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PROOF RESEARCH PAPER

Effects of Stocking Density on Growth, Body Composition, Digestive Enzyme Levels and Blood Biochemical Parameters of *Anguilla marmorata* in a Recirculating Aquaculture System

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Abstract

We conducted a 71-day experiment to investigate the effects of stocking density in a recirculating aquaculture system (RAS) on the growth performance, body composition, digestive activity, and blood biochemical parameters of the marbled eel *Anguilla marmorata*. We stocked marbled eel in 1.0 m^3 tanks at stocking densities of 12, 20, and 28 kg/m^3, which were designated the low-density (LD), medium-density (MD) and high-density (HD) groups. The fish weighed 169.72 ± 2.75 g at the baseline level. Results revealed that the fish in the LD and MD groups had a higher ash content than fish in the HD group. The crude lipid content was significantly lower in the MD and HD groups than in the LD group. The protease activities in the stomach and intestine did not differ among the three groups. The total protein and aspartate aminotransferase levels were higher in the LD group than in the MD group. The alanine aminotransferase level was significantly lower in the LD group. These results indicate that a greater increase resulted in stress and significantly affected the growth of the fish. The optimum stocking density of *A. marmorata* can be achieved at 20.0 kg/m^3 in a RAS.

Keywords: *Anguilla marmorata*, stocking density, body composition, digestive enzyme, blood biochemical.

Introduction

Due to the lack of space, limited fresh water availability, and concerns over pollution, the recirculating aquaculture system (RAS) is a good choice for the aquaculture industry (Badiola, Mendiola, & Bostock, 2012). However, due to the high fixed investment and operational costs involved, a high stocking density is required to maximize the yield and economic benefits (Gang, Tan, Luo, & Sun, 2010; Zhu et al, 2011; Badiola et al, 2012).

Many studies have demonstrated that stocking fish at a high density may have a negative effect on farmed fish. The growth, survival, and food utilization rates have all been shown to decrease with an increase in stocking density (Gang et al, 2010; Zhu et al, 2011). A high stocking density not only increases direct competition among fish for space and food but it may also increase the incidence and mortality of cultured fish (Salas-Leiton et al, 2010). Blood composition, lysozyme, and alkaline phosphatase (ALP) levels are commonly used to measure the impact of stocking density on fish physiology (Montero, Izquierdo, Tort, & Robaina, 1999; Bartons, 2002), and high stocking density has shown to have a negative effect on some blood serum parameters (Gang et al, 2010). However, the effects of stocking density, which is also an area of welfare concern, are complex and appear to involve numerous interacting and case-specific factors (Ashley, 2007). High stocking densities have been shown to have a positive effect on the behavior and production of African catfish (*Clarias gariepinus*), and in African catfish larvae and juveniles increasing densities were found to reduce the occurrence of agonistic behavior (Hecht and Uys, 1997; Hengsawat, Ward, & Jaruratjamorn, 1997). Therefore, it is vital to study the growth performance and physiological responses of the specific cultured species under different stocking densities.

The Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*) are primary breeding species, and their larval resources are in decline (Jacoby *et al.* 2015). Marbled eel (*Anguilla marmorata*) belong to the order Anguilliformes family Anguillidae, and have a much larger adult body size than other Anguillid eels. These eels are widely distributed throughout most of the tropical and sub-tropical western Pacific and Indian Oceans, they are found in the southern islands of Japan, Taiwan, southeastern China, throughout the Indo-Pacific

region, New Caledonia, the islands of Polynesia and French Polynesia, and in the eastern and southwestern Indian Ocean (Luo, Guan, Li, & Jin. 2013b). However, the available information on the aquaculture technology of *A. marmorata* is meager. In order to achieve high-density stocking, resource protection, and commercial utilization of *A. marmorata*, this paper studied the effects of stocking density on the growth, body composition, digestive activity, and blood biochemical parameters of *A. marmorata* cultured in RAS.

