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Effect of stocking density on performances of juvenile turbot (*Scophthalmus maximus*) in recirculating aquaculture systems*

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Abstract Limited information has been available about the influence of loading density on the performances of *Scophthalmus maximus*, especially in recirculating aquaculture systems (RAS). In this study, turbot (13.84±2.74 g; average weight±SD) were reared at four different initial densities (low 0.66, medium 1.26, sub-high 2.56, high 4.00 kg/m^2) for 10 weeks in RAS at 23±1°C. Final densities were 4.67, 7.25, 14.16, and 17.47 kg/m^2, respectively, which translate to 82, 108, 214, and 282 percent coverage of the tank bottom. Density had both negative and independent impacts on growth. The final mean weight, specific growth rate (SGR), and voluntary feed intake significantly decreased and the coefficient of variation (CV) of final body weight increased with increase in stocking density. The medium and sub-high density groups did not differ significantly in SGR, mean weight, CV, food conversion rate (FCR), feed intake, blood parameters, and digestive enzymes. The protease activities of the digestive tract at pH 7, 8.5, 9, and 10 were significantly higher for the highest density group, but tended to be lower (not significantly) at pH 4 and 8.5 for the lowest density group. The intensity of protease activity was inversely related to feed intake at the different densities. Catalase activity was higher (but not significantly) at the highest density, perhaps because high density started to induce an oxidative effect in turbot. In conclusion, turbot can be cultured in RAS at a density of less than 17.47 kg/m^2. With good water quality and no feed limitation, initial density between 1.26 and 2.56 kg/m^2 (final: 7.25 and 14.16 kg/m^2) would not negatively affect the turbot cultured in RAS. For culture at higher density, multi-level feeding devices are suggested to ease feeding competition.

Keyword: turbot; stocking density; growth; protease activity

1 INTRODUCTION

In China, turbot (*Scophthalmus maximus*) aquaculture was first developed in 1999 using fingerlings introduced from Great Britain. Currently, the industry is flourishing, especially around the Shandong Peninsula. Many semi-closed or recirculating aquaculture systems (RAS) have been developed to replace flow-through systems to reduce environmental pollution and to save underground seawater resources (Lei et al., 2005).

Economic considerations have pushed producers to increase stocking density in culture systems (Irwin, et al., 1999; Blancheton, 2000; Gonçalves et al., 2010). High stocking density may lead to poor water quality, and a "crowding effect" that may negatively affect fish health (Santos et al., 2010). In general, high stocking densities also result in heterogeneous growth rates, final weights, and feed intakes, and can even lead to mortality (Irwin et al., 1999; Hosfeld et al., 2009). These are important issues that must be considered for industrial operation of aquaculture

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facilities (North et al., 2006a). Therefore, defining an optimal stocking density at each stage of fish development is critical for balancing fish welfare and farm economy.

Most previous studies of the effect of stocking density on fish performance have focused on swimming species (Ashley, 2007; Rafatnezhad et al., 2008), and few studies have been carried out on turbot (*Scophthalmus maximus*) (Martinez-Tapia and Fernandez-Pato, 1991; Irwin et al., 1999, 2002; Labatut and Olivares, 2004; Gonçalves et al., 2010). In the few existing studies, the ranges of densities investigated were limited compared with those used in the practical operation of fish farms.

In RAS, hormone-related substances (e.g., stress hormones, chemical alarm cues) might accumulate with increasing fish density (Ruane and Komen, 2003; van de Nieuwegiessen et al., 2009). For example, Ruane and Komen (2003) found a high cortisol concentration in the water when the loading density of common carp (*Cyprinus carpio*) increased. Most previous studies related to stocking density in RAS were carried out using a one-loop system (Irwin et al., 1999; Schram et al., 2006). Therefore, interaction of the substances accumu-lating in the rearing water can occur, which may make it difficult to elucidate the direct effect of stocking density.

Feed intake or motivation, which is a well-known indicator of fish welfare (Lall and Tibbetts, 2009), is the first and most important element in the feed-growth loop. It is thought that an increase in digestive enzyme production is related to increase feed intake and nutrient assimilation. For example, in roach (*Rutilus rutilus*), the digestive enzyme activity increases with the feed intake in response to increasing temperature (Hardewig and Van Dijk, 2003). However, little is known about the relationship between digestive enzymes and feed intake at different stocking densities.

