



Evaluation on the potential of betaine, taurine, nucleotide and nucleoside as feeding stimulant for juvenile marble goby *Oxyeleotris marmoratus* through behavioural assays

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Abstract This study was conducted to evaluate the potential of betaine, taurine, inosine (INO), inosine 5'-monophosphate disodium (IMP·Na₂), and guanosine 5'-monophosphate disodium (GMP·Na₂) as a feeding stimulant for juvenile marble goby (*Oxyeleotris marmoratus*) (total length 6.6–8.5 cm) through behavioural assays using agar gel pellets. All fish were conditioned to accept agar gel pellet before the behavioural assays started. Each chemical substance was tested on 50 replicates of individual fish once, and the overall ingestion rate was calculated as the representative data. The pure agar gel pellet was totally rejected by the fish (0 % ingestion rate). Therefore, any added test substance which can significantly improve the fish ingestion of the agar gel pellet can be the potential feeding stimulant. Of all the chemical substances tested at 0.1 M concentration, the ingestion rates of both INO and IMP·Na₂ were the highest (both 100 %) and were significantly higher ($P < 0.05$) than those of the other chemical substances tested. However, INO was identified as the most potent feeding stimulant as it could function perfectly (100 %) even at the lower concentrations tested (0.01 and 0.001 M). The ingestion rates of IMP·Na₂ were found significantly decreased ($P < 0.05$) at the concentrations of 0.01 and 0.001 M (78 and 2 %, respectively). The ingestion rate of GMP·Na₂ at 0.1 M was 60 %, hence higher concentration (>0.1 M) may be required to improve its efficiency as the feeding stimulant. Taurine was not a feeding stimulant, and betaine was neither a feeding stimulant nor feed enhancer for the juvenile *O. marmoratus*.

Keywords Feeding stimulant · Nucleotides and nucleosides · Betaine · Marble goby · *Oxyeleotris marmoratus* · Behaviour

Abbreviations

INO	Inosine
IMP·Na ₂	Inosine 5'-monophosphate disodium
GMP·Na ₂	Guanosine 5'-monophosphate disodium
FS	Feeding stimulant
FT pellet	For-training pellet
PAG pellet	Pure agar gel pellet
TS pellet	Test substance pellet
AAM	Amino acids mixture
CI	Confidence interval

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Introduction

Feeding stimulant (FS) is defined as the substance which has high ingestion rate by fish (Kasumyan and Døving 2003). In aquaculture, dietary supplementation of FS has been commonly practised to pellet-wean (Person-Le Ruyet 1989; Kubitzka and Lovshin 1999; Hirt-Chabbert et al. 2012) and improve the feed intake in fish (Dias et al. 1997; Xue and Cui 2001; Papatryphon and Soares 2000). However, the taste preference in fish is species-specific (Kasumyan and Døving 2003); therefore, the effective FS should be identified prior to the dietary supplementation. Betaine, taurine, nucleotides and nucleosides are amongst the commonly known FS for fish (Kasumyan and Døving 2003; Chatzifotis et al. 2008; Lech and Reigh 2012). In addition, betaine is also known to be a potential feed enhancer for fish, which itself contains no effect of taste stimulant, but it can enhance the flavour of other chemical substances in a mixture (Kasumyan and Døving 2003).

Marble goby (*Oxyeleotris marmoratus*) is a high commercial value fish species in the aquaculture industry in many Southeast Asian countries, including Thailand, Malaysia, Singapore, Vietnam, and China for mass production (Tan and Lam 1973; Tavarutmaneeagul and Lin 1988; Cheah et al. 1994; Luong et al. 2005; Wang et al. 2011). However, there is a feeding problem in the culture of *O. marmoratus* at grow-out stage. The feeding practise for this fish has been continuously relying on the low value fish (Bundit and Jauncey 2008) or live fish, which can easily deteriorate the quality of the rearing water and subsequently cause disease outbreak (Lam et al. 2008; 2014). Such feeding practise has been continued because there has been lack of commercial pelleted feeds for *O. marmoratus* (Cheah et al. 1994; Lin and Kaewpaitoon 2000), and this fish generally rejected pelleted feeds (Rojtinnakorn et al. 2012). Successful weaning generally requires long period of time, and cause high fish mortality due to starvation. Although FS can be applied to wean *O. marmoratus* to accept pelleted feeds, information on the suitable FS for this species is limited. In our previous study, amino acids mixture appeared to be more efficient than single amino acid as FS for the juvenile *O. marmoratus* when determined through behavioural assays (Lim et al. 2015). Apart from this, no study has been conducted to evaluate the effectiveness of other potential FS including betaine, taurine, nucleotide and nucleoside on *O. marmoratus*. Therefore, this study was conducted to investigate the possibility of using betaine, taurine, nucleotide and nucleoside as feeding stimulants for juvenile *O. marmoratus*.

