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Feeding Response of Brown-Marbled Grouper (*Epinephelus fuscoguttatus*) Juveniles to an Amino Acid Mixture: A Preliminary Study of Identifying Feeding Stimulant for Grouper Culture

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ABSTRACT

The present study examined the feeding response of juvenile brown-marbled grouper (*Epinephelus fuscoguttatus*) to an amino acid mixture (AAM). The findings contribute to identifying potential feeding stimulants (FS) for grouper aquaculture. Agar gel pellets were used to deliver the AAM at concentrations of 0% (pure agar gel - PAG), 0.01%, 0.1%, and 1.0% to the fish. The fish's feeding responses were recorded and analyzed, and their preference indices (ranging from 0 to 1) for the AAM were calculated. The PAG pellet was completely rejected by the fish (preference index = 0). This result indicates that any substance added to the agar gel pellets that induce the fish to consume them can be considered a potential feeding stimulant. On the other hand, the AAM was generally well accepted by the fish (preference indices ranging from 0.46 to 1), with the 1% AAM treatment achieving the highest preference index. These results confirm that the AAM is an effective FS for grouper juveniles. Further research on the fish's taste preferences for individual amino acids is strongly recommended to optimize the formulation of the feeding stimulant.

INTRODUCTION

Food selection in fish is mediated by intra-oral taste buds, which are sensitive to various taste substances, particularly amino acids **(Morais, 2017)**. According to **Kasumyan and Døving (2003)**, taste preference in fish is species-specific. Therefore, understanding fish preferences for different taste substances is crucial. This knowledge can contribute to aquaculture by identifying taste substances that function as feeding stimulants—compounds that promote fish ingestion—for aquafeed development **(Morais, 2017)**. Indeed, previous studies have shown that dietary supplementation with feeding stimulants can enhance fish ingestion of less palatable plant-based protein feeds

(Zou *et al*., 2017; Jiang *et al*., 2019) and alleviate challenges associated with weaning **(Lai *et al*., 2018; Li *et al*., 2019)**. To determine the taste substances preferred by fish, behavioral assays can be conducted using agar gel pellets as a medium to deliver the taste substances, followed by an examination of the fish's feeding response **(Kasumyan & Døving, 2003; Lim *et al*., 2016, 2017)**.

Groupers are among the most widely cultured marine fish species in Asian countries, including Malaysia **(Rimmer & Glamuzina, 2019)**. They are highly valued in mariculture due to their premium price in the live reef fish trade and seafood industry **(Kam *et al*., 2024)**. However, feed costs in grouper culture are exceptionally high, accounting for nearly 85% of the total production costs, as reported by **Dennis *et al*. (2020)** in a bioeconomic study on hybrid grouper production in Vietnam. This high cost is primarily due to the heavy reliance on expensive fish meals as the main protein source in formulated feeds for grouper farming **(Shapawi *et al*., 2019; Dennis *et al*., 2020)**.

Although plant proteins are now widely recognized as viable substitutes for fish meal protein in grouper feeds, groupers often reject plant-based feeds due to their poor palatability **(Lim *et al*., 2014)**. To address this issue, feeding stimulant supplementation is essential to improve feed palatability. Unfortunately, there is limited information available on suitable feeding stimulants for groupers. To date, betaine has been identified as a feed enhancer (though tasteless itself, it enhances the flavor of other taste substances) for the brown-marbled grouper (*Epinephelus fuscoguttatus*) **(Lim *et al*., 2016; Jamil *et al*., 2019)**. Additionally, inosine, inosine-5'-monophosphate, and guanosine-5'-monophosphate have shown potential as feeding stimulants for the hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂) **(Lim *et al*., 2017)**. Despite these findings, there is still no information on the use of amino acids, which are widely recognized as key taste substances, as feeding stimulants for groupers. Studies suggest that amino acid mixtures are often more effective as feeding stimulants for fish **(Mackie *et al*., 1980; Lim *et al*., 2015)**. Therefore, this study aimed to examine the feeding response of grouper to an amino acid mixture using behavioral assays.

