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Feeding frequency effect on growth, body composition, feed utilization and ammonia excretion of juvenile grouper *Epinephelus coioides*

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Abstract
Juveniles grouper, *Epinephelus coioides*, of 29.33±1.08 g were stocked 16 fish per tank (31-L) and fed with three feeding frequencies (one, two and three meals daily) for 8 weeks to compare their food consumption, growth, body composition, feed utilization and ammonia excretion. Grouper were hand fed to satiation. Fish fed three times per day showed better food consumption, but fed one meal per day had better food conversion ratio. Fish fed one meal daily had heavier stomach than from the other groups, and tended to eat more per meals compared to fed two and three meals daily. Body composition of fed three meals had the highest body lipid content. Our result showed that a peak of excretion occurred 4 hours after the first feeding session. Increased feeding frequency to two and three lead to a cumulative effect of ammonia excretion. Feeding frequency had no effect on apparent digestibility in grouper. Feeding one time a day to satiation seems to be sufficient for maximal growth in juvenile grouper under our rearing conditions.

Keywords: body composition; feeding frequency; grouper; growth

1. Introduction
The growth and metabolism of fish are influenced by several factors including food availability and quality [1], water temperature [2], stocking density [3], fish size [4], and feeding regimes [5]. In addition, the amount of food consumed and the efficiency of its assimilation affect growth of fish [6]. If fish are underfed, the growth is reduced and dominance hierarchies may arise, resulting in size variation and depensatory growth and lower metabolism [7]. However, overfeeding causes a decrease in feed conversion efficiency, whereas the unconsumed feed deteriorates the water quality. Many authors have studied the relationship between feeding frequency and growth and feed utilization [5, 8]. The increase in feeding frequency may cause an increase in food intake or the same food intake may result in different food utilization. In response to reduction in feeding frequency, mammals may show some physiological adaptations such as development of hyperphagia, improvement in growth and alteration in body composition [7]. These adaptations observed in mammals are also produced in fish [9]. Adequate feedings can improve digestibility, and consequently accelerates protein assimilation, which result in high growth. Thus, information on digestibility of fish is useful and will allow one to estimate proper feeding frequency. Marine teleosts, including grouper are predominantly ammoniatelic, with 75-90 % of nitrogen excretion in the form of ammonia [10]. Exogenous excretion is the resultant energy loss associated with the assimilation and deamination of protein following feeding [7]. Ammonia is toxic to fish and is considered to be a major factor limiting fish biomass and stocking density in intensive culture systems [11, 12]. Quantification of ammonia excretion is therefore important in assessing the environmental impact of culture operations [13].
Grouper culture is a newly developed industry in Asia and around the world. Most of the studies on grouper have been focused on larval rearing and grow-out diet development [14], however the aspects on feeding strategies, stocking density and fish size have been rarely studied. The determination of optimal feeding frequency of fish is needed for their efficient production, because their nutrient requirements are largely influenced by feed allowance. Growth in fish is expected to increase with increasing feeding frequency. It is hypothesized

that higher feeding frequencies may increase opportunities for all fish to feed. Additionally, one may expect that increasing feeding frequency may reduce food utilization and size variation within a tank.

The objective of the present study was to investigate the effects of different feeding frequencies on growth performance, food consumption, size variation, body composition, ammonia excretion and digestibility of grouper when fed to satiation at each meal so that optimal feeding strategies can be developed accordingly.

