Effects of Stocking Density and Feeding Frequency on the Growth Performance, Digestive Enzyme Activity, and Tissues Histology of Hybrid Grouper (*Epinephelus Fuscoguttatus* × *E. Lanceolatus*)

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Abstract

A 6-week 5×2 factorial study was conducted to examine the effects of stocking density and feeding frequency on growth performance, body composition, digestive enzyme activity, and tissue histology of juvenile hybrid groupers raised in an indoor circulating water system. Triplicate groups of fish were reared in tanks following a factorial design consisting of ten treatments including 5 stocking densities (SD) [1.1 kg m⁻³ (0.55 kg fish per tank, SD1.1), 2.2 kg m⁻³ (1.1 kg fish per tank, SD2.2), 3.3 kg m⁻³ (1.65 kg fish per tank, SD3.3), 4.4 kg m⁻³ (2.2 kg fish per tank, SD4.4) and 5.5 kg m⁻³ (2.75 kg fish per tank, SD 5.5)] and 2 feeding frequencies (FF) [3 meals a day (FF3) and 2 meals a day (FF2)]. The resulting 10 treatments were G1(SD1.1,FF3), G2(SD2.2,FF3), G3(SD3.3,FF3), G4(SD4.4,FF3), G5(SD5.5,FF3), G6(SD1.1,FF2), G7(SD2.2, FF2), G8(SD3.3, FF2), G9(SD4.4, FF2) and G10 (SD5.5, FF2). Feed consumption and temperature were recorded throughout the experiment. After 6 weeks, the results indicated that the weight gain rate (WGR) and specific growth rate (SGR) of fish in the G7 group were significantly higher than those of other groups ($P < 0.05$), followed by G1, with G3 being the lowest. Weight gain and specific growth rates were generally higher in fish fed twice a day than those fed three times a day. The variations in protein content between groups were consistent with the muscle protein content trends. Feeding frequency and stocking density had significant effects on serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides (TGs), and cholesterol (CHO) ($P < 0.05$). Regarding immune function, grouper albumin (ALB), serum lysozyme (LZY), superoxide dismutase (SOD), and alkaline phosphatase (AKP) were significantly affected by stocking density and feeding frequency ($P < 0.05$). Pepsin and lipase activities in the stomach, intestine, and liver were also affected. The histological structure of the stomach, liver, and intestine in G1, G2, G7, and G8 fish was relatively normal, whereas those of the remaining groups exhibited varying degrees of damage. Overall, the optimum stocking densities were 1.343 kg/m³ (approximately 10 fish) and 2.004 kg/m³ (approximately 20-30 fish) when the fish were fed 3 and 2 times per day, respectively.

Background

The balance between rapid fish growth, feed utilization efficiency, feeding strategy, and intensive fish-farming practices is a key point in fish aquaculture [1, 2]. Feeding time and frequency have been reported to affect feed intake and growth performance in different fish species including the Australian snapper *Pagrus auratus* [3], pikeperch *Sander lucioperca* [4], African catfish *Clarias gariepinus*, Burchell 1822 [5], flounder *Platichthys flesus* [6] and rock bream *Oplegnathus fasciatus* [7]. Water quality, management procedures, stocking density, feeding frequency, and nutritional conditions are all directly associated with fish stress [8, 9, 10].

Feeding frequency is a well-known regulator of growth performance, feed intake, and chemical composition of fish, and therefore optimizing this factor can substantially reduce aquaculture production costs and prevent water quality deterioration resulting from excess feeding. In practice, fish are fed by most farmers to satiety based on visual observation. However, it is very difficult to identify the point at which fish are satiated and fish are overfed frequently. The stomach and intestine may exceed their capacity as a result of overfeeding, which leads to decreases in digestive efficiency and feed utilization [11]. On the other hand, an insufficient feeding frequency leads to poor growth and high mortality, particularly in intensive systems [12]. For example, sporadic and low feeding rates may lead to slower growth rates, increased hunger, intraspecific aggression, and increased cannibalism [13].

Intensive fish farming practices often result in fish stress and poor health. In contrast, improved feeding conditions not only benefit the welfare of fish but also aquaculture profits [9]. In most fish species, high stocking densities adversely affect fish survival, weight gain, and specific growth rates [14]. These effects are attributed to the competition for food and/or space resulting from food scarcity [15], which leads to the establishment of hierarchy [16]. Additionally, high stocking densities can increase the likelihood of physical injuries, stress, and susceptibility, and can also modulate swimming and aggressive behaviors [17]. There is considerable evidence of welfare decline in rainbow trout *Oncorhynchus mykiss* stocked at high densities; however, excessive aggression and poor feeding rates may also occur when rainbow trout are reared at very low densities [18].

Hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) is among the most widely farmed fish species worldwide, particularly in China. Although some work has been done regarding the effects of temperature, nutrients, and disease on grouper growth [19, 20, 21, 22, 23], studies on the effect of feeding frequency and stocking density on this species in Chinese waters remains scarce. Moreover, the synergistic effects of both parameters have not been characterized. Therefore, our study sought to evaluate the effects of stocking density and feeding frequency on growth performance, body composition, digestive enzyme activity, and histology of juvenile hybrid groupers, with the overarching goal of determining whether these two stressful fish farming practices affect fish physiology synergistically or individually.