Table 1 Types of agar gel pellet with its ingredients and composition prepared in the present study

Treatments	Pure agar gel (PAG)	FT pellet (PAG + Otohime extract)	TS pellet (PAG + test substance)
Ingredients			
Freshwater	10 ml	N/A	10 ml
Agar gel powder ^a	0.2 g	0.2 g	0.2 g
Red food dye ^b	0.01 g	0.01 g	0.01 g
Otohime pellet's extract	N/A	10 ml	N/A
Test substances (0.1 M)			
Betaine (BET)/taurine/inosine/inosine-5'-monophosphate disodium (IMP·Na ₂)/guanosine-5'-monophosphate disodium (GMP·Na ₂) ^c	N/A	N/A	0.117 g/0.125 g/ 0.268 g/0.348 g/ 0.363 g, respectively
Betaine experiment (1.0 %)			
BET/amino acids mixture (AAM) ^d /BET + AAM at ratios (1:3), (1:2), (1:1), (2:1) or (3:1)	N/A	N/A	0.1 g

^a Mermaid Brand, Thailand

^b Ponceau 4R, Meebo Brand, Malaysia

^c All Sigma Brand

^d Mixture of Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, and Valine (all L-isomers, Sigma Brand, each 12.5 %) (Lim et al. 2016)



Methods

Preparation of agar gel pellets

The ingredients, composition and type of the agar gel pellets prepared and used in this study are shown in Table 1. The method of preparing agar gel pellets was similar to those described in Lim et al. (2015). In general, agar gel pellets were prepared by dissolving the agar gel powder (2 % of the freshwater volume) and red food dye (0.1 %) in 10 ml freshwater, boiled the mixture in a microwave oven, poured the boiled mixture into a glass petri dish for hardening, and subsequently cut it into uniform size (approximately 3 mm × 3 mm × 3 mm) of pellet. Three major types of agar gel pellet were prepared in this study, namely the FT (for-training) pellet, the PAG (pure agar gel) pellet, and the TS (test substance) pellet (refer Table 1 for the content). The FT pellet was used to condition the fish to accept agar gel pellet; the PAG pellet (without inclusion of any test substance) was served as the negative control, while the TS pellets contained each test substance, which was betaine, taurine, inosine—INO (nucleoside), inosine-5'-monophosphate disodium—IMP·Na₂ or guanosine-5'-monophosphate disodium—GMP·Na₂ (nucleotides).

In FT pellet, the water extract of the commercial marine finfish pellet (Otohime Brand, EP type, Japan, crude protein and crude lipid levels are 48 and 12 %, respectively) was used to replace the freshwater. The water extract of the Otohime commercial pellet was prepared by soaking the pellet powder (5 g) in 50 ml of freshwater with occasional stirring for about 20 min until the water was turned into dark brown colour, then filtered the homogenate through a 60 µm mesh net to obtain the liquid (extract). All extracts were freshly attained prior to the FT pellets preparation. For the TS pellets, 0.1 M of betaine, taurine, INO, IMP·Na₂ or GMP·Na₂ was dissolved into the boiled mixture of agar gel powder, food dye and freshwater before the mixture was poured into the glass petri dish for hardening. For the test substances which were highly preferred by the fish, they were tested on the fish with lower concentrations (0.01 and 0.001 M) to determine their threshold for promoting the fish ingestion.