Materials and methods

Experimental Facilities

The study was conducted in a RAS at Shanghai Aquacultural Engineering Research Center. Experimental tanks were supplied with fresh water from a recirculating aquaculture system (Figure 1). The RAS was operated with a recirculation rate of 89.3%, a moderate flow-through rate allowed renewal of water to the RAS 17 times per day at a rate of approximately 12 L/min. Nine 1.0 m^3 circular polyester resin tanks (1.5 m in diameter) were used indoors. A drum filter (with a 74 μm mesh) was used to treat the wastewater containing suspended solids, which was collected by an external fecal trap. System overflow was evacuated using a vertical elevating pump for biological filtration. Biofiltration to transform ammonia into nitrate was achieved in a moving bed biofilm reactor. The water was continuously aerated using compressed air root blowers.

Fish Stocking and Management

Prior to the start of the experiments, the eels were cultured for a 14-day acclimation period in laboratory conditions in a RAS. Marbled eel (average weight 169.72 ± 2.75 g) were randomly allocated to one of nine tanks assigned to three levels of experimental stocking densities. Three tanks were designated as low-density (LD) tanks (70 fish/m^3, 12 kg/m^3), three as medium-density (MD) tanks (117 fish/m^3, 20 kg/m^3), and three as high density (HD) tanks (165 fish/m^3, 28 kg/m^3).

During both the acclimation and experimental periods, a certain amount of water was changed, unconsumed food and faeces were removed to maintain water quality. The water quality was maintained at pH 7.0–8.0; ammonia-N (NH_4^+-N) < 5.0 mg/L, nitrite-N (NO_2^--N) 0.25–1.0 mg/L; dissolved oxygen (DO), 6.5–7.5 mg/L, and temperature, 28.0 ± 1.0 °C. Fish were fed ad libitum by hand with commercial feed produced by Gaonong Feed Company, Fujian, China. The feed contained 48% crude protein, 4% crude lipids, 3% crude fiber, 2.9% lysine, 17% ash, and 10% moisture. Uneaten feed was collected 1h after feeding by siphon tap and dried, and the total amount of daily consumed feed was calculated.

Passive integrated transponder (PIT) tags were inserted into the dorsal musculature of 20 randomly selected *A. marmorata* individuals per tank using a standard 12-gauge needle injector. The tags were 11 mm long and 2.86 mm in diameter, and weighted 40 mg in water. The exact tagging procedure was as follows: *A. marmorata* individuals that had been fasted for 24 h prior to the tagging were anesthetized with 100 mg/L of 2-phenoxyethanol in a 30-L bucket. The needle containing the tag was inserted into the dorsal musculature 5–10 mm below the dorsal fin and parallel to the axis of the vertebral column. Care was taken to avoid the vertebral column and the lateral line. During insertion, the angle between the needle and the dorsal body surface was approximately 30°. After penetrating the dorsal musculature, the tag was ejected and the needle withdrawn.

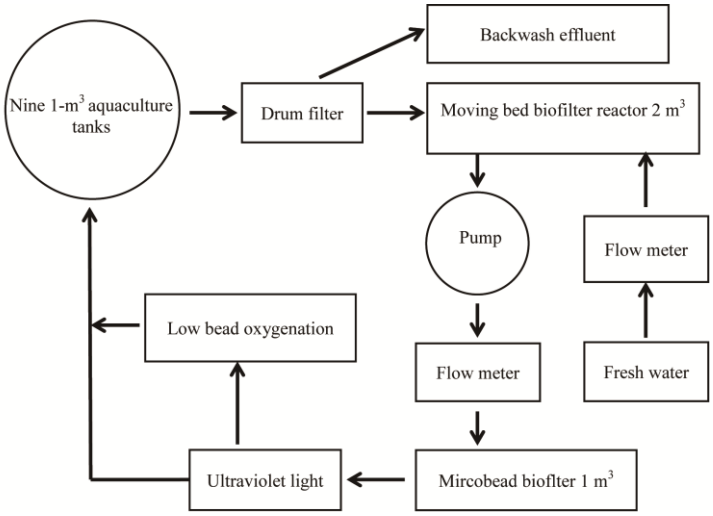

Figure 1. Schematic design of of a recirculating aquaculture system.