2 Methods

2.1 Experimental diets

Diet formulation and proximate analysis [Association of Official Analytical Chemists (AOAC), 1984] [24] details are provided in Table 1. The diet ingredients including fishmeal, soy protein concentrate, casein, shrimp meal, wheat flour, binding agents, soybean oil, fish oil, squid visceral ointment, choline chloride, and monocalcium phosphate were acquired from Guangzhou Rifeng Science & Technology Co., LTD. The mineral and vitamin premixes were purchased from Guangzhou Ashare Aquatech Co., Ltd

All dry ingredients were first thoroughly mixed in a blender, after which the oil was slowly added to the mixture while blending. Deionized water (300 mL kg⁻¹ dry mixture) was then slowly mixed in to achieve a dough-like consistency. This dough was then extruded into pellets (3 mm in diameter) using a twin-screw extruder (South China University of Technology F-26 (II)) and dried in a 60 °C oven until the moisture content was less than 8%. All pellets were sealed in plastic bags and frozen (−20 °C) until used.

2.2 Experimental fish and feeding trial

Prior to the experiment, the use of animals was approved by the "Institutional Animal Care and Use Committee of Hainan Tropical Ocean University" and "Hainan Key Laboratory for Conservation and Utilization of Tropical Marine Fishery Resources" (20161111A1). Juvenile hybrid groupers were acquired from a fish farm (Hainan Chenhai Products Co., Ltd.). The fish were originally kept in a high-quality well water circulation system, after which they were randomly assigned to 30 outdoor circular tanks (500 Litres) that were continuously supplied with 30 liters of water per minute. The following were the inflow water quality parameters: 27.0–28.9 °C; 7.0–8.2 mg L⁻¹ of dissolved oxygen; pH 6.8–7.5; 28.1–31.1‰ salinity; and 0.05–0.1 mg/L total ammonia nitrogen. Feed intake and fish mortality were recorded daily.

Two variables were considered together: feeding frequencies (FF) [3 meals a day (FF3) and 2 meals a day (FF2)] and stocking density (SD) [1.1 kg m⁻³ (0.55 kg fish per tanks, SD1.1), 2.2 kg m⁻³ (1.1 kg fish per tank, SD2.2), 3.3 kg m⁻³ (1.65 kg fish per tank, SD3.3), 4.4 kg m⁻³ (2.2 kg fish per tank, SD4.4), and 5.5 kg m⁻³ (2.75 kg fish per tank, SD5.5)]. Based on this 5 × 2 factorial design, ten treatments were analyzed in triplicate randomly: G1(SD1.1,FF3), G2(SD2.2,FF3), G3(SD3.3,FF3), G4(SD4.4,FF3), G5(SD5.5,FF3), G6(SD1.1,FF2), G7(SD2.2,FF2), G8(SD3.3,FF2), G9(SD4.4,FF2), and G10(SD5.5,FF2). The trial was conducted for 42 days without counting a 15-day pre-trial acclimation period.

2.3 Sample collection

At the end of the growth test, the fish were collected after 24 hours of fasting, anesthetized with tricaine methanesulfonate (MS222) (50 mg L⁻¹), and weighed/measured. Fish numbers and weights per tank were recorded to calculate the feed efficiency ratio (FER), specific growth rate (SGR), survival, and protein efficiency ratio (PER). Eight fish were randomly selected from each tank for proximate analysis, three for whole-body composition tests and five for white muscle components.

The viscera, liver, and intraperitoneal fat of fish were quickly removed and weighed to calculate hepatosomatic index (HSI), viscerosomatic index (VSI), and intraperitoneal ratio (IPR). Blood samples were taken from the tail vein of eight fish in each tank with syringes and centrifuged at 3000 g for 10 minutes at 4 °C.

2.4 Evaluation of growth performance

The main growth performance variables were calculated as follows:

WG (%) = 100 × (final mean weight - initial mean weight)/initial mean weight (g)

Survival (%) = 100 × final fish number/initial fish number

Average body weight gain (BWG, g) = (W_f - W_i)/number of fishes;

Specific growth ratio (SGR, % d⁻¹) = 100 × (ln W_f - W_i)/t;

Weight gain ratio (WGR) = (W_f - W_i)/W_i;

Daily feed intake (DFI, % d⁻¹) = 100 × dry feed intake/[(initial feed weight + final weight)/2 × t];

Condition factor (CF, g cm⁻³) = 100 × (W_f/L³);

Hepatosomatic index (HSI, %) = 100 × (wet weight of the hepatopancreas/W_f);

Intraperitoneal ratio (IPR, %) = 100 × (intraperitoneal fat weigh/W_f);

where W_i and W_f are the initial and final weights (g) of the fish during the experiment. t is the duration of the experiment (d); F is the weight of the feed provided to the fish; L is the average body length (cm) of the fish.

2.5 Biochemical analysis

Proximate composition analyses of diets, fish whole body, and tissues were performed according to the AOAC guidelines (1984)[24]. Moisture analysis was conducted by drying the samples at 105 °C for 24 hours. After acid digestion with an automatic Kjeldahl System (Kjeltec 8400, FOSS, USA), the protein content of the diet and fish samples were analyzed via the Kjeltex nitrogen × 6.25 method. Moreover, the lipid and ash contents of the diets and samples were respectively analyzed with a Soxtec System (Soxtec System 2050; Foss, USA) and combustion at 550 °C for 24 h.

