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Effect of Stocking Density on Water Quality and (Growth, Body Composition and Plasma Cortisol Content) Performance of Pen-Reared Rainbow Trout (*Oncorhynchus mykiss*)

LIU Qun#, HOU Zhishuai#, WEN Haishen*, LI Jifang*, HE Feng, WANG Jinhuan, GUAN Biao, and WANG Qinglong

1) *College of Fisheries*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*
2) *Key Laboratory of Mariculture*, *Ministry of Education*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract The goal of the study was to examine the effect of stocking density on the water quality of culture area, as well as the growth, body composition and cortisol content of rainbow trout (*Oncorhynchus mykiss*). Pen-reared trout were stocked in densities of 40, 60, 80 fish individuals m^{-3} (4.6, 6.6, 8.6 kg m^{-3}, SD1, SD2 and SD3 groups, respectively) for 300 days. Compared to the water from SD1 and SD2, that from SD3 exhibited significantly higher NH_4^+-N content and COD (chemical-oxygen-demand), and a significant reduction of dissolved oxygen in day 180 (40.6 kg m^{-3}). Stocking density was significantly associated with body weight, standard length, VSI (viscerosomatic index), CF (condition factor) and FC (food coefficient) in group SD3, particularly in day 240 and day 300 (45 or 49.3 kg m^{-3}). Increased crude fat and decreased crude protein were displayed in high density group when the density reached to 36 kg m^{-3}. As a cumulative effect of density-related stress, VSI, CF, FC, moisture, and crude protein content varied over time in each density group (SD1, SD2, and SD3). In summary, trout exhibited a better growth performance in low density (26.3 kg m^{-3}) than those reared in high densities (36 and 45 kg m^{-3}). The results indicate that rainbow trout (114.44 g ± 6.21 g, 19.69 cm ± 0.31 cm) initially stocked in 6.6 or 8.6 kg m^{-3} should be lightened to less than 36 kg m^{-3} after an intensive rearing for 240 days.

Key words rainbow trout; stocking density; growth; cortisol; body composition

1 Introduction

Stocking density is a major factor that can determine the productivity of pen-reared fish, and many commercial institutions show great interests in this area (Ashley, 2007; Ellis, 2002). Usually the increasing stocking density can increase energy demand and alter digestive enzyme activities (Bolasina *et al*., 2006), as well as body composition (Toko *et al*., 2007). Crowded pen-rearing condition is considered a chronic stressor that can impede fish growth and lead to elevated physiological stress (Montero *et al*., 1999; Larsen *et al*., 2012). To some species such as the Arctic char (*Salvelinus alpinus*), although high density may have a positive effect (Jørgensen *et al*., 1993), most of the studies have reported that high stocking density impede the overall welfare (Lupatsch *et al*., 2010), body composition (Montero *et al*., 1999; Piccolo *et al*., 2008;), and growth performance of most fish species (North *et al*., 2006; Lin *et al*., 2009; Tolussi *et al*., 2010; Oliveira *et al*., 2012). Previous studies have shown that high density can impair the growth (Costas *et al*., 2008; Santos *et al*., 2010). In early studies of water quality for aquaculture, an increasing stock density is shown to have a negative effect on water quality parameters such as reduction of dissolved oxygen (DO), as well as increase of ammonium (NH_3), nitrate (NO_3^-) and carbon dioxide (CO_2) contents (Fivelstad and Binde, 1994; Fivelstad *et al*., 1995, 1998). In recent years, many reports have indicated that both changes in fish density and water quality may affect fish growth performance (Hosfeld *et al*., 2009; North *et al*., 2006).