MATERIALS AND METHODS

1. Tank system designed for behavioral assay

In this study, the taste preference or feeding response of fish to an amino acid mixture was assessed through behavioral assays. A tank system was specifically designed to facilitate video recording of the fish's responses (Fig. 1). The tank setup followed the design described by **Lim *et al*. (2016, 2017)**. Three fiberglass tanks (150L each) were arranged in a row, with each tank stocked with 20 fish individuals. Each tank served as a replicate. All tanks were supplied with flow-through filtered seawater and aeration to

maintain optimal water quality. As groupers are highly sensitive to observer movements, horticultural shade nets were used to curtain the tanks' perimeters to minimize disturbance. The shade net had a light penetration reduction level of 50%, allowing observers to monitor fish behavior without causing stress. A mobile basket equipped with a horizontal pulley system was installed above the tanks to facilitate video recording. A digital camera (Olympus TG2, Japan) was mounted inside the basket to record the fish feeding response. The camera could be easily moved from tank to tank within the basket, ensuring minimal disturbance and stress to the fish during the recording process.

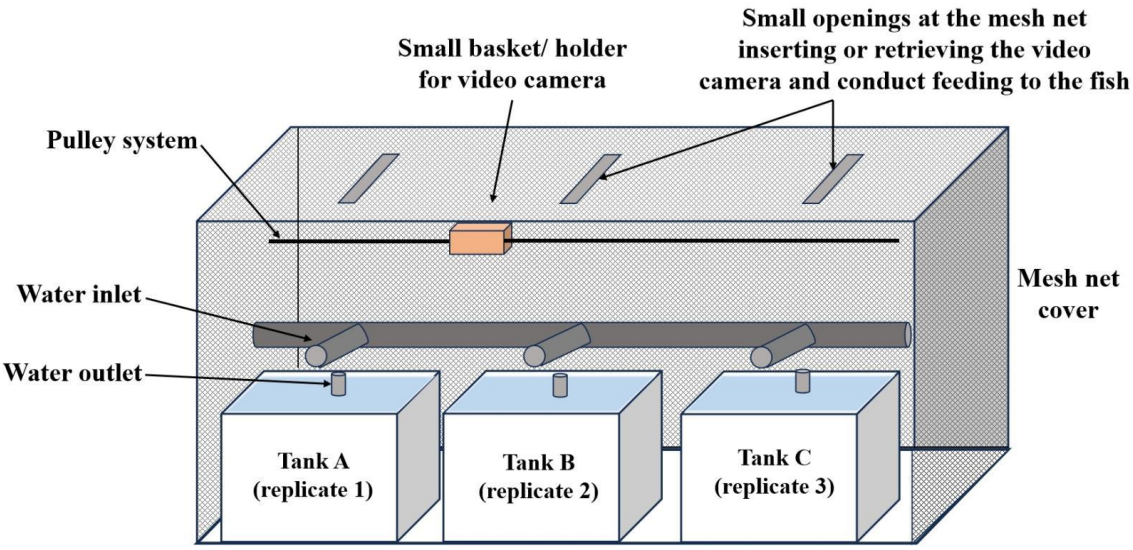

Fig. 1. Tank system used to record the feeding response of the *E. fuscoguttatus* to the agar gel pellet

2. Experimental fish

E. fuscoguttatus juveniles, with a body weight of 12.10 ± 2.70g, were used for the behavioral assays. The experimental fish were purchased from a local fish farmer and acclimatized for one week in tank systems designed for the behavioral assays. During the acclimatization period, the fish were fed commercial feed (Otohime brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to apparent satiation once daily. Flow-through filtered seawater and aeration were also provided.

3. Agar gel pellet preparation

Similar to **Lim *et al*. (2016, 2017)**, agar gel pellets were used as the medium to deliver an amino acid mixture to grouper juveniles in the behavioral assays conducted in this study. The agar gel pellet treatments prepared for this study, along with their

ingredient compositions, are shown in Table (1). The amino acid mixture (AAM) used was adopted from previous work by **Lim *et al*. (2015)**, which identified it as a functional feeding stimulant for a carnivorous fish species.

To prepare the agar gel pellets, agar powder (2% of the seawater volume) and red food dye (0.10%) were mixed with filtered seawater, and the mixture was heated in a microwave until boiling. Different percentages of the AAM (0.01%, 0.10%, or 1.00% of the seawater volume) were then dissolved in the boiled mixture. The solution was poured into glass petri dishes to cool and solidify into agar. Once solidified, the agar was cut into uniform pellets (approximately 1cm × 1cm × 0.50cm). In addition to these treatments, pure agar gel (PAG) pellets, containing no AAM, were prepared as a control, while agar gel pellets with feed essence (FE) were produced to condition the fish to accept agar gel pellets before the behavioral assays. To obtain FE for agar gel pellet preparation, commercial feed (Otohime brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) powder was soaked in filtered seawater (20g/ ml) for 20 minutes until the water turned dark brown. The homogenate was then filtered using a 60μm mesh net.