Plasma biochemistry was characterized with a Modular P800 automatic biochemical analyzer (Roche Diagnostics, Mannheim, Germany) at Guangzhou Kingmed Center for Clinical Laboratory Co., Ltd.

2.6 Digestive enzyme activity

To assess digestive enzyme activity, approximately 1 g of frozen wet heavy tissue samples (gastric, intestinal, and hepatic) were homogenized with 9 mL cold normal saline, then centrifuged at 2,500 rpm for 10 min at 4°C. The final supernatant was divided into subsamples for each enzyme analysis and stored at 4 °C until all the enzyme analyses were completed within 24 hours. Amylase activity was determined with a test kit (Jiancheng Biotech Co., Ltd., Nanjing, China.) according to the manufacturer's instructions. One amylase unit was defined as the activity required to hydrolyze 10 mg of starch in 30 min at 37°C. Lipase activity was determined as described by Li et al. (2005)[25] using olive oil as substrate. Each lipase activity unit was defined as the release of 1 μmol of fatty acids per minute per gram of protein. A slightly modified version of the Anson method was used to determine pepsin activity using casein as the

substrate coupled with the Folin reagent, and total soluble protein was determined using bovine serum albumin as standard using the Bradford method, as described by Brito et al. (2000)[26].

2.7 Tissues histology

The collected specimens were fixed with 10% buffered formalin for at least 24 hours, then dehydrated, paraffin-embedded, and cut into 4 μm thick slices on an RM2135 rotary slicer (Wetzlar Leica, Germany). The sections were stained with hematoxylin and eosin (H&E) and histopathological examinations were performed according to standard methods. All sections were examined using a DFC495 optical microscope.

2.8 Statistical analysis

All data were reported as the mean $\pm$ standard deviation (SD, $n = 3$). Mean values were compared using one-way ANOVA, and variance normality and heterogeneity tests were performed using SPSS19.0 (SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant. The regression model was established using Origin 9.0 (OriginLab Corporation, USA).

3 Results

3.1 Growth performance

Table 2 summarizes the growth and biometry analysis results of juvenile pearl gentian groupers at different stocking densities and feeding frequencies. W_f , BWG, WGR, SGR, and DFI were significantly different among the treatment groups ($P < 0.05$). Specifically, W_f , BWG, WGR, SGR, and DFI in treatments G1, G7, and G8 were significantly higher than those in the other treatments ($P < 0.05$). G7 had the highest W_f , BWG, WGR, and SGR, followed by treatments G1 and G8. Our results indicated that optimal W_f , BWG, WGR, and SGR values were obtained at a stocking density of 2.2 kg m^{-3} when the fish were fed twice a day, followed by a 3.3 kg m^{-3} stocking density when the fish were fed 3 times a day, relatively higher W_f , BWG, WGR, and SGR values were observed at a 1.1 kg m^{-3} stocking density.

The DFI values of juvenile hybrid groupers in the G1-G5 groups were significantly different even at high stocking densities and a 3-times-per-day feeding frequency (Table 2). The DFI in the G3 group was significantly lower than that of the other groups ($P < 0.05$), whereas the highest DFI was observed in the G2 group, followed by G5. When the fish were fed twice per day, the DFI of the juvenile hybrid groupers decreased gradually with increasing stocking density. Overall, the DFI was the parameter that was most affected by stocking density and feeding frequency. No mortality was recorded throughout the experimental trials.

Upon comparing the two feeding frequencies evaluated herein, no significant differences in CF were observed among groups except for G8, which exhibited a significantly higher value, and G6 had the lowest value ($P < 0.05$). The HSI values of the fish in the G4 and G7 groups were significantly higher than those in the G1, G2, G6, and G9 groups ($P < 0.05$) but not significantly different from those in other groups. Feeding frequency and stocking density had no significant impact on fish, except in the G1 and G10 treatment groups.

According to the BWG regression model (y-axis) in response to the stocking density when the fish were fed twice a day (x-axis), the optimal stocking density was 2.004 kg/m^3 . However, based on BWG (y-axis) in response to the stocking density when the fish were fed three times a day (x-axis), the optimal stocking density was 1.343 kg/m^3 .

3.2 Biochemical analysis

3.2.1 Nutrients analyses in whole fish and muscle

As the stocking density increased in fish fed three times a day, the moisture content gradually increased, and protein, fat, and ash contents were gradually reduced, particularly in the G1 group, which was significantly different from the G5 group ($P > 0.05$). In contrast, increasing the stocking density in fish fed twice a day did not significantly affect moisture contents ($P > 0.05$). On the other hand, protein, fat, and ash contents were significantly different between treatments, particularly in the G7 and G8 groups, which were significantly higher than the other three groups ($P < 0.05$).

When the stocking density was increased in fish fed three times a day, the moisture content of the whole fish was no significantly different ($P > 0.05$). Moreover, the protein contents of G2-G5 and G9-10 were not significantly different ($P > 0.05$) but were significantly lower than those of the G1 and G6-7 groups ($P < 0.05$). There was no significant difference in fat content ($P > 0.05$) regardless of feeding frequency with increasing stocking density. The ash contents were the highest in the G1 group, followed by the G6 group, which was significantly higher than the other treatments ($P < 0.05$).