Cortisol is the principal glucocorticoid and plays an essential role in the maintenance of energy balance, immunity regulation and growth when teleost are exposed to exogenous stresses (Mommsen *et al*., 1999; Hori *et al*., 2010). Cortisol treatment affects glucocorticoid response and heat shock protein 90 gene expression in rainbow trout liver (Sathiyaa and Vijayan, 2003; Vijayan *et al*., 2003). Moreover, tilapia (*Oreochromis niloticus*) larvae immersed in cortisol and channel catfish (*Ictalurus punctatus*) injected with glucocorticoids demonstrated a reduction in myostatin gene mRNA abundance (Rodgers *et al*., 2003; Weber *et al*., 2005). Cortisol is also widely

# The authors contributed equally to this work.
* Corresponding authors. E-mail: wenhaishen@ouc.edu.cn
E-mail: lijf@ouc.edu.cn

used as a biomarker of stress to fish. Previous studies have shown that plasma cortisol content in sole (*Solea senegalensis*) significantly increase in high stocking densities. A similar finding has been reported in rainbow trout (Ellis *et al*., 2002; López-Patiño *et al*, 2013; Laursen *et al*., 2013).

Rainbow trout are among the most intensively studied fish in a wide range of subjects such as physiology, genetics and nutrition (Nagler *et al*., 2007; Walock *et al*., 2014; Wiens *et al*., 2014). In China, the trout industry contributed significantly to the aquaculture industry, particularly to the pen-reared aquaculture model fish in rural areas. Most rainbow trout farms in the northwest region of China use pen-reared culture systems in reservoirs with simple facilities for intensive culture. Profitability depends on the facilities expenditure, daily cost and the volume of production. As an effort of reducing cost and boosting productivity, farmers often increase the stocking density that is much higher than the fish experienced in the wild. But whether the increased density causes problems relating to growth and body composition has yet to be systematically documented. The objective of the present study was to determine the effect of stocking density on growth and body composition in pen-reared rainbow trout, and to identify the optimum density for growth performance and productivity. The aim also included the investigating of stocking density on water quality and adaptive progress as was indicated by cortisol content.

2 Materials and Methods

2.1 Experimental Animals and Husbandry

The average body weight of the rainbow trout in stocking conditions was 114.44 g ± 6.21 g, and the standard length was 19.69 cm ± 0.31 cm. Rainbow trout were stocked in 3 m×3 m×3 m net cages in triplicate. All fish were at the same ages. The initial three stocking densities were 4.6 kg m^{-3}, 1080 fishes per cage (40 fishes m^{-3}) for group SD1; 6.6 kg m^{-3}, 1620 fishes per cage (60 fishes m^{-3}) for group SD2; 8.6 kg m^{-3}, 2160 fishes per cage (80 fishes m^{-3}) for group SD3. According to the standards of the aquaculture industry and other information for rainbow trout studies, 40 fishes m^{-3} (4.6 kg m^{-3}) and 80 fishes m^{-3} (8.6 kg m^{-3}) are considered low and high initial stocking densities, and 30 kg m^{-3} as a relatively high density in which growth performance and overall welfare of trout is not impaired (UK Farm Animal Welfare Council (FAWC); Aksakal *et al*., 2011; Larsen *et al*., 2012). Fish were fed on a commercial pellet diet with 40% protein, 26% fat and 14% carbohydrate, twice a day at 8:00 a.m. and 4:00 p.m., respectively. The feeding rate was depended on the body weight of the fish. During the experiment, the fish were fed at 1%–2% of the body weight. Water quality was constantly monitored for factors such as pH, temperature, ammonia nitrogen and dissolved oxygen. Nets of pen-rearing cages were cleaned and maintained every other week. The experiment was carried out from May 2011 to March 2012 for a total of 300 days.

2.2 Water Parameter Measurement

Daily recordings were taken at 09:00 am for water parameters such as temperature (T), dissolved oxygen (DO) and pH using Multi-functional water quality monitor HACH HQ40d 58258-00, chemical oxygen demand (COD) with $KMnO_4$ method, and the content of NH_4^+-N with Nessler's reagent. During the experiment, temperature varied between 8.51 and 18.20℃ and pH between 8.5 and 8.9, and the natural photoperiod was followed.

2.3 Sampling Protocol

Twelve fish were randomly selected each density and mildly anaesthetized in a tank with MS-222 (3-Aminobenzoic acid ethyl ester methanesulfonate) at 40–45 mg L^{-1} (or ppm). Body weight (exact to 0.01 g), standard length (fork length, exact to 1 mm), eviscerated weight (exact to 0.01 g) were measured. Blood was sampled from caudal vein with syringes and stored at 4℃. The blood was then separated by centrifugation (1200 g for 15 min) and stored at −80℃ for cortisol assay. The fish bodies were stored in polyethylene bags and store at −40℃ for the body composition analysis.