2. Materials and Methods

2.1 Experimental conditions and procedures

This experiment was conducted with 29.33±1.08 g juvenile grouper *Epinephelus coioides* stocked at 16 fish /tank and fed with three frequencies (M1: 1-meal /day, 0700 h; M2: 2-meal /day, 0700 h and 2100 h and M3: 3-meal /day, 0700 h, 1500 h and 2100 h) during 8 weeks. The fish were stocked in 31-L (40 × 29 × 28cm) recirculating glass tanks aerated constantly. The fish were fed a formulated diet prepared at the aquaculture department laboratory. Formulations and proximate analyses are listed in Table 1. Part of the feed was marked with 0.5 % chromic oxide (Cr_2O_3), for digestibility determination. Experimental diet was prepared by mixing all dry ingredients in a food mixer for 20 min, followed by fish oil and soybean oil mixture for a further 20 min before adding water and mixing for 15 more min. Following pelleting, the diet was dried overnight in forced-draft oven at 70 ℃ and then stored at 4 ℃ until use. At each meal fish were hand-fed to apparent satiation, judged as the point at which pellets remained on the tank and were not approached by fish for more than two min. Each meal after feeding the uneaten food was removed manually to estimate food consumption. At each two days (alternate), the weight of food consumed by the fish in each tank was measured at each feeding time, and expressed as consumption per day on a body weight basis (% body weight /day). Coefficient of variation (standard deviation/ average) was used as size variation index of fish body weight. The fish were weighed at the beginning of the study and every 2 weeks thereafter.

Table 1: Formulation and proximate composition of the experimental diet

Ingredients	Content (g/ 100g dry matter)
Fish meal	56.5
Wheat gluten	2.7
Corn gluten	1.1
α- Starch	10.0
Fish oil: Soy oil (7:3)	3.5
Wheat flour	15.0
Carboxymethylcellulose	3.0
Squid liver meal	1.0
Calcium phosphate	0.5
Mineral premix[1]	3.0
Vitamin premix[1]	2.0
Choline chloride 50	0.5
α- Cellulose	1.3
Proximate composition[2]	
Moisture	6.8
Crude protein	48.6
Crude lipid	6.9
Ash	14.2

[1]Mineral premix and vitamin premix were obtained from Yi-Cherng Company, Taiwan.
[2]Values are presented in % dry matter.

2.2 Water quality parameters

Several parameters were routinely monitored to ensure good water quality maintained. Water temperature and salinity were measured daily by minimum-maximum thermometer and hand-held refractometer ATAGO (s/Mill-e, Japan), respectively. Dissolved oxygen and pH were measured weekly by dissolved oxygen meter (YSI Model 56, Ohio, USA), Suntex pH/ mV/ Temp. Meter (sp-701, Taiwan) respectively. Ammonia concentration was analyzed by the indophenol blue method described by Harwood & Huysen [15], using a spectrophotometer Hitachi (U-2000 Tokyo, Japan). The ranges of water quality parameters were: temperature, 28-29 ℃; salinity, 34-35 ppt; dissolved oxygen, 4.3 – 5.9 mg/L; pH 7.5-7.9 and TAN < 0.2 mg /L.

2.3 Ammonia excretion measurement

One day before the conclusion of the study, ammonia excretion was measured in each tank. All aquarium surfaces were scrubbed to detach any nitrifying bacteria. For measurement of the ammonia, the recirculating system was stopped for the entire sampling period. Water samples were taken in each aquarium before feeding session and 30 min postprandial and then every 2 h for 24 hour's period. Ammonia excretion was standardized by fish biomass in each aquarium as ammonia production per 100 g body weight per hour. Ammonia concentration was measured using the phonol-hypochlorite method [16].

2.4 Biological parameters

At the end of the growth trial, fish from each tank were collected and anaesthetized with MS-222 at a concentration of 200 mg L^{-1}. Their total body length (TL in cm) and body weight (BW in g) were measured individually to determine the condition factor:

$$CF = 100 \times (BW/TL^3)$$

Three fish from each tank were dissected and muscle, liver, fat from the body cavity, viscera, stomach and gut were removed and weighed to determine the following body indices (muscle ratio (MR), hepatosomatic index *(HIS)*, intraperitoneal fat (IPF), viscerosomatic index(VSI), stomachosomatic index (SSI), and gut relative weight (GRW)):

MR = (muscle weight / body weight × 100),
HIS = (liver weight / body weight × 100);
IPF = (IPF weight / body weight × 100),
VSI = (viscera weight / body weight × 100),
SSI = (stomach weight / body weight × 100)
GRW = (gut weight / body weight × 100).

