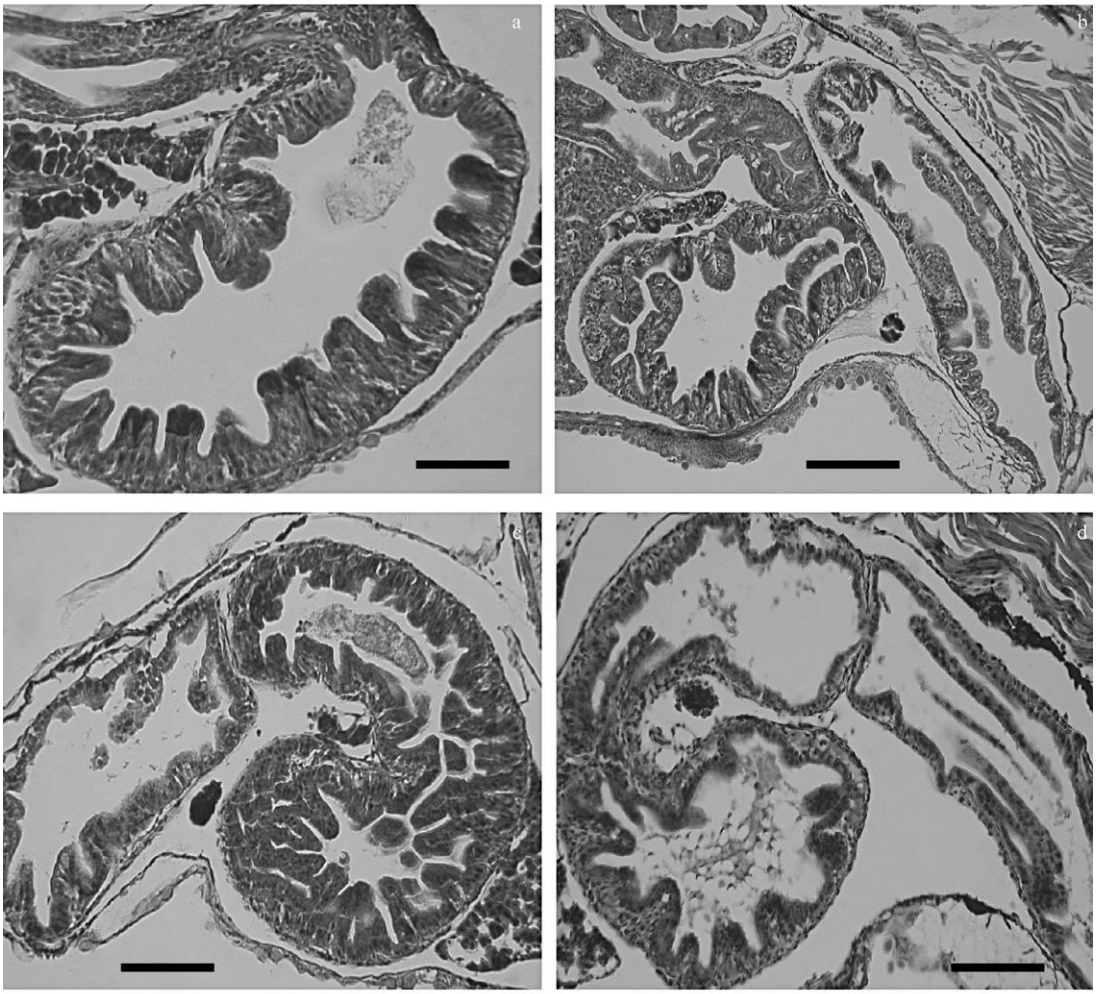


Fig. 4 Photomicrographs showing the digestive tract of red sea bream larvae fed on LF and MD-T at different ages. LF, live food; MD-T, microparticle diet T. Bar= 100μm
a) Larvae fed LF (12 days after hatching); b) Larvae fed LF (18 days after hatching);
c) Larvae fed MD-T (12 days after hatching); d) Larvae fed MD-T (18 days after hatching)

## 2.4 Changes of protein and nucleic acid contents

The values for daily growth rates of protein, DNA and RNA of larvae fed LF were all higher compared to those of fed MDs (Fig. 5). Among MD treatments, larvae fed the MD-T had the highest daily growth rate compared to any of the others, while the performance using MD-U was still somewhat better than the results achieved with diet MD-S.