2.4 Growth and Feed Conversion Ratio

The calculation was conducted with (according t with) the following methods. Coefficient of variation (CV) = 100 × SD/Mean. Food coefficient (FC) = (Feed intake (g)) × $(W_{t2}-W_{t1}-W_{\mathrm{dead}})^{-1}$, W_{t2}, W_{t1} and W_{dead} represent the body weight at sampling time t_2 and t_1 and that of dead fish. The condition factor (CF) = 100 × Body weight × Body length^{-3}, Hepatosomatic index (HSI) = 100 × Liver weight/ Body weight. Viscerosomatic index (VSI) = 100 × Visceral weight/Body weight.

2.5 Plasma Cortisol and Body Composition

The plasma cortisol content was measured using a commercially available solid-phase ^{125}Iodine radioimmunoassay kit (Tianjin Nine Tripods Medical and Bioengineering Co., Ltd. Tianjin, China), following the method described previously (Wen *et al*., 2006; Mu *et al*., 2013). The assay sensitivity reached 0.21 μg dL^{-1} by the kit protocol.

According to the reported experiment on body composition about croaker (*Nibea japonica*), flounder (*Paralichthys olivaceus*), and amur sturgeon (*Acipenser schrenckii*), whole fish body were analyzed with standard methods for moisture, ash, crude fat and crude protein (Han *et al*., 2014; Kim *et al*., 2014; Ni *et al*., 2016).

2.6 Statistical Analysis

The effects of stocking density were evaluated by one-way analysis of variance (ANOVA), while the differences were analyzed via Duncan's multiple comparison range test. It was considered statistically significant when $P < 0.05$. Data are presented as mean ± S.D. All data and results were analyzed and discussed among different densities and within each density. Data analyses were conducted with the SPSS (version 13.0) software.

3 Results

3.1 Parameters of Stocking Density and Water Quality

Variation in densities is shown in Table 1. Densities increased considerably between day 120 and day 180. Mortality in SD3 was 3.35%±0.14%, significantly higher than those in SD1 (2.21%±0.21%) and SD2 (2.48%±0.24%) in day 300.

COD and NH_4^+-N showed significant difference among densities in day 300. Moreover, after peaking in day 120, the NH_4^+-N content decreased in groups of SD1 and SD2, but remained high in group SD3 in day 300. The COD showed significant difference among different densities in day 180 when the densities increased dramatically. Pen-reared cages populated more heavily with fish growth, and consequently, in day 300, content of DO in group SD3 was significantly lower than that in SD1 and SD2. DO content of SD1, SD2 and SD3 was 6.9, 6.3, and 5.4, respectively.

Table 1 Parameters of water quality in various stocking densities measured in different days

Item		Day 0	Day 60	Day 120	Day 180	Day 240	Day 300
	SD1	4.6	8.9	12.0	22.6	26.3	31.1
Density (kg m^{-3})	SD2	6.6	12.7	16.4	31.7	36.0	40.6
	SD3	8.6	15.9	20.0	40.6	45.0	49.3
T (℃)		11.98	18.20	16.19	9.60	8.51	10.19
	SD1	8.5	8.8	8.7	8.6	8.9	8.7
PH	SD2	8.6	8.8	8.7	8.5	8.8	8.7
	SD3	8.7	8.8	8.7	8.5	8.8	8.6
	SD1	0.35±0.08	0.35±0.05	0.53±0.05	0.36±0.10	0.36±0.11	0.33±0.05^a
NH_4^+-N (mg L^{-1})	SD2	0.34±0.08	0.37±0.02	0.55±0.01	0.38±0.07	0.39±0.09	0.36±0.08^a
	SD3	0.36±0.09	0.37±0.07	0.60±0.06	0.43±0.09	0.41±0.08	0.55±0.07^b
	SD1	7.54±0.68	8.63±0.71	8.29±0.62	8.21±0.51^a	9.42±0.68	10.21±0.81^a
COD (mg L^{-1})	SD2	7.73±0.53	8.65±0.69	8.36±0.98	8.23±0.63^a	9.53±0.77	11.61±0.78^a
	SD3	7.86±0.62	8.77±0.84	8.52±0.77	9.71±0.54^b	9.62±0.98	13.28±0.88^b
	SD1	7.5±0.12	6.8±0.31	6.5±0.22	6.7±0.25	7.2±0.16	6.9±0.24^b
DO (mg L^{-1})	SD2	7.6±0.20	6.8±0.17	6.4±0.14	6.3±0.11	6.9±0.22	6.3±0.35^b
	SD3	7.7±0.15	6.5±0.25	6.8±0.41	6.4±0.31	6.9±0.38	5.4±0.31^a