Weight gain (WG), food conversion ratio (FCR), daily food consumption (DFC), meal food consumption (MFC) (%), final size (weight) variation index (SVI) (%) and survival rate (SR) (%) were calculated using the following equations:

$$WG = 100 \times \frac{(\text{Final body weight} - \text{initial body weight})}{\text{initial body weight}}$$

$$FCR = \frac{\text{food consumption}}{\text{body weight increment}}$$

$$DFC(\%) = \frac{\text{food consumption per day}}{\text{body weight}}$$

$$MFC\ (\%) = \frac{\text{food consumption per meal}}{\text{body weight}}$$

$$SVI\ (\%) = 100 \times \text{coefficient of variation}$$

$$SR(100) = 100 \times \frac{\text{number of fish survived}}{\text{number of fish stocked}}$$

2.5 Digestibility and feces production

Digestibility was measured using the remaining fish in each aquarium after the biological measurements. Fish in the same treatment were pooled and transferred to one culture net of dimensions (65 × 30 × 50 cm). The three culture nets were placed in a 2000-L circular tank, to ease the feces collection. The feeding regimes were maintained for a further 30 days. Fish were acclimatized to the marked feed and conditions for 2 weeks before the feces collection. Feces from each tank were continuously collected each 40 min from 1600 to 2100 daily for 10 days. Feces collected from a given cage in the same day were pooled. In addition, feces production was collected every 2 h for a period of 24 hours. Rectangular net placed from beneath each cage was used to collect directly the feces after emission. Fresh and intact feces were selected and oven-dried at 65 ℃, and then stored at – 20 ℃ until chemical analysis. The apparent digestibility coefficient of dry matter (ADC_{dm}) and protein (ADC_{cp}) was calculated using the following formula described by Eusebio *et al.* [17] as follows:

$$ADC = 100 - 100 \times \left(\frac{\%Cr_2O_{3diet}}{\%Cr_2O_{3feces}}\right) \times \left(\frac{\%nutrient_{feces}}{\%nutrient_{diet}}\right)$$

Where: nutrient = dry mater (dm) or crude protein (cp).

2.6 Chemical analysis

Three fish at the start of the experiment and one fish from each tank at the end were frozen for determination of whole body composition**.** Proximate composition was determined for the diet and the whole body fish. Crude protein (CP) content was determined for the feces. Crude lipid (CL), CP, moisture and ash were determined following methods of the Association of Official Analytical Chemists [14], (AOAC, 1990): CP (*N* × *6.25*) was determined by Kjeldahl method after acid digestion using Kjeltec System Tecator. CL was extracted with the methanol-chloroform mixture (1:2) by the method of Folch *et al.* [18]. Moisture was determined by oven drying at 105 ℃ for 24 h and ash was calculated from the weight loss after incineration of the samples for 12 h at 550 ℃ in a muffle furnace. Content of Cr_2O_3 in the diet and feces were determined by the method of Furukawa & Tsukahara [19], using spectrophotometer Hitachi (U-2000 Tokyo, Japan) after acid digestion.

2.7 Statistical analysis

Data were compared using three-way analysis of variance (ANOVA) to test the significance on each effect, followed by Duncan's multiple range test (DMRT) to determine individual mean differences when a significant main effect was found. MFC, digestibility data and ammonia excretion was compared using one-way ANOVA to test the significance on the effect, also followed by DMRT. The significant level was set at $p \leq 0.05$. The survival data were transformed into a normal distribution using the arcsine square root prior to analysis of variance.

3. Results and Discussion

3.1 Growth performance and Feeding

Daily feed consumption (DFC) of fish fed three meals per day was higher than DFC of fed two and one meal per day and there was no difference in DFC between fed one and two meals per day (Table 2). Fish fed one meal per day showed better FCR and poor feed intake compared with fish fed three times daily. A similar result was obtained for sea bass [20], and hybrid striped bass [5]. The better FCR could be attributed to less physiological activity of fish. However, the poor FCR at higher feeding frequency indicates that a portion of the food consumed might have been wasted.

