SCREENING OF POTENTIAL FEEDING STIMULANTS FOR MARBLE GOBY, *Oxyeleotris marmoratus*

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ABSTRACT

The main objective of present study was to screen amino acids as the potential feeding stimulants for marble goby, *O. marmoratus*. Two separated experiment were conducted. In the first experiment, the stimulatory effect of single and mixtures of amino acids on the gustatory sense of *O. marmoratus* were determined by behavioral method. Amino acids were incorporated into the agar gel pellets, fed to the fish and the pellets acceptance ratios were recorded. For single amino acids, L-alanine attained the highest acceptance ratio followed by L-glutamine, L-glycine, and betaine. On the other hand, L-cysteine, L-glutamic acid, L-lysine, L-serine and taurine were totally rejected. The function of betaine, which is a derivative amino acid was studied to determine whether or not it can enhance the feed intake of essential amino acids mixture (EAAM). Betaine and EAAM were originally poorly accepted by the fish, with 0.093 and 0.19 respectively. However, when both of them were mixed, the acceptance ratio of betaine+EAAM agar gel pellet was improved, which was 0.374 although no significant different, indicated that betaine was taste indifferent but it enhanced the palatability of EAAM. Therefore, betaine can be used as feed enhancer for juvenile *O. marmoratus*. Non-essential amino acids mixture (NEAAM) achieved the highest acceptance ratio with value of 0.944, suggesting that NEAAM may be the better feeding stimulant compared to EAAM. Based on these results, a prototype feeding stimulant was formulated. In the second experiment, the effectiveness of this prototype feeding stimulant in shortening the pellet-weaning period of wild-caught juvenile *O. marmoratus* was evaluated. Twelve wild-caught fish (BW=4.13 g to 8.01 g; BL= 7.7 cm to 9.3 cm) are divided into 2 groups equally, each group consisted of 6 fish where each fish was kept separately in aquarium. Two diets of protein and lipid levels were 48 % and 7.8 % respectively were formulated, namely, the fish meal (FM) diet and fish meal + prototype feeding stimulant (FM+FS) diet, and were fed to the fish for 14 days. The fish in the FM+FS treatment was successfully weaned in the shorter period (during first week) than those in the FM treatment (during second week). Such result confirmed that the prototype feeding stimulant was effective in shortening the pellet weaning period of the juvenile *O. marmoratus*.

ABSTRAK

Objektif utama kajian ini ialah mengenalpasti asid amino yang berpotensi sebagai peransang makanan untuk ikan ketutu, *O. marmoratus* juvana. Dua eksperimen telah dijalankan. Dalam eksperimen pertama, kesan peransangan asid amino tunggal dan campuran terhadap deria gustatori telah dikenalpasti melalui kaedah perlakuan ikan. Asid amino ditambah ke dalam pelet gel agar-agar dan diberi makan kepada ikan. Nisbah penerimaan direkodkan. Untuk amino asid tunggal, L-alanine mencapai nisbah penerimaan tertinggi, diikuti dengan L-glutamine, L-glycine dan betaine. L-cysteine, L-glutamik asid, L-lysin, L-serine dan taurine pula tidak diterima sepenuhnya. Fungsi betaine yang merupakan derivative asid amino, telah dikaji untuk mengenal pasti adakah ia mampu meningkatkan pengambilan campuran asid amino yang diperlukan (EAAM). Betaine dan EAAM yang mulanya mencapai nisbah penerimaan yang rendah, iaitu 0.093 dan 0.19 masing-masing, setelah kedua-duanya dicampurkan, nisbah penerimaan untuk pelet betaine+EAAM dipertingkat, iaitu 0.374 walaupun tiada perbezaan siknifikasi. Keputusan ini memperlihatkan betaine ialah bahan bukan perangsang makanan, tetapi ia telah meningkatkan kelazatan EAAM. Oleh itu, betaine boleh digunakan sebagai bahan peningkat kelazatan untuk *O. marmoratus* juvana. Campuran asid amino yang tidak diperlukan (NEAAM) memperoleh nisbah penerimaan yang tertinggi dengan nilai 0.944, dicadangkan NEAAM merupakan perangsang makanan yang lebih bagus daripada EAAM. Berdasarkan keputusan tersebut, satu prototaip perangsang makanan telah dihasilkan. Dalam eksperimen kedua, keberkesanan prototaip perangsang makanan dalam menyingkatkan tempoh melatih *O. marmoratus* juvana tangkapan liar memakan pelet telah dikaji. 12 ikan tangkapan liar (berat badan: 4.13 g-7.99 g; panjang keseluruhan badan: 7.7 cm-9.3 cm) telah dibahagi kepada dua kumpulan, 6 ikan dalam satu kumpulan, dengan setiap ikan diasingkan dalam aquarium masing-masing. Dua jenis diet (tahap protein: 48 %; tahap lipid: 7.8 %) telah diformulasikan, iaitu diet tepung ikan (FM) dan diet tepung ikan + prototaip perangsang makanan (FM+FS), diberi makan kepada ikan selama 14 hari. Kumpulan ikan diet FM + FS telah berjaya dilatih dalam tempoh yang lebih singkat (dalam minggu pertama), berbanding dengan kumpulan ikan diet FS (dalam minggu kedua). Keputusan sebegini mengesahkan bahawa prototaip perangsang makanan adalah berkesan dalam memendekkan tempoh melatih *O. marmoratus* juvana liar memakan pelet.