Analysis of Water Quality Parameters

Water temperature, pH and DO were measured using a YSI556 meter (YSI Incorporated 1725, Yellow Springs, OH, USA). Ammonia-N and nitrite-N were analysed spectrophotometrically following standard methods (Carranzo, 2012).

Method for Determining Experimental Parameters

Each of the tagged eels were weighed on days 15, 29, 43, 57, and 71. Data on initial weight, final weight, total length, liver weight, and feed intake were used to calculate the growth efficiency (GE), specific growth rate (SGR), final stocking density (FSD), feed conversion ratio, and protein efficiency rate as follows:

Daily weight gain (DWG): $DWG = (W_2 - W_1)/(t_2 - t_1)$

Specific growth rate (SGR): $SGR = [(\ln W_2 - \ln W_1)/(t_2 - t_1)] \times 100$

Feed conversion ratio (FCR): FCR = feed consumed (kg)/fish biomass increase (kg)

Protein efficiency rate(PER%)=$100\times(W_2-W_1)/(F\times48\%)$

Growth efficiency (GE): $GE = [(W_2 - W_1)/F] \times 100$

Survival (%) = $100\ (N_f - N_i)/N_i$

where W_1 and W_2 are the weights at times t_1 and t_2 (first and last days of the experiments, respectively), n is the number of fish, F is the weight of total feed (g), L_w is the liver weight (g), N_i is the initial number of fish, and N_f is final number of fish.

The fish were starved for 24 h before they were sampled for digestive activity and Blood biochemical parameters indicators. Three fish were randomly netted from each tank and immediately anesthetized using 100 mg/L of 2-phenoxyethanol in a 30-L bucket. The fish reached stage V of anesthesia within 5–10 min and were subsequently stunned by a blow to the back of the head. Blood was collected from the caudal vein by cutting off the tail. The time from capture to blood collection was <5 min. Each blood sample was placed in a 1.5-mL non-heparinized Eppendorf tube. After clotting, the samples were centrifuged at 4500 rpm for 15 min at 4°C. The serum samples were split into several aliquots, stored at –80 °C, and analyzed on the following day. The stomachs and intestines of the sampled fish were extracted and homogenized thrice using normal saline (1: 3 w/v) at 0 °C for 30 s each time, using an electric blender operating at 8000 rpm. The homogenate was centrifuged at 10,000 g for 30 min at 4 °C to eliminate tissue debris and lipids. The supernatant (enzyme extract) was dispensed into 1.5-mL Eppendorf tubes and stored at –80 °C until enzymatic assay. The back muscle was rapidly excised from the fish and kept at –80 °C.

Serum TP, TG and GLU concentrations were measured using a biochemical analyzer (Mindray Chemistry Analyzer BS-200; Shenzhen, Guangdong Province, China), and the kits used were supplied by Mindray Medical International Limited (Shenzhen, China). The protease activity was assayed according to the method of Lowry *et al.* (Lowry, Rosebrough, Farr, & Randall, 1951), using casein as the substrate and a Folin phenol reagent to obtain a reaction. Serum COR (ng mL−1) concentration was measured as the change in absorbance using an enzyme-linked immunosorbent assay (ELISA; Bio-Tek ELX-750, Bio-Tek Instruments, Inc., Winooski, VT, USA) and expressed as 1 μg of tyrosine or maltose released per min (U/mg protein). One unit of enzyme activity was expressed as 1 μM of tyrosine released per min. Lipase activity was determined by the measurement of fatty acid release caused by the enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil (Borlongan, 1990). Enzyme activities, including those of proteases, were expressed as specific activity in U/mg of protein, and lipase activity was expressed as U/mg of intestinal content (Yanbo and Zirong, 2006).