Table 2: The effect of feeding frequency on weight gain, daily food consumption, food conversion ratio, size variation index and survival rate of grouper[1]

Feeding frequency [2]	WG[5]	DFC[6]	FCR[7]	SVI[8]	SR[9]
M1	176.25±37.03	1.17±0.13^{b}	0.90±0.04^{b}	35.84±4.70^{a}	99.00±2.45
M2	165.75±27.43	1.23±0.08^{b}	0.96±0.06^{ab}	34.46±7.09^{ab}	95.00±5.90
M3	197.55±47.65	1.39±0.10^{a}	0.99±0.07^{a}	28.31±4.34^{b}	95.00±5.89

[1]Values are means and standard deviations of 3 replicates. Within a column, values without a common superscript are significantly different ($p \leq 0.05$).
[2]M1: 1-meal /day, 0700 h; M2: 2-meal /day, 0700 h and 2100 h; M3: 3-meals /day, 0700 h; 1400 h and 2100 h.

The SVI of body weight is used to distinguish size variations, which are induced by competition or hierarchy effects. It has been observed that in fish populations where the growth of some individuals is suppressed by competition or hierarchical effects, the SVI increases ([21]. Our result showed that the SVI decreased with increasing feeding frequency. SVI of fish in the treatment group fed three times daily was much lower than in the other treatment groups. This supports the hypothesis that more frequent feeding yields fish of more uniform sizes. This also suggests that satiation feeding at infrequent intervals may not lead to all fish being satiated, and that more frequent feedings may increase feeding opportunities [22].

Table 3 shows the result of feeding pattern. There was a trend that meal food consumption (MFC) reduced as time progressed or fish grew. M1 always had the highest morning MFC among the three feeding frequencies. M3 had the lowest MFC as fish grew. For M2, morning MFC was higher than evening MFC at stage I, but became the other way around at stage III and IV. For M3 at stage I, morning MFC was higher than afternoon and evening MFC and there was no difference between the latter two. However, MFC became no different among the three meals therafter. Fish fed three times per day consumed larger quantity of food per day than those fed less often, but the individual meal consumption becomes smaller. This is consistent with the studies conducted on other species [5, 23]). Fish accomplished this by increasing stomach volume and became hyperphagic [14].

Table 3: The effect of feeding frequency on meal food consumption[1] of grouper[2]

Period[3] Feeding		Feeding time (h)		
	Frequency[4]	0700	1400	2100
Stage I	M1	$2.45^{a} \pm 0.45$		
	M2	$_{y}1.13^{b} \pm 0.14$		$_{x}1.44^{a} \pm 0.10$
	M3	$_{x}1.35^{b} \pm 0.13$	$_{y}0.91 \pm 0.15$	$_{y}0.97^{b} \pm 0.18$
Stage II	M1	$1.81^{a} \pm 0.31$		
	M2	$0.85^{b} \pm 0.30$		0.58 ± 0.11
	M3	$0.68^{b} \pm 0.17$	0.66 ± 0.10	0.71 ± 0.21
Stage III	M1	$1.67^{a} \pm 0.14$		
	M2	$_{x}1.02^{b} \pm 0.15$		$_{y}0.58 \pm 0.07$
	M3	$0.66^{c} \pm 0.08$	0.58 ± 0.11	0.54 ± 0.06
Stage IV	M1	$1.44^{a} \pm 0.09$		
	M2	$_{x}0.83^{b} \pm 0.04$		$_{y}0.59^{a} \pm 0.09$
	M3	$0.57^{c} \pm 0.10$	0.45 ± 0.10	$0.49^{b} \pm 0.06$

[1]meal food consumption (% body weight per meal) = food consumption per meal / body weight (in the feeding pattern trial).
[2]Values are means and standard deviations of 3 replicates. Within a column, values without a common superscript (a, b, c…), and within a row, values without a common subscript (x, y, z…) are significantly different ($p \leq 0.05$).
[3]Stage I: week 0-2; stage II: week 2-4, stage III: week 4-6 and stage iv: week 6-8.
[4]M1: 1-meal /day, 0700 h; M2: 2-meal /day, 0700 h and 2100 h; M3: 3-meals /day, 0700 h; 1400 h and 2100 h.