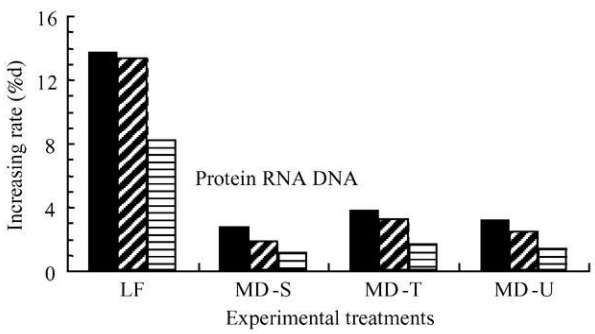


Fig. 5 Calculated averages daily rate of increase of protein, DNA and RNA contents of larval red sea bream raised for 20 days on various diets. Abbreviations were shown in Fig. 2

The value for the RNA/DNA ratios for red sea bream larvae that fed LF and MDs are given in Fig. 6. RNA/DNA ratio for larvae fed LF showed an overall increase from 0.76 at the onset of the exogenous feeding up to 1.64 at the end of the experiment. The same ratios for larvae fed MD-T and MD-S were slight lower at 3 days after hatching and 7 days after hatching, but gradually increased until

10 days after hatching. In contrast, the ratio for larvae fed MD-S showed a slight but continuous increase of the ratio from 3 days after hatching to 10 days after hatching. Thereafter, values remained at a stable level in both MD-U and MD-S treatments reaching final values of 0.91 and 0.86, respectively. In contrast, the RNA/DNA ratio for larvae receiving the MD-T stayed at a stable level until 13 days after hatching, and gradually increased thereafter again until 16 days after hatching, remaining constant thereafter while attaining a final value of 1.01.

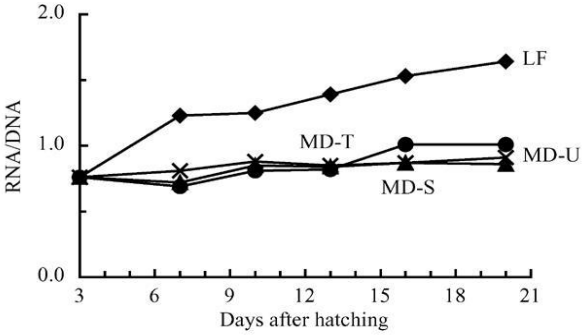


Fig. 6 Age-specific RNA/DNA ratio of larval red sea bream fed LF and MD. Values represent the average of triplicate assays for each treatment. Abbreviations were shown in Fig. 2

## 3 Discussion

The present study demonstrated that the supplementation with powder soybean lecithin and gluten instead of using paste soybean lecithin can assist improving the stability of MDs. Differences in survival between MD-T and other MDs may be related to reduced leaching rates of water-soluble nutrients. The results of the present study agreed with the findings of earlier studies that supplementation with exogenous enzymes had not positive effects on larval growth and survival[12, 13]. However similar studies demonstrated positive results on larval growth and protein utilization[11, 21]. Several factors such as fish age, species and type of the dietary enzymes as well as food habits may play an important role in producing these results. It has been suggested that digestive enzymes at first feeding larvae may not be sufficiently available in early larvae for effective digestion of MDs[22, 23]. Therefore, the idea to provide exogenous enzyme via feed may either show that this working hypothesis is not valid or indicates that higher concentrations of endogenic digestive enzymes are rapidly activated in red sea bream larvae immediately after feeding starts. Therefore, the level of exogenic digestive enzyme in MD to exert an positive effect on red sea bream larval growth may need to be higher[12] or not needed at all.

The nutritional conditions and growth of larval fish have been determined by measuring RNA, DNA and protein contents[24, 25]. The quantity of DNA (as an index of cell number) is considered to be constant in somatic tissues even under a fluctuating environmental condition[26]. However, the quantity of RNA (as an index of protein synthetic capacity of a cell) is directly proportional to protein synthesis and thus to nutrition. Because larval growth is dependent upon protein synthesis, the RNA/DNA ratio has proven to be a useful indicator of the nutritional condition and subsequent fish growth[27, 28]. The present results showed that RNA/DNA ratio of larvae fed MDs were notable lower than those fed with LF. Some studies have indicated that well-fed and fast growing larvae have higher RNA/DNA ratios and wider daily increment on their otoliths than starving larvae[29—31]. Since high percentage of larvae accepted the MD (data not shown) in the present study, the low survival and slow growth in MD treatments could possibly be explained by the low protein synthesis which in turn was probably associated with either leaching of nutritive material out of the pellets, or with poor digestion and assimilation of the MDs.