3.2.2 Serum biochemical analyses

Fish serum biochemical indicators are summarized in Table 4. When the stocking density was increased in fish fed three times a day, AST (aspartate aminotransferase) and CHO (cholesterol) levels increased gradually and were significantly different between the treatment groups, with the G1 group being significantly lower than the other groups ($P < 0.05$). Moreover, when the fish were fed twice a day, the G7 and G8 groups were significantly lower than the other three groups ($P < 0.05$).

Regarding immune function, our study characterized TP (total protein), GLB (globin), ALB (albumin), GLU (glucose), LZY (lysozyme), SOD (total superoxide dismutase), and AKP (alkaline phosphatase), all of which were significantly different between treatment groups. When the fish were fed three times a day, the G4 group had the highest TP, GLB, and ALB values among all tested groups ($P < 0.05$), followed by G5 and G1. Moreover, groups G1 and G2 exhibited significantly higher GLU, LZM, SOD, and AKP values than the other groups. When the fish were fed twice a day, the G7 group exhibited significantly higher TP,

GLB, and LZM values than the remaining groups ($P < 0.05$). The SOD and AKP values of groups G7-G9 were not significantly different, whereas G6 had the highest values.

3.3 Tissues enzyme assays

As demonstrated in Table 5, there was a significant difference between the gastric, intestinal, and hepatic pepsin and lipase activity between groups ($P < 0.05$). Pepsin and lipase activity increased with increasing density regardless of stocking density, with the G5 and G10 groups having the highest values ($P < 0.05$), followed by G4 and G9, whereas the G1 and G6 groups had the lowest values. Additionally, no significant differences in gastric, intestinal, and hepatic pepsin and amylase activities were observed between treatments regardless of feeding frequency ($P > 0.05$).

3.4 Tissues histology

As illustrated in Fig. 1, the vasodilatation of the gastric wall of the G3, G4, G9, and, G10 fish indicated the presence of inflammatory cells in these organisms. Moreover, white vesicles were identified in the cells of fish in the G3, G4, G9, and G10 groups, indicating that the gastric cells in those groups were metamorphosed and became adipogenic. Additionally, gastric tissue sections of other fish groups exhibited varying degrees of inflammation and fat granules except for G7 and G8, which were normal. Figure 2 shows that increasing stocking densities in the fish fed three times a day resulted in lamina propria edemas in the intestinal tissue sections of the G3, G4, and G5 fish, as well as thinner and shorter intestinal villi, some of which were starting to detach. In contrast, the aforementioned symptoms were only identified in the G10 fish that were fed twice a day. Finally, as seen in Fig. 3, vacuolation occurred in the G2, G5, G6, G9, and G10 fish, and nuclear deviations were observed in G3 and G4, whereas the hepatocytes in G1, G7, and G8 were normal.

4 Discussion

Many factors affect fish growth, including diet, fish size, feeding time, stocking density, and feeding frequency. In this experiment, when the fish were fed three times a day, the BWG, WGR, and SGR of fish stocked at a density of 0.55 kg per tank gradually decreased with increasing stocking density and was significantly higher than the other four groups. This suggested that the growth performance of the fish in the 1.10 kg and 1.65 kg per tank stocking densities was better than that of the other groups with the same feeding frequency. In other words, feeding frequency and stocking density influenced the growth performance of this species, which was consistent with previous studies[27, 28]. The poor growth performance observed in the high stocking density was likely due to crowding stress[29].

Additionally, our findings suggested that feeding three times per day instead of two may have affected satiety levels, thereby resulting in food waste. Several studies have reached different conclusions regarding the effect of stocking density on the growth performance of fish depending on the species. For instance, Rowland et al. (2006) [30] reported that stocking density had no significant effects on silver perch. In contrast, high stocking density decreased the final weights of Brook charr according to Vijayan et al. (1990)[31], and Boeuf et al. (1989)[32] reported that lower densities had positive effects on the growth of Atlantic salmon during smolting. According to the quadratic regression model based on the BWG of the hybrid groupers studied herein, when fed twice a day, the function was $y = -0.4872163x^4 + 6.391186x^3 - 28.19146x^2 + 45.84394x - 6.26$, $R^2 = 1$ and we predicted that the optimal stocking density was 2.004 kg/m^3 , whereas when the fish were fed three times a day, the function was $y = -0.6366255x^4 + 9.53982x^3 - 50.46522x^2 + 107.8068x - 55.9$, $R^2 = 1$ and the optimal stocking density was 1.343 kg/m^3 .

Blood can objectively reflect the nutritional metabolism of farmed animals and is an important physiological, pathological, and toxicological indicator. AST and ALT are common indicators of liver cell damage. Specifically, when liver cells are damaged, AST and ALT enzymes will infiltrate into the blood, causing a significant increase in the activity of these enzymes in the blood. Previous studies have linked the occurrence of liver damage with elevated AST and ALT levels [33, 34, 35, 36]. In this study, AST levels increased significantly with stocking density, except at a 2.2 kg/m^3 . Moreover, ALT decreased significantly with increasing stocking density, particularly at 4.4 kg m^{-3} and 5.5 kg m^{-3} in both feeding frequencies. Our findings suggest that although increases in AST were indicative of some degree of liver damage, lower ALT levels may explain the 100% survival ratio, as it is an important indicator of fish health [37].

