

# Betaine is a feed enhancer for juvenile grouper (*Epinephelus fuscoguttatus*) as determined behaviourally

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## ABSTRACT

The present study aimed to determine if betaine can function as a feeding stimulant or feed enhancer for the juvenile grouper, *Epinephelus fuscoguttatus* through a behavioural experiment with video recording. Agar gel pellet was used as the medium to deliver the chemical test substances – betaine (BET), amino acids mixture (AAM) and mixtures of 1:1, 1:2, 1:3, 1:4, 1:5 BET + AAM to the fish. The pure agar gel (PAG) and feed extract pellets were used as the negative and positive control, respectively. From the recorded videos, two parameters were observed: (I) the pellet was consumed or rejected [A] – if consumed, recorded 1; if rejected, recorded 0, and (II) frequency of the pellet has been captured before it was consumed or rejected and ignored [B], and the preference index was calculated through  $[A]/[B]$ . The PAG pellet was totally rejected by the fish (index's value = 0). The index's values of BET and AAM were  $0.17 \pm 0.39$  and  $0.19 \pm 0.31$  (mean  $\pm$  SD), respectively. No significant difference was found among the index's values of PAG, BET and AAM pellets. However, the index's values of all BET + AAM pellets (1:1–1:5) were very high (0.60–0.90), and were significantly higher than those of the PAG, BET and AAM pellets. No significant difference was found among the index's value of 1:1, 1:2, 1:3 and 1:4 BET + AAM treatments. These results concluded that betaine is a feed enhancer for the *E. fuscoguttatus* and little amount of betaine supplementation is sufficient to enhance the flavour of amino acids mixture.

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## 1. Introduction

Epinephelinae groupers are aquaculture-targeted species as a result of its high commercial value and demand. They are carnivorous fish which are often fed with fish meal-based compounded feeds (Williams 2009). However, fish meal's price is hiking due to its increasing demand globally (Tacon & Metian 2008). Alternative protein sources from terrestrial animals and plants are being exploited to substitute the fish meal. However, high inclusion level of alternative protein sources generally deteriorates the feed's palatability which subsequently leads to the reduced fish intake and growth (see review Lim et al. 2014). Although feed palatability can be manipulated by feeding stimulant or feed enhancer supplementation (e.g. Dias et al. 1997; Papatryphon & Soares 2000; Xue & Cui 2001), there is no documented feeding stimulant or feed enhancer for juvenile groupers. Feeding stimulant is defined as the substance which is highly preferred by fish. The food item that contains feeding stimulant is usually consumed by fish at its first capture. Therefore, it can promote high feeding rate in fish. Feed enhancer is the substance that enhances the food flavour and subsequently promotes fish's consumption on the flavoured food, although it is not a stimulant (Kasumyan & Døving 2003).

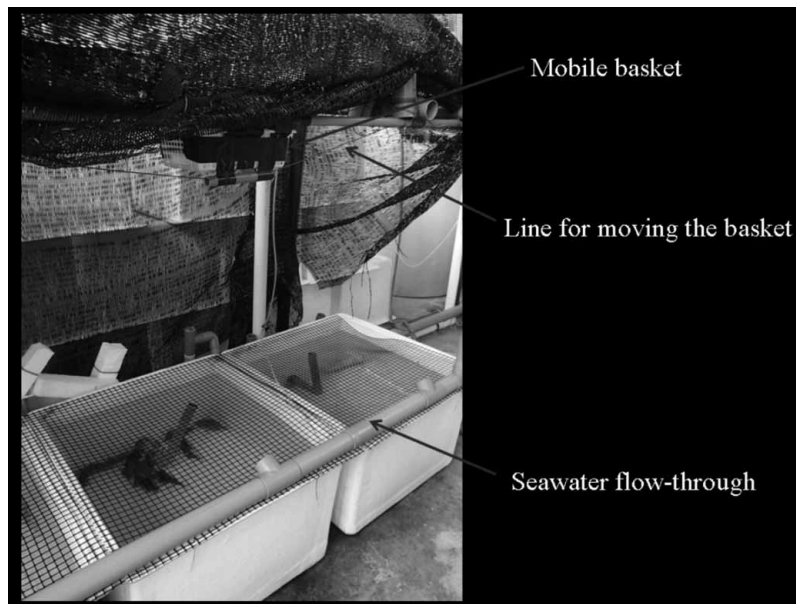
Betaine is a neutral quaternary ammonium compound that can be found in many plants and animals, including marine invertebrates. It was first discovered in sugar beets *Beta vulgaris* and then in other organisms (Craig 2004). In aquaculture, it is a common feed additive or stimulant to promote fish intake (e.g. Fredette et al. 2000; Shankar et al. 2008; Zakipour et al. 2012).