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°C	Degree Celsius
%	Percentage
AOAC	Association of Analytical Communities
BW	Body weight
cm	Centimetre
CMC	Carboxyl methyl cellulose
CMFF	Commercial Marine Finfish
DM	Dry matter
EAAM	Essential amino acids mixture
FM	Fish meal
FM+FS	Fish meal + feeding stimulant
g	Gram
L	Litre
mg/L	Milligram per Litre
ml	Millilitre
mm	Millimetre
NEAAM	Non-essential amino acids mixture
pH	Potential of hydrogen
TL	Total body length

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CHAPTER 1

INTRODUCTION

1.1 Marble goby, *Oxyeleotris marmoratus*

In Malaysia, marble goby (*Oxyeleotris marmoratus*) is known as ikan ubi, ketutu, betutu and ikan hantu in malay language, "soon hock" in the Hakka and Cantonese dialects of the Chinese community. In Sabah, wild *O. marmoratus* are usually harvested from its natural habitat such as paddy field, river and freshwater reservoirs. However, in peninsular Malaysia, this fish were rarely harvested from the wild as the natural sources of *O. marmoratus* have been exhausted (Senoo *et al.,* 1994). Since early 1980's, *O. marmoratus* has been targeted as an aquaculture species in Malaysia and the artificial reproduction has been succeeded (Cheah *et al.,* 1994; Senoo *et al.,* 1994).

Apart from Malaysia, *O. marmoratus* is well known in the aquaculture industry of other Asian countries, such as China, Indonesia, Taiwan and Thailand. In the year of 2000, the total production of marble goby in Malaysia, Thailand and Singapore was about 207 tonnes, which had contributed 74% of the total global production of goboid fish (GALE, 2005). It has high market value and demands mainly due to its lean, firm and boneless flesh (Sompong, 1980). However, the global production of *O. marmoratus* is very low if compared to other farmed freshwater species, such as African catfish, *Clarias gariepinus* and red tilapia, *Oreochromis niloticus. O. marmoratus* has slow growth under rearing condition especially in tank. It has to be cultured in low stocking density and well-aerated condition which causing the production cost to become comparatively high (FAO, 2008). This species exhibits slow eating habits, lower metabolism rate and lower growth rate as compared to other cultured species (Kottelat, 2001). Several studies had been done in favor to provide crucial information for improving the culture techniques and production of *O. marmoratus* (e.g. Tavarutmaneegul and Lin, 1988; Senoo *et al.*, 1994; Amornsakun *et al.*, 2003; Lin *et al*, 2004; Van *et al.*, 2005; Abol-Munafi *et al.*, 2006; Darwis *et al.*, 2008; Senoo *et al.*, 2008; Chew *et al.*, 2010; Idris *et al.*, 2012). However, there is still lacking of commercial diet formulated for *O. marmoratus* up to date.