SOD can eliminate excess free radicals in organisms [38] and modulate the balance between the formation and elimination of free radicals. Therefore, this parameter is an important physiological indicator of antioxidant capacity and non-specific immune function. LZY and AKP are also important indicators of non-specific immunity in the body. AKP is very important for various metabolic processes including phosphorylation and dephosphorylation, as well as nutrient absorption, and its activity can be used as an indicator of fish immune function.

Previous studies have demonstrated that high stocking densities were associated with decreases in SOD activity in some fish [39, 40] and shrimps [41, 42]. LZY is known to disrupt the structure of the bacterial cell wall, and therefore LZY levels can directly reflect lytic capacity, as well as the impact of pathogens and other environmental factors on fish health. In Senegalese sole, high stocking densities reduced plasma LZY levels[43]. A similar pattern was found in juvenile turbot[39]. In our study, high stocking densities had negative effects on SOD, AKP, and LZY concentrations, which may explain why high stocking densities often lead to diseases. Nonetheless, other studies have demonstrated that high stocking densities could increase AKP activity[44, 45].

TG and CHO are key indicators of blood lipid levels. According to medical research, elevated TG and CHO levels are linked to fatty liver. TP includes GLB and ALB, which are involved in protein metabolism. Albumin content generally accounts for more than 50% of total plasma protein content and is known to maintain osmotic pressure balance between blood vessels and tissues, mediate substance binding and transport, protect blood cells and organs, inactivating inflammation, and also acts as an antioxidant and damage repair agent, all of which are closely related to the body's immune function.

In this study, the CHO levels of groupers gradually decreased with increasing stocking density regardless of feeding frequency, meaning that higher stocking densities resulted in more liver cell damage, which would negatively affect fish digestion and fat translation. Moreover, TP and ALB showed a gradual

increase, whereas GLB and GLU decreased gradually with increasing stocking density, indicating that the vital activities represented by the above indicators were gradually weakened as the stocking density increased.

Previous studies have shown that the detection and analysis of digestive enzyme activities can directly reflect the physiological status of fish digestion [46, 47, 48]. In the present study, increases in stocking density had a positive effect on gastric, intestinal, and hepatic pepsin and lipase activities regardless of feeding frequency. However, these results were not consistent with a previous study in which the lipase, trypsin, and amylase activities of *Oreochromis niloticus* fingerlings were all depressed in high-density conditions (600 organisms m^{-3}) [44]. This indicated that the fish exhibited better digestive physiology at an appropriate stocking density, which may also explain why the fish had higher growth rates in the corresponding density range. However, the high stocking density in our study may have elicited crowding stress, thereby requiring additional energy expenditure to resist the stress, which in turn required an increased digestive performance. We therefore speculated that increased digestive enzyme activity may be a protective mechanism in high-density conditions; however, this hypothesis must be further explored in future studies.

In this study, feeding frequency and stocking density were found to significantly influence gastric, intestinal, and hepatic tissue histology. When the fish were fed three times a day, even low stocking densities (e.g., 1.1 kg m^{-3}) resulted in pathological signs or symptoms, which was also observed in fish that were fed twice a day and stocked at 2.2 kg m^{-3} and 3.3 kg m^{-3} densities. These results were consistent with those of Refaey et al. (2018)[49], who reported that a high stocking density (300 fish m^{-3}) resulted in intestine and muscle histological changes.

5 Conclusions

Based on observations of grouper growth performance, digestive enzyme activity, and tissue histology at different stocking densities and feeding frequencies, the optimum stocking density was 1.343 kg/m^3 (approximately 10 fish) when the fish were fed three times a day, and 2.004 kg/m^3 (approximately 20–30 fish) when the fish were fed twice a day.

Abbreviations

SD: stocking densities; FF: feeding frequencies; WGR: weight gain rate; SGR: specific growth rate; FER: feed efficiency ratio; PER: protein efficiency ratio; HIS: hepatosomatic index ; VSI: viscerosomatic index; IPR: intraperitoneal ratio; BWG: body weight gain; CF: condition factor; H&E; hematoxylin and eosin ; DFI: Daily feed intake; AST: aspartate aminotransferase; ALT; alanine aminotransferase; TGs: TP: total protein; GLB: globin; ALB: albumin; GLU: glucose);triglycerides; CHO: cholesterol; ALB: albumin; LZ: lysozyme; SOD: superoxide dismutase; AKP: alkaline phosphatase; AOAC: association of official analytical chemists; MS222: tricaine methanesulfonate; SD: standard deviation; W_i : initial weights; W_f : final weights; WW: wet weight.

Declarations

-Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. The use of animal was approved by the Institutional Animal Care and Use Committee of Hainan Tropical Ocean University.

-Consent for publication

Not applicable

-Availability of data and material

All data generated or analysed during this study are included in this article.

-Competing interests

The authors declare no conflicts of interest.