Stocking density can also affect the level of oxidative stress of the fish (Braun et al., 2010). Greater oxidative stress may increase the production of relative oxygen species (ROS), further altering cellular function. ROS are controlled by antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD) (Ahmad et al., 2000). Most of time, deteriorated water quality also accompanies increased stocking density, which may promote pathogen and bacterial infections. In fish, lysozyme (LZM) is an important immune factor used to eliminate pathogens (Budiño et al., 2006). However, how these biochemical enzymes respond to stocking density is poorly understood (Braun et al., 2010).

The goals of this study were to evaluate the following in *S. maximus* juveniles reared at different stocking densities in RAS: (1) growth indexes, including weight, food conversion rate (FCR), specific growth rate (SGR), and coefficient of variation (CV) of body weight; (2) the relationship between feed intake and protease and amylase activity in the digestive tract; and (3) biochemical parameters, including SOD, CAT, and LZM. The knowledge about turbot performance at different densities gained in this study should provide useful information that will be applicable to industrial fish farming operations.

2 MATERIAL AND METHOD

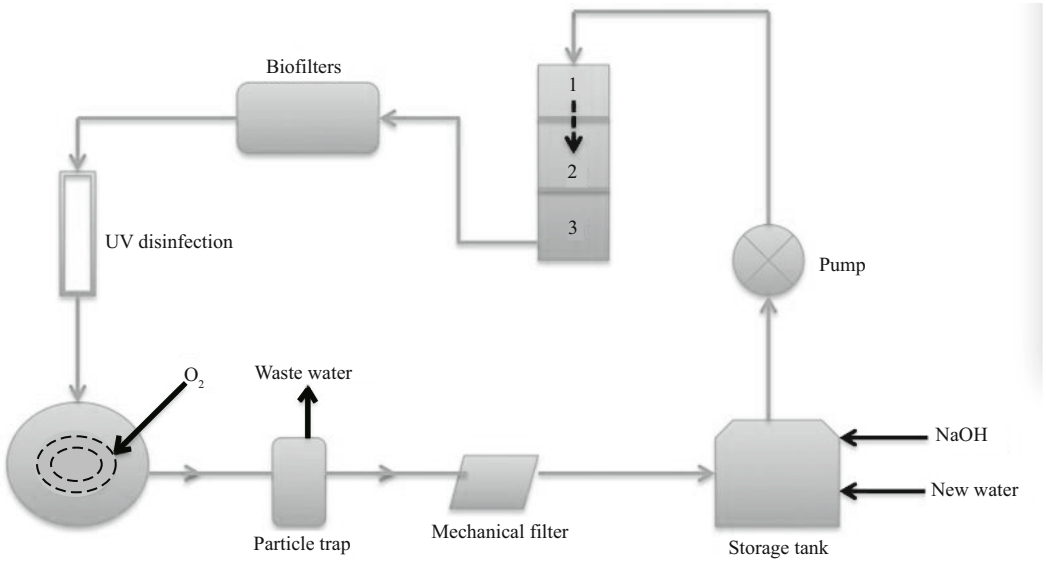
2.1 Source of fish

Turbot were supplied by Shandong Oriental Ocean Sci-Tech Co., Ltd (Shandong, China) and were first stored in a flow-through system for 3 months. Healthy and intact juveniles were selected for acclimation at a density of 170 fish per tank in the experimental RAS for 1 month, by which time a normal appetite was recovered. After biometric measurements were taken, the fish were distributed into 12 RAS to begin the experiment. The initial fish weight was 13.84±2.74 g (average±SD), and the CV for initial weight was ~0.2.