To measure the moisture content of fish muscles, the muscles were dried in an oven at 105°C until a constant weight was attained. Ash was obtained by incinerating the sample at 550°C. The fish muscles were dried in a vacuum freeze dryer until a constant weight was attained, and then subjected to a composition analysis. The crude protein content was determined by measuring the nitrogen (N, 6.25) content and by using an elemental analyzer (Elementar Trading Co., Ltd). The crude lipid content was determined using the chloroform/methanol extraction technique described previously by Folch (Folch, Lees, & Stanley, 1957).

Statistical Analysis

Data are expressed as means ± standard error (SE). The data were analysed by one-way ANOVA for repeated measures and tested by Duncan's multiple range test. Normality and homogeneity of distribution were estimated by the Kolmogorov–Smirnov goodness of fit test and the Bartlett–Box F-test. The level of significance was set at $P < 0.05$ for all statistical tests. All statistical analysis was performed using the computer package SPSS 20.0 for Windows.

Results

Growth Performance

Figure 2 illustrates the growth of *A. marmorata* from days 1-71. In the first 43 days, LD and MD weighed more than HD individuals. On days 51-71, LD individuals weighed more than MD and HD individuals. But there were no significant differences in weight among the three groups. The final density of fish reared at LD, MD, and HD increased from 12.0 to

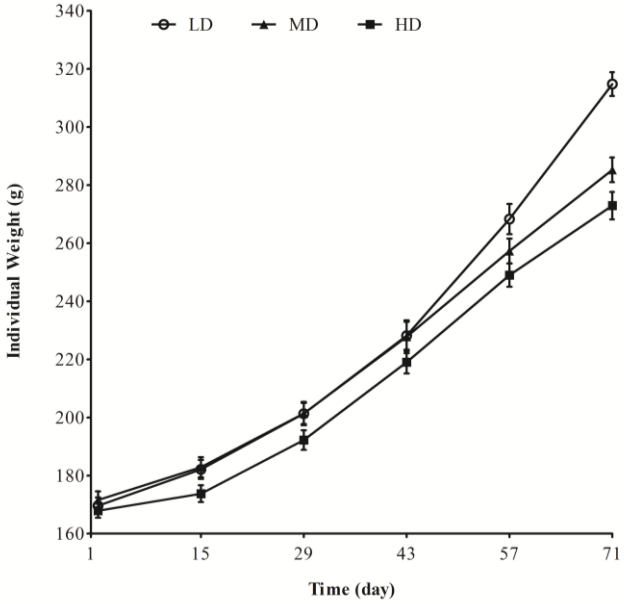

Figure 2. Growth of *Anguilla marmorata* stocked at three rearing densities and cultured for 71 days.

20.72 ± 1.13 kg/m^3, from 20.0 to 32.51 ± 0.70 kg/m^3, and from 28.0 to 43.76 ± 0.79 kg/m^3 respectively, and the final body weights were 314.81 ± 20.37, 285.31 ± 8.38, and 272.95 ± 9.16 g, respectively (Table 1). Analysis of the fish growth parameters revealed that the GE was significantly higher in the LD group than in the MD and HD groups. In contrast, there were no significant differences in the parameters between fish in the MD and HD groups. The FI was higher in the LD and MD groups than in the HD group. The SGR, FCR, and FER did not significantly differ among the stocking densities.

Body Composition

There was no significant difference in the moisture content (% wet basis) and protein content in the muscle of *A. marmorata* grown at different stocking densities (Table 2). The ash content was significantly higher in the LD group than in the other two groups, but there were no significant differences between the MD and HD groups. The crude lipid content in the LD group was significantly higher than those in the MD and HD groups.

Digestive Activity

There were no significant differences in the protease activity in both the intestine and stomach of the fish from the three groups ($P > 0.05$). The intestinal lipase activity was significantly lower in the MD group than in the HD group ($P < 0.05$) (Table 3).