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-Authors' contributions

Yan Chen performed the conduct of Experiments, analyzed and interpreted the data regarding growth performance and biochemical analysis of this species, and was a major contributor in writing the manuscript. Huanghai & Jun Ma analyzed and explained the relevant data regarding the tissues enzyme assays and tissues histology of this fish. All authors read and approved the final manuscript.

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Tables

Table.1 Formulation and proximate composition of the experimental diet.

Ingredients	%
Fishmeal	50.00
Soy protein concentrate	12.00
Casein	4.00
Shrimp meal	3.50
Wheat flour	20.5
Binding agents	2.00
Soybean oil	1.50
Fish oil	1.50
Squid visceral ointment	1.50
Vitamin premix ²	1.00
Mineral premix ³	1.00
Choline chloride	0.50
Monocalcium Phosphate	1.00
Proximate composition	%, dry matter basis
Crude protein	43.84
Crude lipid	8.20
Gross energy (MJ/kg)	17.56

Proximate composition values represent means $\pm$ standard deviation of triplicate samples, rounded to the nearest 0.1 g.

1. Obtained from Guangzhou Rifeng Science & Technology Co., LTD (Guangzhou, China).

2. Vitamin mixture (mg kg^{-1} diet): retinol acetate, 38.0; cholecalciferol, 13.2; α -tocopherol, 210.0; thiamin, 115.0; riboflavin, 380.0 pyridoxine 88.0; pantothenic acid, 368.0; niacin acid, 1030.0 biotin, 10.0; folic acid, 20.0; vitamin B12, 1.3; inositol, 4000.0; ascorbic acid, 500.0 (Ding et al., 2010).

3. Mineral mixture (mg kg^{-1} diet): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3568.0; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 25568.0; KCl, 3020.5; $\text{KAl}(\text{SO}_4)_2$, 8.3; CoCl_2 , 28.0; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 353.0; Calactate, 15968.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 9.0; KI, 7.0; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 63.1; Na_2SeO_3 , 1.5; $\text{C}_6\text{H}_5\text{O}_7\text{Fe} \cdot 5\text{H}_2\text{O}$, 1533.0; NaCl, 100.0; NaF, 4.0 (Ding et al., 2010).

Table.2 Growth and biometry of juvenile pearl gentian groupers (mean $\pm$ S.D., n=3)

Group	Growth performance			Biometric indices						
	W _i (g)	W _f (g)	BWG(g)	WGR (%)	SGR(%/d)	DFI (%/d)	Survival(%)	CF(g/cm ³)	HIS (%)	IF
G1	11.03±0.09	28.88±0.003b	17.85±0.11b	1.62±0.02b	2.29±0.02b	2.65±0.01d	100.00±0.00	1.53±0.16bcd	1.83±0.50c	0
G2	11.10±0.05	24.89±0.02d	14.79±0.03c	0.89±0.04d	2.15±0.01cd	2.91±0.04a	100.00±0.00	1.67±0.24abc	1.98±0.84bc	1
G3	11.22±0.18	21.14±0.06f	9.92±0.59f	0.65±0.04e	1.51±0.08g	2.23±0.01e	100.00±0.00	1.56±0.23abcd	2.72±0.83ab	1
G4	11.28±0.10	22.76±0.12ef	11.48±0.12e	0.85±0.03d	1.67±0.02f	2.73±0.02c	100.00±0.00	1.76±0.37ab	2.87±0.87a	1
G5	11.02±0.07	21.62±0.02f	10.59±0.06f	0.73±0.01e	1.60±0.01fg	2.82±0.03b	100.00±0.00	1.72±0.29abc	2.13±0.64abc	1
G6	11.03±0.04	23.96±0.06de	13.39±0.28d	1.27±0.06c	1.95±0.06e	2.74±0.01c	100.00±0.00	1.38±0.16d	1.53±0.86c	0
G7	11.12±0.06	33.86±0.21a	23.69±0.11a	2.33±0.01a	2.86±0.01a	2.67±0.01d	100.00±0.00	1.67±0.15abc	2.95±0.62a	1
G8	11.17±0.07	30.18±1.72b	17.63±0.38b	1.29±0.02c	2.26±0.04bc	2.10±0.01f	100.00±0.00	1.80±0.24a	2.20±0.90abc	1
G9	11.32±0.07	26.79±0.08c	15.47±0.08c	1.26±0.01c	2.05±0.01de	1.96±0.01g	100.00±0.00	1.49±0.24cd	2.00±0.54bc	1
G10	11.17±0.03	26.64±0.22c	15.10±0.36c	1.24±0.03c	1.99±0.05e	1.86±0.01h	100.00±0.00	1.58±0.15abcd	2.80±0.73ab	1
ANOVA (<i>P</i> value)										
Breeding density	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.047	0.012	0
Feeding frequency	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.221	0.948	0
Interactions	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.032	0.003	0

Values (mean ± S.D.) with different letters within the same row are significantly different ($P<0.05$). The absence of letters indicates no significant difference between treatments.

ANOVA: Two-way analysis of variance

Table 3 Proximate composition of muscle and whole body of juvenile pearl gentian groupers (mean ± S.D., n=3).