However, the function of betaine as a stimulant in fish can be species specific (Kasumyan & Døving 2003). Betaine does not improve the feed intake of some fish species, such as coregonid *Coregonus schinzi palea* Cuv. Et Val. (Dabrowski & Kaushik 1985), Chinook salmon *Oncorhynchus mykiss* (Hughes 1991, 1993) and Piaçu *Leporinus macrocephalus* (Normandes et al. 2006). Also, it is a deterrent to Atlantic salmon parr *Salmo salar* (Mearns et al. 1987). Nevertheless, betaine has synergistic properties to enhance the flavour of amino acids and function as a feed enhancer, although it can be tasteless to the fish (Ohsugi et al. 1978; Goh & Tamura 1980; Mackie et al. 1980; Mackie 1982). Therefore, the stimulant function of betaine in the targeted fish should be clarified before it is supplemented to fish feeds. Nonetheless, it is unknown whether betaine is a feeding stimulant, feed enhancer or a deterrent for juvenile groupers. The taste effects of chemical substances on fish can be determined behaviourally through observations on the feeding responses of fish-fed mediums (such as agar gel pellets) contained with the chemical test substances (Kasumyan & Døving 2003). Therefore, the present study aims to make clear if betaine can function as a feeding stimulant or feed enhancer in the juvenile grouper, *Epinephelus fuscoguttatus* through behavioural experiment.

## 2. Materials and methods

### 2.1. Source of fish

Sixty individuals of juvenile grouper *E. fuscoguttatus* (body weight  $13.8 \pm 0.9$  g and mean  $\pm$  SD) were purchased from a



**Figure 1.** Tank system set-up for the behavioural experiment with labels.

fish farmer. The fish were acclimatized in a 3-tonne fibreglass tank for 3 days, supplied with flow-through seawater and aeration, and fed with commercial feed for marine finfish (Otohime brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) twice daily in the morning and afternoon (approximately at 0800 and 1400 hours). The fish were transferred to the experimental tanks set-up for the behavioural experiment after the acclimatization period.

## 2.2. Experimental tank system set-up

Figure 1 shows the tank system set-up for the behavioural experiment. Three fibreglass tanks (150 L each) were prepared. Each tank contained 20 individuals of fish, and was considered as a replicate. All tanks were equipped with the seawater flow-through system, and aeration. The tanks were also curtained with black mesh net to avoid disturbance because the fish were sensitive to the observer's movement. However, the observer was still able to see the fish through the mesh net. Above the tanks, a mobile basket with a horizontal pulley system was installed. A digital camera (Olympus brand, TG2 model, Japan) was placed inside the basket to record the fish feeding activities. Inside the basket, the digital camera could be moved easily from tank to tank without stressing the fish.

## 2.3. Preparation of agar gel pellets

Table 1 presents types of agar gel pellet prepared for the experiment and its composition. For the preparation, agar gel powder (2% of the seawater volume) and red food dye (0.1%) were first dissolved in the filtered seawater and heated in a microwave until boiled. The chemical test substance (betaine, amino acids mixture (AAM) or betaine + AAM, 1%) then was dissolved in the boiled mixture. The mixture was poured into a glass petri dish for hardening, cut into uniform size of pellets (approximately 1 cm × 1 cm × 0.5 cm) and stored in a refrigerator (4°C)

until further use. To get the liquid extract, the commercial feed (Otohime brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) powder was soaked in filtered seawater for approximately 20 min until the water turned into dark brown, then the homogenate was filtered through a 60 µm mesh net to obtain the liquid. All agar gel pellets were kept in refrigerator not more than 4 days to maintain its freshness.

## 2.4. Experimental procedure and data analysis

Preliminary observation was conducted to understand the fish feeding behaviour by feeding them with the pure agar gel (PAG) and feed extract (FE) pellets, which were the negative and positive control, respectively. Interesting behaviour was observed. All fish ignored the next agar gel pellet after a given PAG pellet was captured but rejected by several fish; in opposite, feeding frenzy happened after an FE pellet was consumed by a fish. These results showed that the fish feeding is highly influenced by their social learning through vision. Based on this condition, the experimental procedure was designed.

The fish were trained to confirm that they will recognize the agar gel pellets as food before the experiment started. The training was carried out by feeding the FE pellet to the fish once in a day at evening. At the end of each training session, commercial feed was given to the fish as the reward and to compensate their nutritional requirement. The experiment started after the fish consumed all FE pellets given in each training session, continuously for three sessions. In the experiment, a piece of test agar gel pellet from any type (the PAG, betaine, AAM or betaine + AAM) was given to the fish in the midst of feeding them with the FE pellets. At the same time, the observer read out the type of the given test agar gel pellet hence it was voice-recorded in the video. Until the feeding frenzy stopped, the camera was moved to the next tank and the same experimental procedure was repeated. The aeration and flow