Lack of suitable artificial pellet for *O. marmoratus* is one of the constraints in the culture of *O. marmoratus*. Mass mortality usually occurred during the pellet-weaning period because many fish rejected most of the self-formulated feeds and died in starvation; it is not easy to invent a successful artificial feed for marble goby, because the fish rejected most of the self-formulated feed (Bundit, 2007; Rojtinnakorn *et al.*, 2012). In such situation, supplementing with suitable feeding stimulant in the formulation of diet can be helpful in solving this problem (Kubitza *et al.*, 1997).

1.2 Feeding Stimulants

Feeding stimulants are substances which can promote the fish to ingest food. Supplementation of feeding stimulant into diets hence can promote the feed intake by the fish (Kasumyan and Døving, 2003).

Usually, chemical substances including amino acids, nucleotides, nucleosides, quaternary amines, organic acids and others were shown to evoke stimulatory effect on a number of fish species (Carr and Derby, 1986; Marui and Caprio, 1992; Kasumyan and Døving 2003). Among these chemical substances, amino acids are the most commonly used feeding stimulant for aquatic species, but the preference of fish towards different amino acids is species-specific. For instance, tryptophan was highly stimulating to *Trachunus japonicus* (*Ikeda et al.*, 1988), L-alanine is the most palatable for common carp, *Cyprinus carpio* (Kasumyan and Morsi, 1996), whereas L-leucine and L-isoleucine were stimulants for rainbow trout, *Salmo gaidneri* (Jones, 1989). However, which amino acid can be the potential feeding stimulant for *O. marmoratus* is still an unknown.

Betaine is a unique type of amino acid derivative, which can function as feeding stimulant, feed enhancer or taste indifferent substance. It always elicits positive synergistic properties with different types of amino acids in many fish species. For example, in some species including atlantic cod (*Gadus morhua*), rainbow trout (*Onchorhyncus mykiss*), gibel carp (*Carassius auratus gibelio*) and Dover sole (*Solea solea*), betaine alone was a taste-indifferent substances as it did not result in the increase of feed intake, but it can enhance the feed intake when it was mixed with other L-amino acids which were feeding stimulants (Mackie *et al.*, 1980; Jones, 1989; Johnstone and Mackie, 1990; Franco *et al.*, 1991; Dias *et al.*, 1997; Papatryphon and Soares, 2000; 2001; Xue and Cui, 2001; Yamashita *et al.*, 2006).

To date, it is unknown that how is the effect of betaine to the gustatory sense of *O. marmoratus*. Therefore, in the experiment 1 of present study, 9 single amino acids and 2 mixtures of amino acids were screened for *O. marmoratus*. Betaine alone and betaine + amino acids mixture were also tested on *O. marmoratus*, to study whether it can function as a feeding stimulant, feed enhancer or it is taste-indifferent to the fish.

1.3 Behavioral Method to Study Taste Preference of *O. marmoratus*

Behavioral approach is one of the methods to study taste preference of fish through observation on the feeding responses of fish towards the offered test substance, which the feeding response generally classified into approach, capture, rejection and ingestion (Linstedt, 1971; Mearns *et al.*; Jones, 1989; Stradmeyer, 1989).

In the present study, behavioral method was chosen to study the taste preference of *O. marmoratus* for amino acids. *O. marmoratus* is slow in motion and less active which eased the observation on the behavior of the fish. Hence, it was suitable to study their feeding behavior with the observation method. Two feeding behavior actions, which were the food rejection and ingestion, were the key observation points to determine the acceptance level of the test substances by *O. marmoratus*.

1.4 Feeding Stimulant Used for Pellet Weaning

Juvenile fish can often weaned successfully to moist or dry artificial diet. Palatability of the diet is among the prime factors attribute to the success in weaning (Kubitza and Lovshin, 1999). During the fish weaning to formulated diet, palatable ingredients were always used to improve flavor and attractiveness of the diet, which resulted in improvement in feed ingestion. Inclusion of feeding stimulants into formulated diet was revealed to attain higher weaning success by increasing the acceptance and ingestion of the diet, just like in the cases of the early weaning of Barramundi (*Lates calcarifer*) larvae and largemouth bass (*Micropterus salmoides*) larvae (Kubitza *et al.*, 1997; Curnow, 2006). Therefore, in the second experiment of the present study, a prototype feeding stimulant was formulated based on the result of experiment 1 and its effectiveness in shortening the pellet-weaning of wild-caught juvenile *O. marmoratus* was evaluated.