Group	Proximate composition of muscle (% WW ^a)				Proximate composition of whole body(% WW)			
	Moisture	Protein	Fat	Ash	Moisture	Protein	Fat	Ash
G1	75.70±0.33de	20.05±0.37abc	1.34±0.06bcd	1.53±0.01c	67.28±0.13e	19.53±0.11a	6.00±0.42ab	6.77±0.10a
G2	77.32±0.11bcd	18.71±0.13cd	1.31±0.06cde	1.54±0.01b	71.64±0.07ab	17.78±0.04b	5.06±0.46ab	5.32±0.01d
G3	78.69±0.21ab	17.20±0.20e	1.16±0.07ef	1.46±0.01e	71.21±0.12b	18.12±0.07b	4.05±0.06b	5.28±0.01d
G4	78.47±0.26ab	17.65±0.23de	1.43±0.03bc	1.46±0.01e	71.73±0.19ab	18.21±0.25b	4.23±0.06ab	4.84±0.01f
G5	79.85±0.39a	16.36±0.29e	1.39±0.05bcd	1.33±0.01g	71.24±0.09b	18.23±0.25b	4.77±0.05ab	5.08±0.03e
G6	77.48±1.54bc	19.06±1.31bc	1.03±0.07f	1.49±0.01d	66.94±0.23e	20.65±0.30a	7.49±1.87ab	6.38±0.03b
G7	76.51±0.23cde	20.28±0.32ab	1.74±0.03a	1.44±0.01f	68.00±0.20d	20.61±0.16a	5.41±0.07ab	5.49±0.05c
G8	75.18±0.21e	21.05±0.10a	1.48±0.06b	1.54±0.01b	68.07±0.21d	20.93±0.19a	8.58±3.78a	5.32±0.03d
G9	76.22±0.17cde	20.37±0.13ab	1.38±0.02bcd	1.57±0.01a	71.78±0.19a	18.07±0.47b	4.37±0.07ab	4.98±0.01e
G10	77.25±0.18bcd	19.68±0.16abc	1.24±0.04de	1.46±0.01e	70.56±0.22c	18.12±0.12b	4.36±0.06ab	5.25±0.01d
ANOVA(<i>P</i> value)								
Breeding density	0.005	0.012	< 0.001	< 0.001	< 0.001	< 0.001	0.295	< 0.001
Feeding frequency	< 0.001	< 0.001	0.139	< 0.001	< 0.001	< 0.001	0.157	0.250
Interactions	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.377	< 0.001

Values (mean ± S.D.) with different letters within the same row are significantly different ($P<0.05$). The absence of letters indicates no significant difference between treatments.

^a WW: wet weight.

Table.4 Serum biochemistry (mean $\pm$ S.D., n=3).

Group	Items								
	AST	ALT	TP	GLB	ALB	TG	CHO	GLU	LZM
G1	17.33 $\pm$ 0.58h	269.00 $\pm$ 8.39a	30.50 $\pm$ 0.06e	21.03 $\pm$ 0.12bc	9.47 $\pm$ 0.07f	0.99 $\pm$ 0.01fg	1.65 $\pm$ 0.01h	22.60 $\pm$ 0.08b	91.41 $\pm$ 4.82e
G2	52.33 $\pm$ 0.58d	152.00 $\pm$ 3.46b	26.97 $\pm$ 0.09h	17.27 $\pm$ 0.13f	9.70 $\pm$ 0.06ef	1.31 $\pm$ 0.02c	1.87 $\pm$ 0.04g	20.45 $\pm$ 0.16c	76.67 $\pm$ 5.09f
G3	33.00 $\pm$ 1.00f	138.33 $\pm$ 6.57b	29.37 $\pm$ 0.15fg	19.37 $\pm$ 0.09d	10.00 $\pm$ 0.10e	0.90 $\pm$ 0.03h	2.38 $\pm$ 0.02f	9.73 $\pm$ 0.04e	58.89 $\pm$ 4.84gh
G4	53.33 $\pm$ 0.58d	52.67 $\pm$ 1.76c	36.87 $\pm$ 0.17a	21.53 $\pm$ 0.07a	15.33 $\pm$ 0.15a	1.03 $\pm$ 0.03ef	2.76 $\pm$ 0.01d	8.57 $\pm$ 0.04e	53.33 $\pm$ 3.33h
G5	60.00 $\pm$ 0.00c	47.67 $\pm$ 2.33c	31.30 $\pm$ 0.10d	17.17 $\pm$ 0.07f	14.13 $\pm$ 0.15b	0.78 $\pm$ 0.03i	2.63 $\pm$ 0.01e	12.70 $\pm$ 0.10d	66.67 $\pm$ 0.00fg
G6	21.00 $\pm$ 1.00g	267.67 $\pm$ 2.03a	29.07 $\pm$ 0.03g	20.67 $\pm$ 0.15c	8.40 $\pm$ 0.15g	1.53 $\pm$ 0.02b	1.44 $\pm$ 0.03i	2.05 $\pm$ 0.03f	166.67 $\pm$ 0.00c
G7	39.00 $\pm$ 1.73e	258.33 $\pm$ 9.94a	33.20 $\pm$ 0.15b	21.97 $\pm$ 0.07a	8.23 $\pm$ 0.19g	0.93 $\pm$ 0.03gh	3.56 $\pm$ 0.03b	13.63 $\pm$ 0.19d	285.55 $\pm$ 5.37a
G8	18.00 $\pm$ 1.00h	108.67 $\pm$ 5.04b	29.73 $\pm$ 0.23f	21.43 $\pm$ 0.35ab	8.30 $\pm$ 0.15g	1.65 $\pm$ 0.01a	3.37 $\pm$ 0.01c	38.37 $\pm$ 1.39a	201.11 $\pm$ 1.92b
G9	69.67 $\pm$ 1.15b	36.67 $\pm$ 0.33c	30.70 $\pm$ 0.26e	18.43 $\pm$ 0.18e	12.27 $\pm$ 0.09d	1.08 $\pm$ 0.02e	2.78 $\pm$ 0.01d	22.87 $\pm$ 0.08b	277.78 $\pm$ 9.62a
G10	77.33 $\pm$ 0.58a	43.67 $\pm$ 1.33c	32.10 $\pm$ 0.10c	18.83 $\pm$ 0.09e	13.27 $\pm$ 0.12c	1.19 $\pm$ 0.03d	4.10 $\pm$ 0.07a	22.55 $\pm$ 0.24b	108.33 $\pm$ 8.34d
ANOVA(P value)									
Breeding density	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Feeding frequency	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interactions	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Values (mean $\pm$ S.D.) with different letters within the same row are significantly different ($P<0.05$). The absence of letters indicates no significant difference between treatments.