2.2 Experimental design

2.2.1 RAS structure

Twelve RAS were set up at four stocking densities at the Rare Sea Products and Mariculture Laboratory of the Institute of Oceanology, Chinese Academy of Science (Yantai, China). Each RAS consisted of one 550 L (1 m diameter by 0.7 m height) cylindrical fish tank (water depth 0.5 m), a particle trap, a mechanical filter, a pump, a storage tank, a foam fraction, a degassing unit, biofilters, and a UV disinfection system (Fig.1) (Sun et al., 2011). A pump in the water reservoir was used to control the flow though the protein skimmer, degassing unit, biofilters, UV disinfection system, and finally the fish tank. Oxygen was injected into fish tanks via an air stone. The water residence time in the fish tanks was 1 h, and the renewal rate of water in the loop was 1–2 m^3/kg feed. A 12-h light: 12-h dark photoperiod was used.



1: Foam fraction; 2: Degassing unit; 3: Pumping tank

Fig.1 The structure of recirculating aquaculture system

2.2.2 Density distribution

After acclimation, the fish were distributed into 12 RAS at four stocking densities (37, 74, 148, and 222 fish per tank); each density was tested in triplicate. As is typical for flatfish, density was expressed in kg/m^2 and in percent of the tank bottom covered by fish bodies. The initial densities were 0.66 kg/m^2 (low density), 1.26 kg/m^2 (medium density), 2.56 kg/m^2 (sub-high density), and 4.00 kg/m^2 (high density). No differences in weight and CV for initial weight were found among the four densities. The experiment lasted for ten weeks and biometrics were done on the first and last day of the period.

2.3 Food and feeding regimen

Fish were fed EP5 and EP6 semi-dry pellets (Marubeni Nisshin Feed Co., Ltd., Japan). The pellet composition was as follows: protein≥48.0%; lipid≥13.0%; fiber≤2.0%; ash≤16.0%. During the acclimation period, the fish were fed at 1.5% of the biomass every day at 8:00 am and 8:00 pm. Over the experimental period, fish were fed twice a day. The feeding procedures were as follows: (1) increase light intensity for 30 min before feeding; (2) feed to satiation (until about 10 pellets are left on the bottom); (3) quantify the uneaten pellets 20 min after feeding and remove uneaten pellets. To meet the fishes' natural feeding pattern, the speed with which pellets were distributed was initially rapid and then slowly decreased as the fish returned to the bottom.

2.4 Sampling procedure and parameters for analysis

2.4.1 Sampling

All fish were starved for 1 day before sampling. At day 70, five individuals from each RAS were carefully transferred to a 50-L tank containing ~20 mg/L AQUI-S^{TM} (AQUI-S, Lower Hutt, New Zealand) so that they would be slightly anaesthetized. They were weighed quickly, and blood was taken from the caudal vein using heparinized syringes. Samples were centrifuged immediately for 15 min at 5 000 r/min at 4°C. The fish were killed by a blow to the head and dissected on ice to obtain the whole intestinal tract. Separated plasma and intestinal tract were frozen in liquid nitrogen for 2 d and then stored at -80°C until analysis.

2.4.2 RAS water quality

Water temperature, salinity, pH (measured with a JENCO Model 6010 pH meter, China), and dissolved oxygen (measured with a WTW OXI 7300, Germany) in fish tanks were checked and adjusted twice daily. Ammonia and nitrate concentrations in water samples from fish tank drain tubes were tested three times per week using a spectrophotometer (Unico UV-2000, USA). No diseases were found and no chemical

treatments were used during the experimental period.

Over the experimental period, the water temperature was kept at 23±1°C, salinity was 29, and pH was between 7.2 and 7.58. The dissolved oxygen concentration in the fish tanks was kept at 7.5±0.2 mg/L, which was greater than 100% saturation. Total ammonia nitrogen (TAN) concentration was maintained between 0.9 and 3.2 mg/L, un-ionized ammonia nitrogen (NH_3-N) concentration was between 0.03 and 0.09 mg/L, and nitrite concentration was below 2 mg/L.