All agar gel pellets were stored in a refrigerator (4°C) and used within four days to maintain freshness.

Table 1. Composition of the agar gel pellets prepared for this study

Treatments	**Pure agar gel (PAG)**	**Feed essence (FE)**	**Amino acids mixture (AAM)**		
			0.01%	**0.10%**	**1.00%**
Ingredients (in g or ml)					
Filtered seawater	10 ml	-	10 ml	10 ml	10 ml
Agar gel powder [1]	0.20 g	0.20 g	0.20 g	0.20 g	0.20 g
Red food dye [2]	0.01 g	0.01 g	0.01 g	0.01 g	0.01 g
Commercial feed essence [3]	-	10 ml	-	-	-
Amino acids mixture [4]	-	-	0.001 g	0.01 g	0.10 g

[1] Mermaid Brand, Thailand

[2] Ponceau 4R, Meebo Brand, Malaysia

[3] Otohime Brand, EP type, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan

[4] Alanine, Arginine, Glutamine, Glycine, Proline, Serine, Tyrosine, and Asparagine (all L-isomer, Sigma Brand, each 12.5%) **(Lim *et al*., 2015)**

4. Fish conditioning

The grouper juveniles in the tanks were conditioned to accept agar gel pellets using FE pellets before the behavioral assay began. Following the method described by **Lim *et al*. (2016, 2017)**, FE pellets were fed to the fish once daily in the evening (at approximately 1600). After the fish consumed all the FE pellets, they were rewarded with

commercial feed (Otohime Brand, Japan) at the end of the conditioning session. If the fish did not ingest the FE pellets, they would starve, and the conditioning session was repeated the following day. The fish were considered well-conditioned and ready for the behavioral assay when they consistently ingested all the FE pellets in three consecutive conditioning sessions. In this study, all tanks of fish were successfully conditioned within 4–5 days, with no observed mortality.

5. Behavioral assay procedures

Prior to the behavioral assay, the flow-through seawater and aeration supplies were turned off to ensure clear video recording. A few pieces of feed essence (FE) pellets were initially fed to the fish. Then, during feeding, a single agar gel pellet from one of the treatments (either the pure agar gel [PAG] pellet or one containing any concentration of the amino acid mixture [AAM]) was introduced. The assay ended once the fish feeding frenzy ceased, after which the camera was moved to the next tank, and the same procedure was repeated. After completing the assays in all tanks, any uneaten or remaining agar gel pellets were removed from the tanks using a hand net. The fish were then fed with commercial feed to apparent satiation, and aeration and flow-through seawater supplies were resumed.

Each agar gel pellet treatment was tested in each tank of fish five times, in a random sequence. To prevent learned or memorized responses, only one treatment was tested, and only one test was performed per day. In total, each agar gel pellet treatment was tested 15 times during the study.

6. Video analysis

The fish's preference for the tested agar gel pellets was evaluated through recorded videos. Following the evaluation method described by **Lim *et al*. (2016, 2017)**, two parameters were observed from the videos:

i. Ingestion or rejection of the test pellet ([A]): If the pellet was ingested, a value of 1 was recorded; if rejected, a value of 0 was recorded.

ii. Frequency of snapping at the test pellet before ingestion or total rejection ([B]).

The preference index was calculated by dividing [A] by [B]. Thus, the maximum and minimum values of the index were 1 and 0, respectively. If the test pellet was ingested, a higher frequency of snapping before ingestion resulted in a lower index value, indicating a lesser preference for that pellet.

The data were analyzed using the non-parametric Kruskal–Wallis test, followed with the Conover–Inman post hoc analysis test for pairwise comparisons. Significant difference was assumed in all tests when $P < 0.05$. Both statistical tests were conducted using SYSTAT 13 (Systat Software, Inc.).