1.5 Objectives of Present Study

The objectives of the present study included:

1. To determine the suitable amino acids as potential feeding stimulant for marble goby, *O. marmoratus*.

2. To determine the function of betaine to the gustatory sense of *O. marmoratus*.

3. To evaluate the effectiveness of the prototype feeding stimulant in shortening the pellet-weaning period of the wild-caught juvenile *O. marmoratus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Biological Features of Marble Goby, *Oxyeleotris marmoratus*

Marble goby, *O. marmoratus* is the largest freshwater fish in the family Eleotridae (Kottelat, 2001). This species can grow up to 50 cm and achieves sexual maturity around 7cm in body length. However, the maximum body length of wild-caught *O. marmoratus* which ever recorded was 65 cm. *O. marmoratus* has an elongated, brownish body covered with long dark blotches and dusty bands. Its body may sometimes changes into paler or darker when the light intensity of its surrounding changes. The obvious difference between the true goby and marble goby is that the true goby has cup-like pelvic fins which joined together to form a sucker, whereas the marble goby has separated pelvic fins; marble goby is known as sleeper goby (Bundit, 2007). In the wild, *O. marmoratus* can be found in rivers, swamps, reservoirs and canals along the Mekong and Chao Phraya Basins, Malaysia, Indochina, Philippines and Indonesia (Kottelat *et al.,* 1993). This fish is carnivorous as it feeds mainly on macrofauna such as prawns, benthos, small fish, crabs and aquatic insects (Yap, 1988; Robert 1993). It is inactive which likes to stay at the bottom of water body motionlessly for a long time. It is a bottom-feeder fish which always half-bury in the substrate, waiting patiently to ambush the passing–by prey (Kelvin and Peter, 2002). Its feed consumption is greatly influenced by the seasonal availability of prey (Vu *et al.,* 2005).

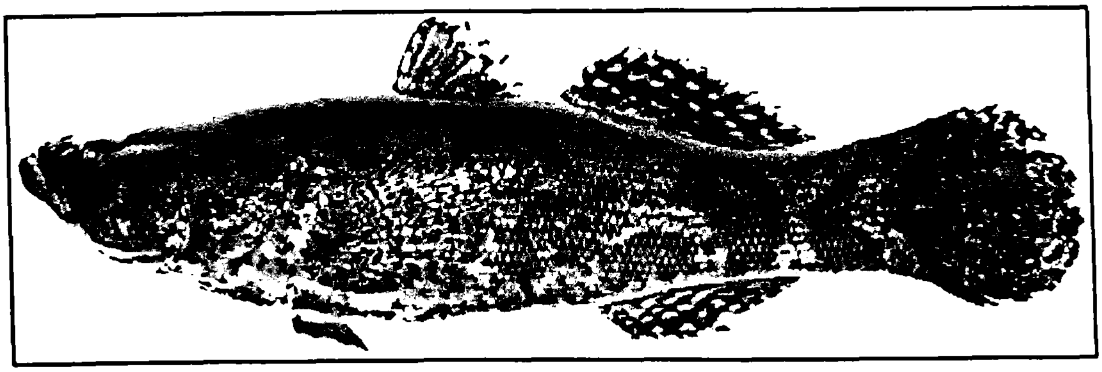

Figure 2.1 The image of marble goby, *O. marmoratus.*

2.2 Betaine as Feeding Stimulant/ Feeding Enhancer

Betaine is a non-toxic amino acid derivative (Kettunen *et al.*, 2001), which is also known as glycine betaine or trimethylglycine. It is found in abundance within marine invertebrates (Meyers, 1987), plants and microorganism. It is also contributes a major components in the diet of marine carnivorous fish (Konosu *et al.*, 1966). It is extremely water soluble and diffusible. The roles of betaine within the aquatic animals are helping in the methyl group donation, osmoregulation, prevents unusual water loss, prevent enzyme inhibition and so on.