3.2 Biological parameters

Except on condition factor (CF) and stomachosomatic index (SSI), feeding frequency had no effects on the other biological parameters (Table 4). M1 had the lowest CF. There was no difference in CF between M2 and M3. M1 had the highest SSI. No difference in SSI was found between M2 and M3.

Table 4: The effect of feeding frequency on biological parameters of grouper[1]

Feeding Frequency	CF	HSI	VSI	IPF	MR	GRW	SSI
M1	1.4 ± 0.1^{b}	2.7±0.6	10.9±1.5	1.7±0.3	31.9±4.2	0.8±0.1	0.8 ± 0.1^{a}
M2	1.4 ± 0.1^{b}	3.1±1.4	10.8±1.5	1.6±0.7	31.8±4.2	0.7±0.1	0.7 ± 0.1^{b}
M3	1.5 ± 0.1^{a}	3.1±0.7	11.7±1.1	1.9±0.5	36.1±1.9	0.7±0.1	0.7 ± 0.1^{b}

[1]Values are means and standard deviations of the replicates (n=3). Within a column, values with different superscripts are significantly different ($P < 0.05$).

Table 5: The effect of feeding frequency, stocking density and fish size on body composition of juvenile grouper[1]

Feeding frequency[2]	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
Initial	71.70 ± 0.55	17.61 ± 0.52	5.38 ± 0.28	4.28 ± 0.24
M1	69.16 ± 0.57	18.84 ± 0.75	$5.71^{b} \pm 0.68$	4.73 ± 0.36
M2	69.91 ± 0.57	18.57 ± 0.41	$5.47^{b} \pm 0.53$	4.88 ± 0.50
M3	69.77 ± 1.14	18.81 ± 0.49	$6.41^{a} \pm 0.48$	4.48 ± 0.23

[1]Values are means and standard deviations of the replicates (n=3). Within a column, values with different superscripts are significantly different ($P < 0.05$).
[2]Feeding frequencies were: M1, one meal per day; M2, two meals per day and M3: three meals per day.

Fish fed once daily showed the highest SSI. The higher SSI observed in fish fed one meal per day indicated that these fish become hyperphagic, which is inducsed by the time deprivation of food [24]. However, the intake compensation might not be possible as indicated by the lower daily food consumption compared to fish fed three meals per day. Hyperphagia is usually accompanied by increase in gastric capacity, which result in an increase in the stomach weight of animals fed intermittently [21]. In mammals, hyperphagia is accompanied by consistent increases in the weight of the stomach, intestine and liver of fed animals [25].

3.3 Proximate composition

At the end of this study fish, appeared to have lower moisture, but higher CP, CL, and ash than fish before the rearing (Table 5). Fish fed three times daily had the highest lipid content. Crude protein, moisture and ash contents did not differ significantly among the three groups. The higher lipid content and condition factor of the fish fed three times daily suggest that these fish gain more fatness compared to fish allowed less frequent access to food. In addition, an increase of lipid content without growth improvement of the fish that were fed thrice a day compared with fed once or twice a day suggested that juvenile grouper fed three meals per day at satiation would be overfed. This is in consistent with many studies, which reported that increase of feeding frequency in several species of fish led to a high body lipid content [14, 26, 27].

3.4 Ammonia excretion

The rate of ammonia excretion increases rapidly in response to feed intake [20, 28], and the majority of the nitrogen excreted is derived from deamination of amino acids from dietary proteins [29, 30]. Hourly ammonia-nitrogen excretion rates increased 4 h after the morning feed. Table 6.