2.4.3 Analysis methods and parameters measured

2.4.3.1 Growth parameters

Growth parameters were calculated using the following equations:

Stocking density (kg/m^2) = B/A

Specific growth rate (SGR %/day) = (ln W_f – ln W_i)×100/n

Feed conversion ratio (FCR)=$F/(B_f - B_i)$

Coefficient of variation of body weight (CV)=SD/(W_f or W_i)

Percent of covered area (PCA)%=(A_f/A)×100

A_f (m^2) = (102.5W+3 595.0)×10^{-6}, when W<100 g (Irwin et al., 1999)

Voluntary feed intake = F_w/N

B is the biomass in each system; A is the bottom area of the fish tank; A_f is the area of the fish body; W_f and W_i are the final and initial average body weight, respectively; n is the number of days; N is the number of fish; F is the total feed consumption over the experimental period; F_w is the feed consumption every week in each system; B_f and B_i are the final and initial biomass, respectively; and SD is the standard deviation for weight.

2.4.3.2 Digestive enzymes

The whole intestinal tracts were thawed on ice and the surface water was removed by blotting. They were then homogenized using an electrical homogenizer, at a proportion of 1 g of tissue to 9 mL of iced ultrapure water. The homogenates were centrifuged at 6 000 *g* for 30 min at 4°C. The supernatant was collected and then frozen at -80°C. The homogenization and digestive enzyme analysis were completed within 24 h.

The protease activities at different pH levels were estimated using the casein-hydrolysis method (Furné et al., 2008; Gao et al., 2008) with slight modification of the pH buffers. The different pH buffers used were: 0.05 mol/L glycine-HCl for pH 3.0; 0.2 mol/L citrate-0.1 mol/L phosphate for pH 4.0 and 7.0; 0.05 mol/L Tris-HCl for pH 8.5 and 9.0; 0.1 mol/L glycine-NaOH for pH 10.0. For each test at a different pH, 0.4 mL pH buffer, 1 mL casein at 1% (w/v), 0.4 mL extract, and 0.1 mL ultrapure water were mixed and covered for 10 min at 37 °C. Next, 1 mL trichloroacetic acid (TCA, 30% w/v) was added to stop the reaction. Blank tubes containing only the extract and TCA were used as the control. The samples were centrifuged at 3 000 *g* for 10 min. The supernatant was mixed with 5 mL sodium carbonate (0.4 mol/L) and 1 mL Folin reagent and then covered for 15 min at 37°C. Finally, the absorbance of the samples was measured spectrophotometrically at 680 nm. One unit of protease was defined as the quantity of the released tyrosine (mmol) per unit of protein (mg/mL) generated in 10 min.

The concentrations of protein and amylase activity were measured using kits purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China) (Wang et al., 2008).

2.4.3.3 Plasma

Activities of total SOD (which includes CuZn-SOD and Mn-SOD), LZM, and CAT in plasma were determined spectrophotometrically using kits purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China) (Wang et al., 2008; Zhong et al., 2009). Total SOD was determined following Ji (1991), CAT according to Góth (1991), and CAT following Chen and Ji (1992).

2.5 Statistical analysis

The results were expressed as Mean±SD. The analyses were conducted using SPSS version 16.0 for Windows (SPSS, Chicago, IL, USA). The data were first analyzed using Levene's F-test for homogeneity. Differences in parameter values among stocking densities were estimated using one-way analysis of variance (ANOVA) followed by Tukey's HSD comparison test. The percent data were analyzed after arcsine transformation. Differences were considered statistically significant at P<0.05.

3 RESULT

3.1 Voluntary feed intake and growth indicators

After 10 weeks of culture, the stocking densities increased from the initial values of 4.00 (222 fish), 2.56 (148 fish), 1.26 (74 fish), and 0.66 (37 fish) kg/m^2 to the final values of 17.4 (282% PCA), 14.1 (214% PCA),

Table 1 Growth indicators of juvenile turbot (*Scophthalmus maximus*) reared in four stocking densities over 10 weeks

Stocking density	Initial density (kg/m^2)	Final density (kg/m^2)	Final PCA (%)	Final weight (g)	FCR	CV initial	CV final
High	4.00±0.15^a	17.4±0.76^a	282±6.5^a	62.24±2.27^c	0.66±0.03	0.20±0.02	0.35±0.01^a
Sub-h	2.56±0.08^b	14.1±0.70^b	214±7.2^b	75.61±3.78^b	0.67±0.02	0.20±0.02	0.30±0.01^{ab}
Medium	1.26±0.06^c	7.25±0.23^c	108±3.0^c	76.57±3.10^b	0.66±0.01	0.19±0.01	0.29±0.02^{ab}
Low	0.66±0.05^d	4.67±0.53^d	82±5.5^d	99.11±11.32^a	0.66±0.02	0.20±0.01	0.28±0.02^b

P<0.05: significant difference; non-shared characters indicate significant differences. Values are mean±S.D.