**Table 1.** Types of agar gel pellet prepared for the behavioural experiment and its composition.

Treatments	Pure agar gel (PAG)	Feed extract (FE)	Betaine (BET)	Amino acids mixture (AAM)	BET + AAM (ratio 1:1)	BET + AAM (ratio 1:2)	BET + AAM (ratio 1:3)	BET + AAM (ratio 1:4)	BET + AAM (ratio 1:5)
<i>Ingredients (in g or ml)</i>									
Filtered seawater	10 ml	-	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Agar gel powder (g) <sup>a</sup>	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Red food dye (g) <sup>b</sup>	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Liquid extract of pellet feed <sup>c</sup>	-	10 ml	-	-	-	-	-	-	-
Betaine <sup>d</sup>	-	-	0.1 g	-	5.0 × 10 <sup>-2</sup> g	3.0 × 10 <sup>-2</sup> g	2.0 × 10 <sup>-2</sup> g	1.5 × 10 <sup>-2</sup> g	1.5 × 10 <sup>-2</sup> g
Amino acids mixture <sup>e</sup>	-	-	-	0.1 g	5.0 × 10 <sup>-2</sup> g	7.0 × 10 <sup>-2</sup> g	7.5 × 10 <sup>-2</sup> g	8.0 × 10 <sup>-2</sup> g	8.5 × 10 <sup>-2</sup> g

Notes: <sup>a</sup>Mermaid Brand, Thailand.

<sup>b</sup>Ponceau 4R, Meebo Brand, Malaysia.

<sup>c</sup>Otohime Brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan.

<sup>d</sup>Sigma Brand.

<sup>e</sup>Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, and Valine (all L-isomer, Sigma Brand, each 12.5%).

through seawater supplies were temporarily off for video recording before the experiment started. After the experiment, the uneaten/remaining agar gel pellets were removed from the tanks using a hand net. The aeration and flow-through seawater supplies were resumed, and all fish were fed with the commercial feed until the apparent satiation level.

To analyse the fish preference for the agar gel pellets tested, two parameters were observed from the video: (I) the test pellet was consumed or rejected [A] – if consumed, recorded 1, if rejected, recorded 0, (II) frequency of the pellet has been captured before it was consumed or totally rejected [B]. An index of preference was calculated through dividing [A] by [B]. Therefore, the maximum value of the index is 1, while the minimum is 0. If the pellet were consumed, the higher the frequency of capturing before the ingestion will result in the lower value of the index, indicating the lesser preference of the fish for that pellet.

The non-parametric statistical analysis method, Kruskal–Wallis Test was used to compare these data as the data were not normally distributed. If Kruskal–Wallis Test shows significant difference among treatments, the non-parametric Conover–Inman Test will be done as a post hoc test for pairwise comparisons. Both tests were carried out using computer software SYSTAT 13 (Systat Software, Inc.).

### 3. Results and discussion

This is the first report on the taste effects of betaine on juvenile grouper. The preference index's values for the agar gel pellets tested in the present study are given in Table 2. The *P* value from Kruskal–Wallis Test was 0.00 (*P* < .05), indicating that there are significant differences among the index's value of the agar gel pellets. Therefore, the index's values were analysed using Conover–Inman Test. The index's value for the PAG pellet was 0 because it was totally rejected by the fish. Therefore, any added chemical test substance that made the agar gel pellet palatable can be a potential feeding stimulant for the fish. The index's values for the betaine (BET) and AAM pellets were very low, which were 0.17 ± 0.39 and 0.19 ± 0.31 (mean ± SD), respectively. The fish rejected most of the BET and AAM pellets, and consumed only a few of them after many times of capturing them in mouth. No significant difference was found among the index's values of PAG, BET and AAM. However, the index's values of all ratios of betaine + amino acids mixture (BET + AAM) pellets were very high (0.60–0.90), and were significantly higher than those of the PAG, BET and AAM. Most of the BET + AAM pellets, especially those of ratio 1:1 and 1:2 were

**Table 2.** Preference index of the juvenile groupers for different types of agar gel pellet.

Types of agar gel pellet	Total trial	Preference index (Mean ± SD)
Pure agar gel (PAG)	12	0.00 <sup>a</sup>
Betaine (BET)	12	0.17±0.39 <sup>a</sup>
Amino acids mixture (AAM)	12	0.19±0.31 <sup>a</sup>
BET + AAM 1:1	12	0.90±0.23 <sup>b</sup>
BET + AAM 1:2	12	0.88±0.23 <sup>bc</sup>
BET + AAM 1:3	12	0.75±0.39 <sup>bc</sup>
BET + AAM 1:4	12	0.79±0.40 <sup>bc</sup>
BET + AAM 1:5	12	0.60±0.45 <sup>cd</sup>

Note: Different superscripted alphabets indicate significant difference at level 0.05.

consumed immediately by the fish. These results evidenced that betaine can enhance the flavour of the AAM, and it is a feed enhancer to the juvenile grouper, similar to puffer *Fugu pardalis* (Ohsugi et al. 1978), Dover sole *Solea solea* (Mackie et al. 1980) and red sea bream *Chrysophrys major* (Goh and Tamura 1980). In addition, little amount of betaine (four times lesser than the AAM amount) is sufficient to effectively enhance the flavour of the AAM because no significant difference was found among the index's values of BET + AAM 1:1, 1:2, 1:3 and 1:4. Therefore, supplementing a little amount of betaine into groupers diets could be effective to improve the diets palatability and promote fish intake. This hypothesis will be elucidated in the future study. In conclusion, betaine is a feed enhancer for the juvenile grouper *E. fuscoguttatus*. Little amount of betaine is sufficient to enhance the flavour of the AAM.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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