The gustatory systems of many fish species were found to be very sensitive towards betaine (Yoshii *et al.*, 1979; Goh and Tamura 1980b; Hara *et al.*, 1999). Since betaine is found in great amount in fish food organism which are normally eaten by the carnivorous fish, the adding of betaine into fish feed will probable create the taste or the smell of the nature prey organism. Previous studies revealed that betaine can function as feeding stimulants, feed enhancer or taste-indifferent substance in different fish species (Table 2.1). Yet, betaine had not found to elicit negative feeding response in fish species (Jones, 1989; Duston, 1993; Harpaz, 1997).

Betaine performs synergistic effect when combined with other amino acids. It can improve the feed intake of a fish although it is not the feeding stimulant for the fish. This means the betaine acts as feeding enhancer which increases the palatability of other ingredients within a feed causes the fish to increase their feed intake. Such situation can be seen in the study of Mackie (1982), who found that for plaice, the exclusion of betaine which was not a feeding stimulant for plaice from the synthetic squid mixture, can totally terminate the activity of the other non-amino acid groups. As in the study of Goh and Tamura (1980b), the palatability of glycine or alanine for red sea bream improved after adding the taste-indifferent betaine into the diet. Carr and Chaney (1976), Carr *et al.* (1977) and Carr (1982) discovered that a solution containing either betaine alone or the amino acids alone was only 2 % to 9 % as effective as the extracts of flat-head grey mullet and blue crab for pinfish and pigfish, but the mixture of betaine with amino acids was at least 9 times as effective as any of the other components within the extract used alone. The examples of the functions of betaine were summarized in Table 2.1.

Table 2.1 Examples of the different functions of betaine on different fish species.

Species	Substances	Functions	References
Turbot (*Scophthalmus maximus*)	Betaine + trimethylamine	Taste-indifferent	Mackie and Adron (1978)
Red seabream (*Pagrus major*)	Betaine	Taste-indifferent	Goh and Tamura (1980a)
	Betaine+ alanine and glycine	Feed enhancer for amino acids	
Pigfish (*Orthopristis chrysopterus*)	Betaine + amino acids	Feeding stimulant	Carr and Chaney (1976); Carr *et al.* (1977); Carr (1982)
Pinfish (*Lagadon rhomboids*)			
Puffer (*fugu perdalis*)	Betaine	Feeding stimulant	Hidaka (1982)
Plaice (*Peuronectes platessa*)	Betaine + amino acids	Feeding stimulant	Mackie (1982)
Chinook salmon (*Onchorynchus tshawytscha*)	Betaine	Taste-indifferent	Hughes (1991, 1993)
Dover sole (*Solea solea*)	Freeze dried diet + Betaine	Feeding stimulant	Mackie and Mitchell (1980b)
Jack mackerel (*Trachurus japonicus*)	Betaine+ trimethylamine oxide	Taste-indifferent	Ikeda *et al.* (1988)
Rainbow trout (*Onchorynchus mykiss*)	Betaine	Taste-indifferent	Jones (1997)
Largemouth bass (*Micropterus salmoides*)	Betaine	Taste-indifferent	Kubitza *et al.* (1997)
Pike perch (*Sander lucioperca*)	Betaine+biomar	Feeding stimulant	Yilmaz and Ablak (2003)

2.3 L-Amino Acid as Feeding Stimulant

Amino acids are the basic units which build up a protein molecule. Amino acids are needed by living organism for maintenance, growth, reproduction and strengthen immunity. In aquatic animals, amino acids are needed to perform other vital roles, included help in pigmentation, appetite regulation, osmoregulation, anti-oxidative defense, ammonia removal, endocrine system regulation, metabolic regulation, protein synthesis, cell signaling, metamorphosis and energy provision.

Amino acids generally can be classified into two major classes, which are the essential amino acid (EAA) and non-essential amino acid (NEAA). EAAs are obtained by the animals through the diets as they cannot be synthesized or are synthesized insufficiently *de novo* by animals relative to requirements. NEAAs are referred to the amino acids which can be synthesized adequately by the animals. There are 10 types of EAA for fish and other aquatic animals: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. NEAAs for aquatic animals include the alanine, asparagine, aspartate, glutamate, glycine, serine and tyrosine.

Amino acid exists in two types of enantiomers, known as the L-amino acids or D-amino acids. They are the mirror image of each other. The taste properties of amino acids are highly stereospecific. L-amino acids are found to be highly palatable for certain fish species, but the corresponding D-amino acids might be deterrent or indifferent for the same fish species. These phenomena had been revealed in rainbow trout (Adron and Mackie, 1978), plaice (Mackie, 1982), European sea bass and European eel (Mackie and Mitchell, 1983). Examples of L-amino acids which had been identified to induce positive stimulatory effect on the feeding response of fish are listed in the table 2.2.