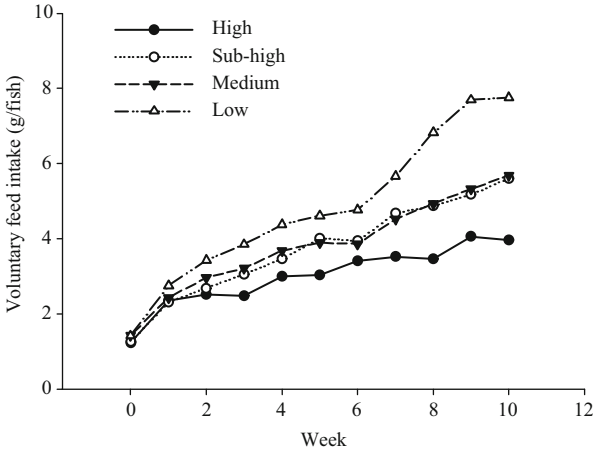


Fig.2 Weekly voluntary feed intake per fish of juvenile turbot (*Scophthalmus maximus*) at four stocking densities

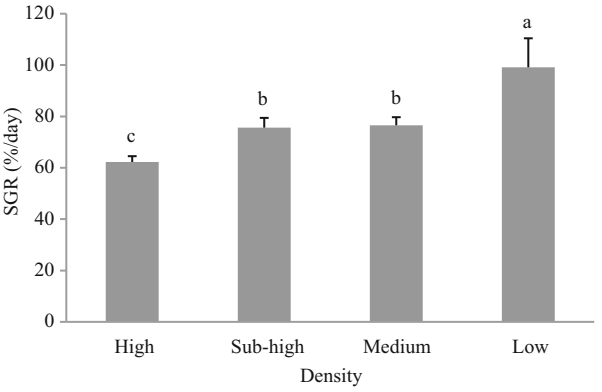


Fig.3 The Specific Growth Rate (%) of juvenile turbot (*Scophthalmus maximus*) at different densities

Non-shared characters indicate significant differences.

7.25 (108% PCA), 4.67 (82% PCA) kg/m^2 (Table 1). No mortality occurred during the experimental period.

The voluntary feed intake during the 10-week period showed that differences first appeared at the second week and became significant from the third week (P<0.05) (Fig.2). The feed intake decreased with increasing stocking density, and the differences

Table 2 Protease activities at different pH and amylase activities in digestive tract of turbot (*Scophthalmus maximus*)

	Stocking density			
	High	Sub-h	Medium	Low
pH 3	3.79±1.82^a	1.58±0.53^b	2.83±1.71^b	4.64±1.94^a
pH 4	2.05±1.40^{ab}	2.72±1.35^{ab}	3.05±1.36^a	1.73±1.09^b
pH 7	5.76±2.43^a	2.34±1.19^b	2.81±0.94^b	2.75±2.05^b
pH 8.5	3.95±0.83^a	1.86±0.80^{bc}	2.07±0.71^b	1.20±0.43^c
pH 9	7.52±1.34^a	3.31±1.38^b	3.52±0.84^b	3.32±0.91^b
pH 10	12.41±2.91^a	5.63±1.66^b	5.45±1.88^b	5.38±2.27^b
Amylase	1.68±0.57	1.50±0.17	2.11±0.25	1.95±0.78

Values are U/mg protein, and expressed as mean±S.D.; different letters (a, b, c) indicate significant differences

Table 3 Plasma parameters of juvenile turbot (*Scophthalmus maximus*) at the end of 10-week period

	LZM	CAT	SOD
High	9.41±0.76	2.70±1.64	62.76±0.43
Sub-h	9.37±0.85	1.64±0.42	61.16±7.00
Medium	8.32±0.10	1.96±0.73	64.66±1.70
Low	9.13±0.88	1.66±1.01	65.24±2.76

Values are U/mL and expressed as mean±S.D.; LZM: lysozyme; CAT: catalase; SOD: superoxide dismutase

increased over time. The final average weight and SGR significantly decreased with increasing stocking density (P<0.05) (Fig.3, Table 1). The CV also increased with increasing density by the end of the experiment (P<0.05). No significant difference was found for FCR among the four stocking densities (P>0.05) (Table 1).