Notes: COD=Chemical oxygen demand; DO=Dissolved oxygen. Data are presented as mean±S.D. $n=24$ for density; $n=3$ for water quality. Different superscripts in same day indicate significant difference.

3.2 Growth, Standard Length, and Coefficient of Variation

Table 2 presents growth performance of rainbow trout in different stocking densities. The highest body weight was observed in group SD1 in day 300. In day 120 and day 180, the body weight increased rapidly. Both weight and standard length demonstrated significant difference between SD1 and SD3 after 240 days density treatment. Moreover, the trout in higher density group displayed greater coefficient of variations in weight and standard length, especially in day 240 and day 300.

Table 2 Growth, Standard length and coefficient of variation of rainbow trout in various stocking densities measured in different days

Item		Day 0	Day 60	Day 120	Day 180	Day 240	Day 300
	SD1	114.42±4.10	221.37±6.84	298.48±8.15^b	562.15±18.90	654.18±21.65^c	773.57±20.10^c
Weight (g)	SD2	114.70±4.30	220.71±7.86	285.01±7.28^b	550.90±17.70	625.63±24.32^b	705.57±18.25^b
	SD3	114.22±4.90	211.17±6.44	265.62±10.40^a	539.22±16.12	597.66±19.83^a	654.77±20.78^a
	SD1	3.58	3.09	2.73	3.36	3.31	2.60
CVW	SD2	3.75	3.56	2.55	3.21	3.89	2.59
	SD3	4.29	3.05	3.92	2.99	3.32	3.17
	SD1	20.31±0.58	24.51±0.71	26.81±0.83	33.26±1.36	36.05±1.38^c	38.20±1.64^c
SL (cm)	SD2	20.29±0.52	24.33±0.67	25.35±0.76	31.18±1.52	33.21±1.48^b	36.58±1.60^b
	SD3	20.31±0.59	24.13±0.65	25.41±0.88	30.71±1.37	30.88±1.72^a	32.22±1.42^a
	SD1	2.86	2.90	3.10	4.09	3.83	4.29
CVSL	SD2	2.56	2.75	3.00	4.87	4.46	4.37
	SD3	2.90	2.69	3.46	4.46	5.57	4.41

Notes: CV=Coefficient of variation; CVW=CV of weight; SL=Standard length; CVSL=CV of standard length. Data are presented as mean±S.D. $n=24$. Different superscripts in same day indicate significant difference.

3.3 Growth and Food Coefficient Among Different Densities

HSI demonstrated no considerable difference among three stocking densities (Fig.1A). VSI in SD2 and SD3 was significantly higher than that in SD1 from day 120 to day 300 (Fig.1B). The CF of SD3 showed significant up-regulation in day 180, 240 and 300 (Fig.1C). During the period from day 180 to day 300, SD3 was significantly higher in FC than other two groups (Fig.1D).