Another set (seven types) of agar gel pellets was prepared with the method modified from Lim et al. (2016) to test the taste effect of betaine as a feed enhancer. These agar gel pellets contained 1 % of betaine, amino acids mixture (AAM) or different rates of betaine + AAM as shown in Table 1. The preparation method for these agar gel pellets were similar to the preparation of the TS pellets as described above. All prepared agar gel pellets were stored in a refrigerator (4 °C) for not more than four days to maintain their freshness.

Experimental fish

Fifty tails of juvenile marble goby (*O. marmoratus*) (total length 6.6–8.8 cm) were obtained from the fish hatchery of Borneo Marine Research Institute (BMRI). The fish were first acclimatised in a 40 L glass aquarium in the indoor wet laboratory of BMRI, supplied with aeration. The fish were adapted to feed Otohime commercial marine finfish pellet. Otohime is well-known for its fine quality, and it is commonly used to culture high commercial value of carnivorous marine fish, such as groupers. Tank cleaning was done, and about 50 % water in the aquarium was exchanged daily. After all fish were successfully adapted to feed on the Otohime commercial pellet in about 30 days, each fish were transferred to a transparent 3 L acrylic aquarium with 1 L water and aeration supply. Subsequently, these fish were conditioned to accept agar gel pellet.

Fish conditioning

Before the behavioural assays started, the fish were individually conditioned to accept agar gel pellet using the FT pellet. The conditioning method was similar with that described by Lim et al. (2015). All fish were firstly starved for 24 h, and three pieces of FT pellet were given to the fish daily. Those fish which accepted the given FT pellets were subsequently rewarded with a few pieces of Otohime commercial pellet. On the other hand, no Otohime commercial pellet was given to those fish which rejected or did not show any response to the FT pellets. These fish were starved and the conditioning continued the next day. The fish were considered ready for the behavioural assays only when they consumed all the FT pellets in the continuous three days of training. In this study, all fish (50 individuals) were successfully trained to accept the FT pellets after 11 days of conditioning. No fish mortality was observed throughout the conditioning period.



Behavioural assays

The behavioural assays started after all fish were successfully conditioned to accept agar gel pellet. Each treatment of TS pellet was fed to 50 replicates of the individual fish. The procedure of the behavioural assays in this study was adopted from that of Kasumyan and Morsi (1996) with modifications. The behavioural assays began by providing a piece of FT pellet to the fish to trigger its feeding desire, followed with a TS pellet, and then another piece of FT pellet. The last piece of FT pellet was served to recover the fish acceptance to agar gel pellet because the fish may be conditioned to reject agar gel pellet if any of the chemical substances tested was a deterrent to the fish. During the behavioural assays, the feeding responses of each fish to the given TS pellet (ingested or rejected) were observed and recorded. If the fish did not show any response to the given TS pellet (the TS pellet was not captured in mouth by the fish), that particular assay was not counted, and it will be repeated the other day. Each TS pellet was tried only once on each fish to avoid adaptation of the fish to the chemical substance tested, and only one test to the fish in each day. The PAG pellet was used as the negative control in the behavioural assay, and it was tested on the fish as the other TS pellets were. After each of the successful assay, the fish was rewarded with the Otohime commercial pellet until apparent satiation level. Tank cleaning was conducted and about 50 % of water in each aquarium was exchanged in the next day at least 4 h before the behavioural assays started. Throughout the experimental period, the water temperature in aquarium ranged from 28 to 29 °C.

Data collection and analysis

In this study, the feeding response of juvenile *O. marmoratus* to each treatment of agar gel pellet was observed for 50 replicates. As the feeding response observed was a binary data (ingested or rejected the TS pellet), binomial test was used for the data statistical analysis. The total ingestion rate for each treatment of agar gel pellet was calculated using the formula “ $A/B \times 100\%$ ” where A represented the total ingestion observed, while B represented the total replicates (50), and its 95 % confidence interval (CI) was calculated following the Clopper–Pearson exact method (Clopper and Pearson 1934). The range of the 95 % CI of agar gel pellet treatment was compared to each other; if the ranges of 95 % CI between two agar gel treatments were not overlapped, significant difference was assumed and vice versa (Sauro and Lewis 2012).