No differences were found between the sub-high and medium density groups for feed intake (Fig.2) or the growth indexes, including weight, SGR, FCR, and CV of weight (P>0.05) (Fig.3, Table 1).

3.2 Digestive enzymes

At the end of the experiment, protease activities in the turbot digestive tract were tested at pH 3, 4, 7, 8.5, 9, and 10 (Table 2). At pH 7, 8.5, 9, and 10, the protease activities for the high density group were significantly higher than those for the other groups (P<0.05). At pH 4 and 8.5, the low density group had a tendency for lower protease activity compared with the others, but the difference was not statistically significant (P>0.05). For the sub-high and medium groups, no significant differences were measured on the protease activity (P>0.05). No significant differences were found for amylase activity among the different densities (P>0.05).

3.3 Plasma parameters

When turbot plasma was analyzed at the end of the experiment, no statistically significant differences were found among the different densities for LZM and total SOD (Table 3). However, CAT activity was higher (but not significantly) at the highest density compared with the others.

4 DISCUSSION

During the 10-week experiment, no mortality occurred at any of the four densities. All water parameters at the outlets were within the safe range for juvenile turbot. NH_3-N concentration was below 0.108 mg/L (Skøtt Rasmussen and Korsgaard, 1996) and nitrite was below 13.07 mg/L (Qu et al., 2007). It would have been ideal to measure the water quality close to the bottom where turbot spend most of their time, as the water quality values there may have differed from the water quality of the outlets. However, these measurements were not made because of technical problems.

4.1 Growth and feed intake

In this experiment, a fixed number of turbot was used to exclude handing effects, which may have a stronger impact than density itself (Braun et al., 2010). Increased stocking density negatively affected turbot growth, as indicated by SGR, final weights, and CV for final weight on day 70. The fish at the lowest density had the fastest growth rate, the highest final weight, and the lowest average body weight dispersion (CV for weight). Fish at the highest density had the slowest growth rate, the lowest final weight, and the highest size dispersion. Previous studies have reported an inverse relationship between density and growth among flatfish, including turbot, California halibut (*Paralichthys californicus*), and Dover sole (*Solea solea*) (Martinez-Tapia and Fernandez-Pato, 1991; Irwin et al., 2002; North et al., 2006b; Schram et al., 2006; Merino et al., 2007). In those studies, no significant difference in average weight was found between groups at densities of 0.25 and 0.5 kg/m^2 (Martinez-Tapia and Fernandez-Pato, 1991), whereas noticeable differences were found for SGR, final weight, and CV for final weight between the lowest and highest groups at densities of 0.7, 1.1, 1.5, and 1.8 kg/m^2, after 45 d (Irwin et al., 1999).

In the current study, in which a large density range was used, an independent impact of density on related parameters appeared. Although the stocking density of the sub-high group (2.56 kg/m^2) was twice that of the medium group (1.26 kg/m^2) group, the growth indexes (weight, SGR, FCR, and CV) were similar. Between 1.26 and 2.56 kg/m^2 (final densities: 7.25 and 14.16 kg/m^2), the related growth indexes were independent of the density.

Because a strong positive correlation between food consumption and growth exists (Jobling and Baardvik, 1994; Irwin et al., 2002) and because FCR was the same at all four densities tested, the diet intake was the main cause for the observed differences in growth (Carter et al., 1996). Competition for food is believed to be the main reason for growth differences when food is limited (Irwin et al., 1999). In the current study, food was not limited. However, when stocking density increased, negative social interactions among fish increased, especially when they were struggling for food at the beginning of the feeding process. This resulted in decreased feed intake even though food was not limited.