^a WW: wet weight.

AST: Aspartate aminotransferase; ALT: Alanine transaminase; TP: Total protein; GLB: Globulin; ALB: Albumin; TG: Triglyceride; CHO: Cholesterol; GLU: Glucose; LZM: Serum lysozyme; SOD: Superoxide dismutase; AKP: Alkaline phosphatase

Table.5 Digestive enzyme activities (mean $\pm$ S.D., n=3).

Group	Gastric			Intestinal		Hepatic			
	Pepsin (U/mg prot)	Amylase (U/mg prot)	Lipase (U/mg prot)	Pepsin (U/mg prot)	Amylase (U/mg prot)	Lipase (U/mg prot)	Pepsin (U/mg prot)	Amylase (U/mg prot)	Lipase (U/mg prot)
G1	17.23	0.68±0.00	18.08±0.12f	1.54±0.01d	0.26±0.00	30.61±0.13ef	4.66±0.01d	0.10±0.00	2.23±0.04d
G2	18.48	0.71±0.00	19.11±0.11e	1.62±0.01c	0.25±0.00	31.76±0.11d	5.03±0.01cd	0.11±0.00	2.56±0.04cd
G3	20.67	0.77±0.00	20.0±0.14ad	1.68±0.01bc	0.24±0.00	32.33±0.16c	5.37±0.05bc	0.12±0.00	2.65±0.08c
G4	22.32	0.80±0.00	20.71±0.21ab	1.76±0.02ab	0.23±0.00	33.07±0.20b	5.44±0.00bc	0.12±0.00	2.90±0.03ab
G5	24.21	0.84±0.00	21.18±0.25a	1.78±0.01ab	0.22±0.00	34.80±0.12a	5.94±0.01b	0.13±0.00	2.97±0.05a
G6	17.38	0.70±0.00	17.63±0.15f	1.57±0.02cd	0.28±0.00	30.33±0.21f	4.87±0.01dc	0.10±0.00	2.12±0.04e
G7	19.48	0.74±0.00	18.65±0.20e	1.63±0.01c	0.23±0.00	30.85±0.10e	5.19±0.01c	0.11±0.00	2.24±0.06d
G8	22.57bc	0.80±0.00	19.65±0.19d	1.72±0.01b	0.22±0.00	31.42±0.09d	5.61±0.01cd	0.11±0.00	2.68±0.06c
G9	23.62	0.83±0.00	19.84±0.16d	1.77±0.01ab	0.22±0.00	32.23±0.12c	5.88±0.01bc	0.12±0.00	2.79±0.06b
G10	25.03	0.86±0.00	20.61±0.25bc	1.80±0.02a	0.21±0.00	33.21±0.12b	6.24±0.00a	0.14±0.00	2.88±0.04ab
ANOVA(Pvalue)									
Breeding density	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Feeding frequency	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interactions	< 0.001	0.136	0.734	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Values (mean ± S.D.) with different letters within the same row are significantly different ($P<0.05$). The absence of letters indicates no significant difference between treatments.

^a WW: wet weight.

Figures

Figure 1

H&E stained gastric tissue sections of juvenile pearl gentian grouper. (A-J) Gastric tissue sections of fish in groups G1 to G10, respectively (H&E, 400)

Figure 2

H&E stained intestinal tissue sections of juvenile pearl gentian grouper. (A-J) Intestinal tissue sections of fish in groups G1 to G10, respectively (H&E, 400)

Figure 3

H&E stained hepatic tissue sections of juvenile pearl gentian grouper. (A-J) Hepatic tissue sections of fish in groups G1 to G10, respectively (H&E, 400)

Figure 4

Quadratic regression model based on body weight gain (y-axis) in response to fish stocking density when fed twice a day (x-axis).

Figure 5

Quadratic regression model based on body weight gain (y-axis) in response to fish stocking density when fed three times a day (x-axis).

Supplementary Files

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