Blood Biochemical Parameters

Table 4 presents the blood parameters of fish in the LD, MD, and HD groups (12.0, 20.0, and 28.0 kg/m3 respectively) measured at the end of the 71-day experiment. There was no significant difference in the cortisol (COR), total cholesterol (TC), triglyceride (TG), urea, and ALP levels among the three groups. The glucose (GLU) and albumin (ALB) levels were significantly higher in the LD group compared to the MD and HD groups ($P < 0.05$). The total protein (TP) and aspartate aminotransferase (AST) levels were higher in the fish reared at LD than in fish reared at MD ($P < 0.05$), but were not significantly different to the HD group ($P > 0.05$). The ALT level was significantly higher in the HD group than in the LD group ($P < 0.05$).

Discussion

The experiment was performed in a RAS in which each tested stocking density was maintained at a stable level with consistent water quality parameters such as ammonia, pH, dissolved oxygen, and temperature. Food was not a limiting factor during the experiment; therefore, the differences among test groups could be due to on physiological stresses caused by stocking conditions.

Mortality is an important indicator of adaptation of fish to the environment. In several studies, high stocking density resulted in injury or death of fish (Ellis, 2002, Ashley, 2007). However, no mortality was observed in any of the selected stocking densities in our current study. Similar results have been reported for farmed fish cultured with good water quality, e.g., rainbow trout, sea bass, red porgy (*Pagrus pagrus*), African catfish (*Clarias gariepinus*), and Nile tilapia (*Oreochromis niloticus*) (Hengsawat et al, 1997; North et al, 2006; Sophie *et al.* 2009; Laizcarrión *et al.* 2012).

Stoking density is connected to production

Table 1. Growth performance of *Anguilla marmorata* in the experiment.

Parameters	Stocking density (kg/m^3)		
	12.0	20.0	28.0
Initial mean weight (g/fish)	169.62 ± 2.89	171.60 ± 2.95	167.93 ± 2.42
Initial number (fish/tank)	70	117	165
Final mean weight (g)	314.81 ± 20.37^a	285.31 ± 8.38^{ab}	272.95 ± 9.16^b
Initial total length (cm/fish)	40.52±0.09	40.69±0.11	40.63±0.12
Final total length (cm/fish)	47.01±0.33	46.71±0.56	47.46±0.14
Survival rate (%)	100%	100%	100%
Final stocking density (kg/m^3)	20.72 ± 1.13^a	32.51 ± 0.70^b	43.76 ± 0.79^c
Specific growth rate (% /d)	0.91 ± 0.09	0.81 ± 0.04	0.74 ± 0.03
Growth efficiency (%)	80.49 ± 9.10^a	62.55 ± 3.48^b	56.27 ± 2.84^b
Feed conversion ratio	1.52 ± 0.19	1.74 ± 0.05	1.80 ± 0.11
Protein efficiency rate (%)	141.90 ± 20.02	120.06 ± 3.28	116.80 ± 7.77
Feed intake (g/individual)	182.49 ± 0.87^a	180.44 ± 1.15^a	168.64 ± 4.14^b

Note: Values (means ± SE) within the same row with different letters are significantly different ($P < 0.05$).

Table 2. Moisture, lipid, protein, ash and carbon contents of *Anguilla marmorata* at the end of the experiment.

Parameters	Stocking density (kg/m^3)		
	12.0	20.0	28.0
Moisture content (%)	66.32 ± 0.86	69.04 ± 1.00	69.50 ± 1.62
Ash content (%)	2.38 ± 0.22^a	1.92 ± 0.66^a	1.52 ± 0.45^b
Crude protein content (%)	17.10 ± 0.18	17.52 ± 0.37	17.79 ± 0.47
Crude lipid content (%)	10.89 ± 0.70^a	8.75 ± 0.68^b	8.13 ± 0.69^b

Table 3. Digestive activity of *Anguilla marmorata* in three stocking densities at the end of the experiment.

Parameters	Stocking density (kg/m^3)		
	12.0	20.0	28.0
Stomach protease activity (U/mg)	24.39 ± 2.40	28.20 ± 1.90	32.34 ± 5.26
Intestinal protease activity (U/mg)	728.15 ± 8.28	654.95 ± 9.70	719.61 ± 7.67
Intestinal lipase activity (U/mg)	64.70 ± 2.65^{ab}	53.40 ± 5.73^b	65.04 ± 6.44^a

Table 4. Blood biochemical parameters of *Anguilla marmorata* in three stocking densities at the end of the experiment.