The sub-high and medium density groups exhibited the same feed intake, and no significant differences were found for SGR or other growth parameters. This finding supports the premise that feed intake plays a key role in culture. It may be related to the turbot's benthic habits. Under conditions of good water quality and enough food, turbot can tolerate crowding within a certain density range. Thus, the increased social behavioral interactions and density did not affect the voluntary feed intake or growth.

To summarize, competition for food and social interactions generally lead to decreased feed intake and heterogeneous growth at higher densities, even without food limitation. However, in the present study

no negative impacts were found at densities between 1.26 and 2.56 kg/m^2 (final density: between 7.25 and 14.15 kg/m^2). At high densities, multi-level feed devices to disperse competition stress and enable fish to acquire enough food should be used (Boujard et al., 2002).

4.2 Digestive enzymes

Amylase and protease activities of the digestive tract at different pH values were examined at the end of the experiment to identify the relationship between feed intake, digestive enzymes, and high stocking density. Amylase activities were the same at different densities. However, fish at the high density had the highest protease activity and the lowest feed intake, whereas those at the low density seemed to have the lowest protease activity at certain pH values and the highest feed intake.

The assimilation process of fish is directly linked to feed intake (Clark et al., 1995; Lall and Tibbetts, 2009). For roach, digestive enzyme activity increased with the increasing feed intake that occurred in response to elevated temperature (Hardewig and Van Dijk, 2003). In contrast, for yellowtail kingfish (*Seriola lalandi*), protease activity was higher when feed intake decreased during winter compared with summer (Miegel et al., 2010). Thus, the relationship between feed intake and digestive enzyme activity appears to depend on species and conditions. In a study of the impact of density on the digestive enzymes of Japanese flounder (*Paralichthys olivaceus*) juveniles, swimming juveniles were lighter and had a significantly higher trypsin activity than those that were settled on the bottom at the same density (Bolasina et al., 2006). Trypsin activity of those swimming fish decreased 2 d after transfer to a lower density. It remains poorly understood how adaptation of fish behavior regulates feed intake, digestive activity, and assimilation. There is a complicated regulation of energy balance and food intake by peripheral organs (e.g., digestive tracts) and the brain (Lin et al., 2000). Furthermore, when under nutrient limitation stress, fish develop a compensatory strategy, prompting better assimilation efficiency (Miegel et al., 2010). This process may explain why fish at the high density in this study had the highest protease activities even though feed intake was lower. For the sub-high and medium density groups, protease activities did not differ significantly, which was consistent with feed intake and growth index results.

4.3 Plasma parameters

In this study, no significant difference was found among densities for total SOD and LZM. Increasing density was found to possibly induce the oxidative effect in *Salminus brasilliensisi* (Braun et al., 2010), but no information about how stocking density induces oxidative stress in turbot is available. CAT activity of the high-density group was higher than that of the other groups in this study, but the difference was not statistically significant. The same response was reported when goodeid fish (*Girardinichthys viviparus*) were exposed to polluted water (Vega-López et al., 2008). Susceptibility of antioxidant enzymes to stress varies among different fish species (Hasspieler et al., 1994). Thus, high density may have begun to induce the oxidative impact on turbot, but not significantly. Further investigations are necessary to clarify this issue.

5 CONCLUSION

1) Different stocking densities result in heterogeneous growth rates, weights, and feed intake due to social interactions, even without food limitation. Under conditions of good water quality and enough food, no negative impact of density was found between 1.26 and 2.56 kg/m^2 (final 7.25 and 14.16 kg/m^2). The use of multi-level feeding devices to decrease the stress of competition for food at high stocking densities is recommended. In general, turbot are suitable for culture in RAS at a relatively high density (17.47 kg/m^2).

2) It seems that protease activity was inversely related to feed intake in the present study. The reason for this result is poorly understood, and further studies are needed.

3) High stocking density may induce the oxidative effect in turbot. A stocking density of less than 17.47 kg/m^2 is recommended for turbot juveniles in case an oxidative impact impairs fish health at higher densities.

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