RESULTS AND DISCUSSION

The preference indices of *E. fuscoguttatus* juveniles for the tested agar gel pellets are presented in Fig. (2). The preference index for the pure agar gel (PAG) pellet was zero because none of it was ingested. Although the PAG pellet was repeatedly captured in the mouth by several fish, it was ultimately rejected. In contrast, the preference indices for all agar gel pellets containing the amino acid mixture (AAM) ranged from 0.46 to 1.00, significantly higher ($P < 0.05$) than that of the PAG pellet. Moreover, the index increased with AAM concentration, rising from 0.46 at 0.01% AAM to 0.76 at 0.10%, and 1.00 at 1.00%. While no significant difference ($P > 0.05$) was observed between the preference indices for the 0.10% and 1.00% treatments, both were significantly higher ($P < 0.05$) than the 0.01% treatment. These findings confirm that the tested AAM was preferred by the fish.

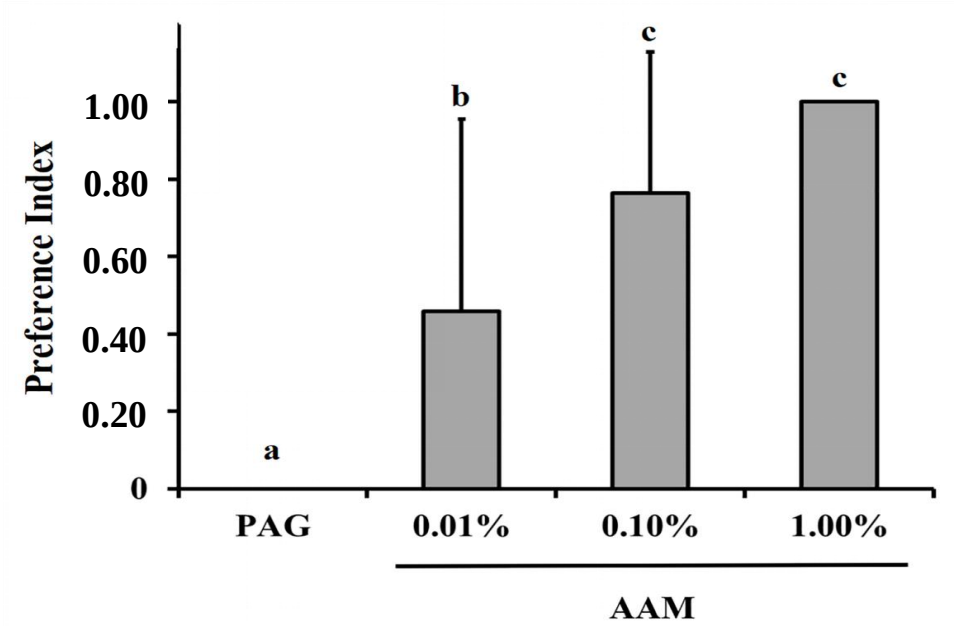

Fig. 2. Preference index of the *E. fuscoguttatus* juveniles to the agar gel pellets. Vertical bars indicate standard deviation of the data

According to **Kubitza and Lovshin (1999)**, carnivorous fishes generally prefer neutral and basic amino acids over acidic ones because the chemical composition of their

natural prey, such as crustaceans, mollusks, and fishes, is dominated by neutral and basic amino acids. In the present study, the formulated AAM contained L-alanine, L-glycine, L-proline, L-serine, L-asparagine, L-glutamine, L-tyrosine, and L-arginine, all of which are neutral or basic amino acids. None were acidic, and the AAM was highly accepted by *E. fuscoguttatus* juveniles, aligning with the findings of **Kubitza and Lovshin (1999)**.

Currently, there is limited information on the evaluation of single or mixed amino acids as feeding stimulants for groupers. Using experimental and analytical protocols similar to those employed in this study, **Lim *et al.* (2016)** reported that *E. fuscoguttatus* juveniles had a preference index of only 0.19 ± 0.31 (out of 1.00) for a mixture of L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, and L-valine. This indicates that the AAM tested in the present study was a more effective feeding stimulant. Notably, the amino acids tested by **Lim *et al.* (2016)** were essential amino acids, while those used in this study were non-essential. These results suggest that non-essential amino acids may be more gustatory stimulative than essential amino acids for groupers. However, there is still no detailed information on the specific taste preferences of groupers for individual amino acids. Formulating feeding stimulants can be costly if too many amino acids are included, but this issue can be addressed by optimizing formulations to use only the most effective amino acids. Therefore, further research on the taste preferences of *E. fuscoguttatus* juveniles for individual amino acids is strongly recommended.

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