Parameters	Stocking density (kg/m^3)		
	12.0	20.0	28.0
Cortisol (ng/mL)	27.97 ± 1.69	25.71 ± 2.44	29.63 ± 2.14
Glucose (mg/dL)	4.61 ± 0.35^a	3.45 ± 0.07^b	3.79 ± 0.28^b
Total protein (g/L)	42.42 ± 1.39^a	37.61 ± 0.76^b	40.35 ± 1.68^{ab}
Total cholesterol (mm/L)	16.22 ± 0.80	15.30 ± 0.85	14.65 ± 0.84
Triglycerides (mm/L)	15.47 ± 1.96	17.45 ± 1.47	22.98 ± 4.01
Albumin (g/L)	18.12 ± 0.50^a	16.34 ± 0.30^b	15.13 ± 0.68^b
Urea (mmol/L)	0.47 ± 0.03	0.51 ± 0.04	0.52 ± 0.04
Alanine aminotransferase (U/L)	4.53 ± 0.56^b	5.93 ± 0.71^{ab}	8.27 ± 1.45^a
Aspartate aminotransferase (U/L)	183.85 ± 7.24^a	114.88 ± 5.05^b	133.34 ± 1.73^{ab}
Alkaline phosphatase (U/L)	546.72 ± 3.91	487.70 ± 2.01	542.11 ± 3.72

parameters when commercial farming conditions are developed (Ashley, 2007). Growth is one of the most well-studied physiological parameters in relation to social interactions (Sloman and Armstrong, 2002). It can be measured easily and used as an indicator of social stress. The individual weight of A. marmorata from days 1–43 showed that the LD and MD had no significant influence on weight; however, increasing the stocking density further to HD affected the growth of fish. The rate of growth was lower in the MD group than in the LD group from days 57–71, and there were no significant differences in the individual weight between the LD group and other groups. Thus, a slight increase in density has a smaller impact on fish, but a greater increase in the stocking density resulted in a significant stress on the fish. The results of the current study showed that the final individual weight, growth efficiency, and feed intake

significantly differed under different experimental stocking after 71 days, whereas the SGR, feed conversion ratio, and protein efficiency rate showed no difference between different stocking density groups. These findings suggested that LD stocking might not alter the growth of A. marmorata, but stocking fish at HD could affect the growth. This result is consistent with some density trials of Jade perch (Luo, Liu, & Tan, 2013a), rainbow trout (Bagley, Bentley, & Gall, 1994; North et al, 2006), Atlantic salmon (Camilladiesen, Jannicke, Sigurdo, Sveinung, & Sigurdo, 2009), and Senegalese sole (Salas-Leiton, Anguis, Manchado, Canavate, 2008; Costas, Aragao, & Dias, 2013). This crowding stress may led to aggression and competition for food and space among fish. It could also resulted in greater difficulty in accessing feed and increased energetic cost of feeding (Marchand and Boisclair, 2011).

Limited information is available regarding the body composition of A. marmorata grown in different stocking densities. Previous studies have investigated the effects of stocking density on the body composition of African catfish, vundu catfish, and rainbow trout (Trenzado, Morales, & Higuera, 2006; Toko, Fiogbe, Koukpode, & Kestemont. 2007). The protein content of most fish species tends to slightly increase or remain more or less stable with increasing body weight (Ramseyer, 2002). In our study, fish grown at different stocking densities showed no significant differences in the moisture content and crude protein content after 71 days. Although the individual weights of fish grown at LD was larger than those of fish grown at HD, the stocking density might not alter the protein content in A. marmorata. Body composition is strongly related to body weight of A. anguilla (Heinsbroek et al, 2007). Data from the present study revealed that increasing stocking density influenced body composition, resulting in increased moisture and protein levels and decreased lipid and ash content at high stocking density. There were no significant differences in the stomach and intestinal protease activity across different densities. The intestine lipase activity significantly differed between the MD and the LD or HD groups. This difference may be because of the different ingestion rates of fish grown at the three densities. Similar results were reported in farmed Japanese flounder, where the fish were found to have a significant reduction in intestinal lipase activity when grown under high stocking density conditions (Bolasin, Tagawa, Yamashita, & Tanaka, 2006). It is likely that HD fish survive by reducing metabolic activities to curb energy consumption, as high intestinal lipase and protease activities increase energy consumption.