Table 6: Effect of feeding frequency on ammonia excretion for grouper[1] (mg NH_3-N/100g body weight/ h)

	Hours (h)					
FF	0-4	4-8	8-12	12-16	16-20	20-24
M1	1.51^{a}±0.26	1.28±0.24	0.84±0.32	1.10±0.16	0.32^{b}±0.04	0.77±0.00
M2	1.04^{b}±0.12	0.92±0.76	0.63±0.11	1.23±0.05	1.39^{a}±0.41	1.25±0.37
M3	1.18^{b}±0.15	1.24±0.40	1.47±0.45	1.52±0.27	0.61^{ab}±0.22	0.70±0.17

[1]Values are means and standard deviations of the replicates (n=3). Within a column, values with different superscripts are significantly different ($P < 0.05$).
[2]Feeding frequencies were: M1, one meal per day; M2, two meals per day and M3: three meals per day.

The ammonia excretion seemed to be related to the amount of food intake by the fish. The daily dynamics of changes in the ammonia nitrogen release rate, found in this study, reflected a gradual increase in the fish metabolic activity of feed, starting about 2 h after the feed was offered. Most studies have found that peak excretion of ammonia occurs 6-8 h after feeding [25]. When studying *Lates calcarifer*, [31] found the maximum NH_3-N release as early as 3 h after the start of feeding. [32], reported that feeding American eels once a day, increased the ammonia excretion to a peak 4 h following feeding and returned to pre-feeding level 10 h thereafter, this support our result that feeding grouper to satiation once a day, increased the ammonia excretion to a peak 4 h following feeding and returned to pre-feeding level 20 h thereafter. In addition, [33] found that following the single meal morning feed, the excretion rate in Sockey salmon rose sharply to a peak, falling rapidly thereafter to the early morning base level. Feeding twice a day, had a cumulative effect on ammonia excretion and rates after a 24 h period remained higher compared to pre-feeding levels. This is in consistent with the founding of [34]. Feeding thrice a day, had a cumulative effect of ammonia excretion and rates after a 24 h period remained higher compared to pre-feeding levels.

3.5 Digestibility

The result of digestibility is presented in Table 7. Feeding one, two or three meals per day to satiation did not result in differences in apparent digestibility of dry matter and protein in juvenile grouper.

Table 7: The effect of feeding frequency on apparent digestibility in juvenile grouper[1]

Feeding frequency[2]	Dry matter	Protein
M1	68.99 ± 2.86	91.70 ± 1.09
M2	70.30 ± 2.13	92.07 ± 0.75
M3	70.23 ± 2.94	92.57 ± 0.10

[1]Values are means and standard deviations of the replicates (n=6). Within a column, values with different superscripts are significantly different ($P < 0.05$).
[2]Feeding frequencies were: M1, one meal per day; M2, two meals per day and M3: three meals per day.

Apparent dry matter digestibility describe how efficiency the feeds is digested and how much their nutrient composition can be made available to the fish for maintenance and growth. Feeding frequency was reported to have no effects on apparent digestibility by many authors [35, 36]. Similar result was found in our study, which showed that the apparent digestibility coefficient of dry matter and protein was not influenced by feeding frequencies. Apparent protein digestibility of different feeding frequency for grouper in this study was high and ranged from 91.70 % to 92.57 %. Eusebio *et al*. [14] reported ADP value of 90.6 % in grouper fed on formulated feed based on Chilean fish meal. Jayaram & Shetty [37] reported ADP value of 91.88 % in rohu fish. The ADM in this study ranged from 69 % to 70.3 %, Eusebio *et al*. [14] found similar result in grouper.

During non-feeding most of the time, fish were motionless on the bottom of the aquarium. Occasionally, some fish were seen swimming. Fish fed lower frequencies, were more active during feeding and may developed food anticipatory activity (FAA), prior to feeding. In study of Chen & Purser [38], they found that Greeback flounder fed lower frequencies, were more active during feeding and developed food anticipatory activity, prior to feeding, which they suggest benefits the fish by optimizing feed intake and feeding efficiency.

4. Conclusion

Feed intake and lipid deposition increased by increasing the number of meals from one to three, without enhancing the growth, thus feeding one times a day to satiation seems to be sufficient for maximal growth in juvenile grouper under our rearing conditions. The results of this study could be relevant advantageously to grouper farming strategies for maximum production

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