Takii et al.[32] reported that red sea bream larvae, which suffered a high mortality and achieved a poor growth by 45 days after hatching, showed a low RNA/DNA ratio that was close to two. In the present study, we observed that RNA/DNA ratio for larvae fed MD were close to one by the 20 days after hatching, at which larval survival rates drastically dropped to a range between 13.3 and 20.7%. However, larvae fed LF, which has RNA/DNA ratio of 1.6 by 20 days after hatching, showed a high survival rate of 86.3%. Sato et al.[33] reported that mean RNA/DNA ratios in unfed Japanese sardine larvae were 1.2 after 3 days of starvation. Clemmesen[34] also indicated that RNA/DNA ratios decrease to about 1 by starvation for 7 days to 2 weeks in various species. As mentioned above, although red sea bream larvae were not deprived of food, larvae fed MDs as well as starved larvae had a declining protein synthesis capacity most likely due to poor digestion of MDs. Therefore, we regard a value

close to 1.0 as an index that the nutritional point of no return for red sea bream larvae has been reached and that such index can be used in evaluating rearing effectiveness with MDs for first feeding larvae.

The findings are supported by the histological observation on the intestine epithelium in larvae fed MDs and this was most obvious during 12—18 days after hatching. It is suggested that larvae were not able to digest food well and did not effectively absorbs the nutritional content of the MD to support their growth and survival and consequently large mortalities in MD treatments resulted during this period.

This study focused mainly on the question of leaching and supplemental enzyme effects. Although the overall results provide good evidence, the absolute values for each of the treatments may not be as accurate as measured. This may simply be due to the fact that the digestible energy basis may have varied slightly between the various preparations, causing their own effects. Nevertheless, the trends observed are certainly useful to support the answers given to the scientific working hypothesis of the study.

In summary, this study demonstrated that supplementation of peptidase into MDs could not facilitate the digestion of MDs and did not convincingly improve survival and growth of red sea bream larvae. Substitution of paste soybean lecithin by powder soybean lecithin and gluten may have reduced leaching of nutritive components and consequently improved larval growth performance. The efficiency of MD for larvae could also be evaluated by biochemical method through measuring protein and nucleic acids content. The present study showed that the frequently used index (RNA/DNA ratios) of the point of no return for red sea bream larvae rearing on the MDs from first feeding were about 1.0.

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# 真鲷仔鱼用微粒子饲料中肽酶与大豆卵磷脂添加效果的研究

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摘要: 本研究以酪蛋白分解物为蛋白源配制三种微粒子饲料MD-S、MD-T和MD-U对真鲷开口仔鱼进行饲养试验。以MD-S的配方为基准, MD-T采用粉状大豆卵磷脂和麸质代替液状大豆卵磷脂; MD-U则另外添加0.1%的肽酶。结果表明, 微粒子饲料在水中浸泡15min后, MD-T的溶出率(35.5%)低于MD-S(46.8%)和MD-U(45.8%); 实验结束时(20日龄), 仔鱼的成活率以生物饵料(轮虫)组为最高(86.3%), 其次是MD-T组为20.7%, 显著高于($P<0.05$) MD-S组(13.3%)和MD-U组(13.6%); 生物饵料组的仔鱼全长(6.14±0.49mm)显著大于微粒子饲料组(4.23±0.30mm~4.46±0.30mm), 各微粒子饲料组之间仔鱼的全长并不存在显著差异($P>0.05$)。在孵化后第12d, 微粒子饲料组的仔鱼肠上皮细胞发育良好, 但至孵化后第18d, 仔鱼肠上皮细胞大部分萎缩、并发生脱落。鱼体的蛋白质、DNA与RNA日间增长率微粒子饲料MD-T组高于MD-S和MD-U组, 但都低于生物饵料组。由此可见, 微粒子饲料中添加肽酶并无助真鲷仔鱼对其消化吸收; 可是, 使用粉状大豆卵磷脂与麸质代替液状卵磷脂能增强微粒子饲料的黏合性, 可减少其营养成分的溶出率, 从而提高微粒子饲料的饲育效果。

关键词: 真鲷仔鱼、微粒子饲料、肽酶、大豆卵磷脂