The evaluation of biochemical and hematological parameters might be useful to diagnose fish physiological status. Researchers often measure the serum COR level as an indicator of stress in fish due to its responsiveness to acute stressors, its relative ease of measurement, and its functional significance in the physiological processes that affect fish health (Barton and Iwama, 1991). The serum COR levels in this study showed no significant difference across the three stocking densities, which was similar to the results reported for *Salvelinus fontinalis* (Vijayan, Ballantyne, & Leatherland, 1990). Hsieh concluded that serum GLU is a transient indicator of high stress levels (Hsieh et al, 2003). When the stress factors continue, the serum GLU levels may fall to preexisting levels. Thus, serum GLU can be used only as an indicator of an acute response to an initial stress. In the current study of chronic stress over a 71-day period, the serum GLU showed different responses in the three groups. Stocking density resulted in no significant difference of GLU level between MD and HD, but the GLU level of the LD group was significantly increased. Prolonged stress may eventually exhaust glycogen stores and the serum GLU level may decrease (Rolf et al, 2002); Therefore, increasing the stocking density could be considered a type of stressor for A. marmorata. As a blood index in the diagnosis of fish health, nutrition, and disease, the TP content has important biological significance. ALB is synthesized by hepatic parenchymal cells. The decrease in the TP content may be due to the inhibition of protein synthesis. In response to stress, the TP content in the blood may have been used as energy. Decrease in the ALB content may be due to liver cell lesions, kidney disease, or malnutrition, which would consequently lead to excessive loss of protein.

TG is also an important source of energy during acclimation to different stocking densities (Herrera et al, 2009; Laizcarrión et al, 2012). The increase in serum TG under high density conditions could be related to lipid mobilization to cope with an increased energy demand when GLU stores are drastically utilized (Sheridan and Mommsen, 1991; Albalat et al, 2005; Huising et al, 2006), as shown by our results. It then follows that MD and HD lead to consumption of GLU, TP, and ALB, inducing a shift towards urea.

The ALT, AST, and ALP levels are used as indicators of liver function and renal function. Under normal circumstances, the activities of these enzymes are low and relatively constant, but their serum levels tend to increase in response to liver damage or environmental stress (Luo et al, 2013a). Although the ALP activity showed no significant difference among the experimental groups, the AST activity was the highest in the LD showed the highest AST activities compared to other treatments. Moreover, ALT activity was significantly higher in the HD as group than in the other groups. A significant increase in the serum ALP, AST, and ALT activities is considered as the response of the organism to stressors (Hao, Ling, & Hong. 2014). Another study showed that the fish are motion-adaptive to stocking density. This process involves consumption of energy, resulting in differences in growth (Gang *et al.* 2010), as observed in the present study.

Conclusion

Here, we studied the effects of stocking density on the growth, digestive activity, body composition and blood biochemical parameters of A. marmorata cultured in RAS. No deaths were observed among the three densities. There were no significant differences in the stomach protease activity and intestinal protease activit, but the intestine lipase activity and body composition, under different experimental stocking densities. There were no significant differences in moisture content and protein content, but the ash content and crude lipid content. Nevertheless, there was no significant in COR, TC, TG, urea, and ALP levels, but not in GLU, ALB, TP, AST and ALT levels, among the groups. The results indicated that A. marmorata growth showed a limited increase with increasing in stocking density, and the stress level increased when the stocking density was high. Blood biochemical parameters of A. marmorata seemed impaired at both the lowest and highest densities in this experiment. Thus, the optimum growth rate of A. marmorata could be achieved at 20.0 kg/m3 in RAS.

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