Results and discussion

This is the first report on the feeding response of juvenile *O. marmoratus* to betaine, taurine, inosine (INO), inosine-5'-monophosphate disodium (IMP·Na₂), and guanosine-5'-monophosphate disodium (GMP·Na₂). In this study, the pure agar gel pellet (PAG) was totally rejected by the fish, hence its ingestion rate was 0 %.

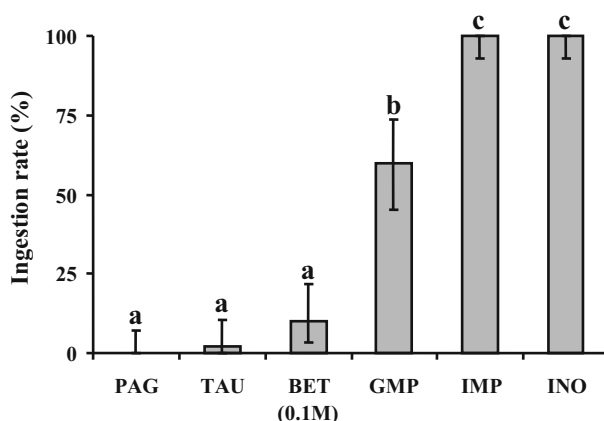


Fig. 1 Ingestion rates of the juvenile *O. marmoratus* on different treatments of TS pellet with the test substance concentration level at 0.1 M

Therefore, any added test substance which was able to significantly improve the fish ingestion rate on the agar gel pellet can be considered as the potential feeding stimulant (Lim et al. 2015; 2016). Figure 1 shows the ingestion rates of the juvenile *O. marmoratus* on different treatments of TS pellet. Among the 5 chemical substances tested in 0.1 M concentration, the ingestion rates of betaine (5 %) and taurine (2 %) were very poor and showed no significant difference ($P > 0.05$) with that of the PAG. The ingestion rate of GMP·Na₂ was moderately high (60 %) and significantly higher ($P < 0.05$) than those of PAG, betaine, and taurine. However, the ingestion rates of INO and IMP·Na₂ were perfected (100 %) as all replicates of fish ingested the INO and IMP·Na₂ TS pellets, and these values were significantly higher ($P < 0.05$) than those of the other TS pellet treatments (PAG, betaine, taurine, and GMP·Na₂). These results showed that INO and IMP·Na₂ were the most potential feeding stimulants for juvenile *O. marmoratus* although the application of higher concentration (>0.1 M) of GMP·Na₂ may improve the fish ingestion. Indeed, both IMP and INO were also reported as the feeding stimulant for several fish species, including turbot *Scophthalmus maximus* (Mackie and Adron 1978), brill *Scophthalmus rhombus* (Mackie and Mitchell 1985), and rainbow trout *Salmo gairdneri* (Mearns et al. 1987), but they were not to Atlantic salmon parr *Salmo salar* (Mearns et al. 1987) and tiger puffer *Takifugu pardalis* although they were identified to stimulate the taste buds on the lips of *T. pardalis* through electrophysiological recording (Hidaka et al. 1978). In other reports for yellowtail *Seriola quinqueradiata* (Takeda 1980), jack mackerel *Trachurus japonicus* (Ikeda et al. 1988; 1991) and largemouth bass *Micropterus salmoides* (Kubitza et al. 1997), IMP·Na₂ or IMP was an effective feeding stimulant, but INO was not. Apparently, the feeding responses of fishes to nucleotides and nucleosides are species-specific as suggested by Li and Gatlin III (2006).

Considering the highest ingestion rate of INO and IMP·Na₂ in the juvenile *O. marmoratus*, these chemical substances were further tested on the fish with the lower concentrations (0.01 and 0.001 M) to detect their threshold to the fish. Figure 2 shows the fish ingestion rates of TS pellets with different concentrations of INO and IMP·Na₂. The ingestion rates of IMP·Na₂ at 0.001 and 0.01 M were 2 and 78 %, respectively; these values were significantly lower ($P < 0.05$) than that of the 0.1 M IMP·Na₂ (100 %). On the other hand, the ingestion rates of INO at 0.001 and 0.01 M were all 100 %, similar with that of the 0.1 M INO. These results suggested that INO was more effective or suitable than IMP·Na₂ to be used as a feeding stimulant for juvenile *O. marmoratus* in term of their functional dosage. Such results were in agreement with those reported on the marbled rockfish *Sebasticus marmoratus* by Takaoka et al. (1990).