Table 2.2 Examples of L-amino acids which induced positive feeding response on fish.

Substances	Species	References
L-Alanine	Seabream (*Pagrus major*)	Kasumyan and Døving (2003)
	Channel catfish (*Ictalurus punctatus*)	Kohbara *et al.* (1992)
	Atlantic Salmon (*Salmo salar*)	Hidaka (1992)
L-Cysteine	Nine-spine stickleback (*Pungitius pungitius*)	Mikhailova and Kasumyan (2006)
	Carp (*Cyprinus carpio*)	Kasumyan and Morsy (1996)
L-Glutamic acid	Atlantic charr (*Salvelinus alpinus erhytrinus*)	Kasumyan and Sidorov (1995b)
L-Glutamine	Three-spine stickleback (*Gasterosteus aculeatus*)	Mikhailova and Kasumyan (2006)
L-Glycine	Sole (*Solea solea*)	Mackie *et al.* (1980)
L-Lysine	Carp (*Cyprinus carpio*)	Goh and Tamura(1978)
L-Leucine	Rainbow trout (*Oncorhyncus mykiss*)	Jones (1989)
Phenylalanine	Caspian Brown Trout (*Salmo trutta caspius*)	Kasumyan and Sidorov (1995a)
L-Serine	Puffer (*Fugu pardalis*)	Hidaka (1982)
L-Tyrosine	Platyfish (*Xiphophorus maculatus*)	Kasumyan, unpublished
L-Valine	Minnow (*Phoxinus phoxinus*)	Kasumyan, unpublished
L-amino acids mixture	Puffer (*Fugu pardalis*)	Hidaka *et al.* (1975)
	Eel (*Anguilla anguilla*	Takii *et al.* (1987)

2.4 Methods to Study fish Taste Preference

Several behavioral methods have been developed to study the taste preference of fish. For example, a polyurethane disc impregnated with chemical substances in the attempt to study the taste preference performed by Sutterlin and Sutterlin (1970). Others than that, Jones (1989, 1990) applied cotton pellet soaked in solutions containing test substance in the study of rainbow trout's taste preference. Mackie and Adron (1978) applied another method by incorporating several compounds into freeze dried pellets. However, applications of many of these methods do not distinguish between gustatory sensory system and olfactory system. Some authors had conducted their studies using purified starch gel as the medium to place the test stimulants (Hidaka *et al.*, 1978; Ohsugi *et al.*, 1978; Murofushi and Ina 1981; Hidaka 1982; Kasumyan 1992). Radiographic (Toften *et al.*, 1995) or radioisotopic methods (Jobling *et al*, 1995) had been applied in the comparison of food ingredients' palatability.

Another behavioral approach had been invented by Mearns *et al.* (1987), which use agar gel as transporter of test stimulants. This method had been applied and had been found to differentiate taste and smell in the study done by Kasumyan and Sidorov (1993a, b).

CHAPTER 3

METHODOLOGY

Total of 2 experiments were conducted in the Wet Laboratory of Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah. The experiment on determining the potential feeding stimulants for marble goby, *O. marmoratus* was done first followed by the next experiment which was to compare the pellet weaning period for wild juvenile *O. marmoratus* using fish meal (FM) diet and fish meal containing prototype feeding stimulant (FM +FS) diet.

3.1 Experiment I: Determination of Potential Feeding Stimulants for *O. marmoratus*.

3.1.1 Fish Specimens

A total of 30 specimens of reared *O. marmoratus* with total body length of 14 cm to 20 cm were acquired from the Wet Laboratory of BMRI. The fish were reared temporarily in a glass aquarium with capacity of 300 liters. The rearing aquarium was aerated by air stone and was equipped with a tank filled with dead coral stones, which functioned as biological filter. The diet of fish was switched from self-made moist pellet to commercial marine finfish (CMFF) pellet (Otohime Brand, Marubeni Nisshin, Japan). The fish was fed to satiation with CMFF pellet once per day in the evening.

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