Betaine is commonly known to possess synergistic effects with other chemical substances, especially amino acids; hence it can be a feed enhancer to some fish species (Kasumyan and Døving 2003). In this study, betaine was neither a feeding stimulant nor a feed enhancer to the juvenile *O. marmoratus*. Figure 3 shows the ingestion rates of the fish to the agar gel pellets with betaine, amino acids mixture (AAM) or betaine + AAM at different rates. Inclusion of betaine to the AAM did not improve the ingestion rates of the TS pellets despite changes in the ration of betaine and AAM. Such results were in contrast with those reported on brown-marbled grouper *Epinephelus fuscoguttatus*, where the ingestion rate of the agar gel pellet significantly ($P < 0.05$) increased after the inclusion of betaine to the AAM, even in a small portion (Lim et al. 2016). Indeed, betaine was also rejected by *S. salar* as reported by Mearns et al. (1987) through behavioural assays.

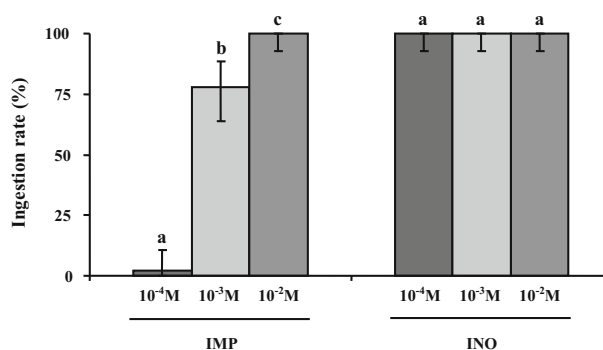


Fig. 2 Ingestion rate of the juvenile *O. marmoratus* on the TS pellets with inosine (INO) or inosine 5'-monophosphate disodium (IMP·Na₂) at different concentration levels



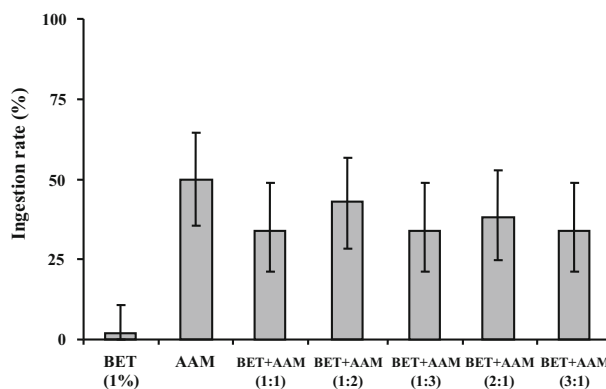


Fig. 3 Ingestion rate of the juvenile *O. marmoratus* on the TS pellets with 1 % betaine, amino acids mixture (AAM) or different rates of betaine + AAM

Conclusions

In conclusion, INO was the most potent feeding stimulant for the juvenile *O. marmoratus* as it was still effective to promote fish ingestion even at low concentration 0.001 M. IMP·Na₂ was an effective feeding stimulant for the fish only at the concentration level of at least 0.1 M. Higher concentration of GMP·Na₂ (>0.1 M) may be required to improve its efficiency as the feeding stimulant. Taurine was not a feeding stimulant, and betaine was neither a feeding stimulant nor feed enhancer to the juvenile *O. marmoratus*. Further study should be conducted to evaluate the effectiveness of dietary INO supplementation to improve the pellet-weaning progress of juvenile *O. marmoratus*.

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Author contribution statement Leong-Seng Lim contributed the original idea of this study, designed the experimental methods and wrote the manuscript. Sian-Kang Jason Lai provided ideas to refine the experimental methods, conducted the experiments, collected and analysed the data. Annita Seok-Kian Yong and Rossita Shapawi provided ideas to refine the experimental methods and proof-read the manuscript. Gunzo Kawamura involved in designing the experimental methods, statistical analysis and proof-read the manuscript.

Compliance with ethical standards

Conflict of interest No competing interests.

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