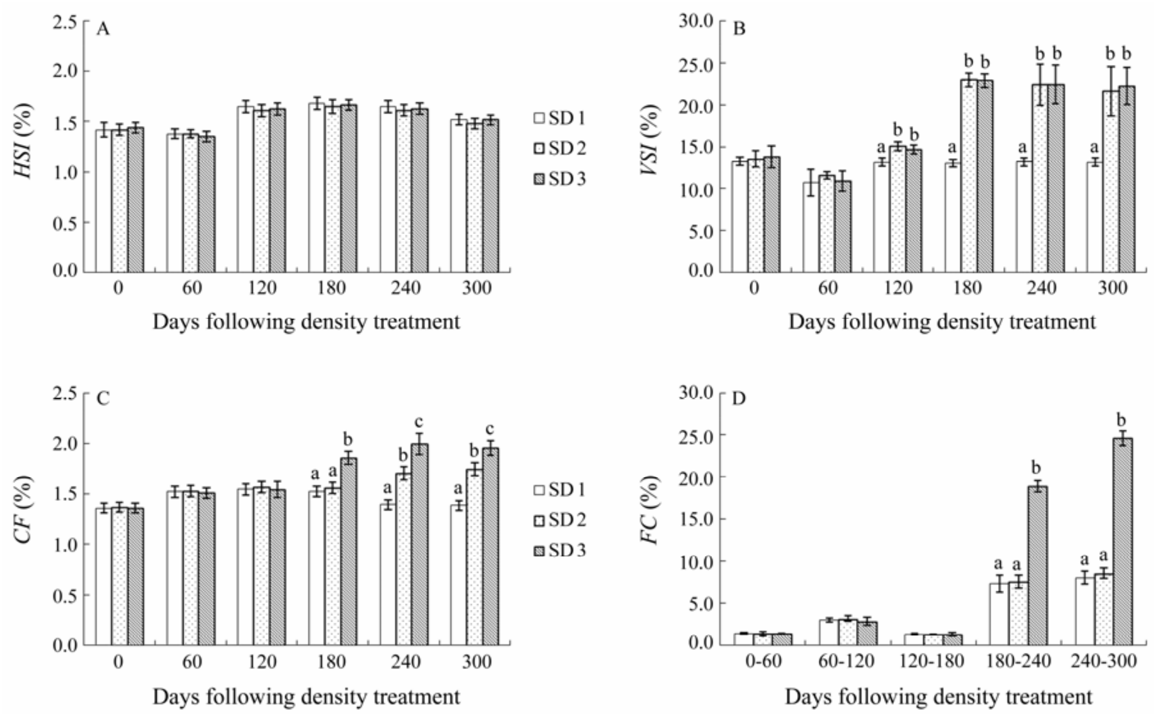

Fig.1 Changes of HSI (hepatosomatic index, A), VSI (viscerosomatic index, B), CF (condition factor, C), FC (food coefficient, D) of rainbow trout in SD1, SD2 and SD3. Data are presented as mean±S.D. $n=12$. Different superscripts in same day following density treatment indicate significant difference.

3.4 Growth and Food Coefficient Within Densities

HSI levels showed significant increases in day 120 compared to that in day 60 within the three densities. In addition, HSI decreased significantly between day 240 and day 300 (Fig.2A). VSI showed significant reduction between day 0 and day 60 within the three density populations. Within SD2 and SD3, VSI in day 0 was significantly lower than those in day 180, 240, and 300 (Fig.2B). CF showed a significant increase in day 60 within the three density populations. CF of SD2 and SD3 increased significantly in day 240 (Fig.2C). FC increased significantly in day 60 to 120 and subsequently decreased. FC increased significantly and continuously in day 240 and day 300 within SD2 and SD3 (Fig.2D).

3.5 Body Composition Among Different Densities

Effects of stocking density on the body composition are shown in Fig.3. Moisture content of SD1 and SD2 was markedly lower than that of SD3 in day 240 and day 300 (Fig.3A), and ash content of SD1 was significantly higher than those of the other two groups in day 60 (Fig.3B). Significant differences were observed among these three densities from day 240 to day 300 with lower crude fat levels in SD1 (Fig.3C). As the stocking density increased, however, the mean crude protein level of SD2 and SD3 were significantly lower than that of SD1 from day 240 to day 300 (Fig.3D).

3.6 Body Composition Within Densities

In SD3, moisture content increased to the same level as day 0 in day 300, whereas moisture content in SD1 and SD2 in day 300 were significantly lower than the values in day 0 and day 60 (Fig.4A). Ash content demonstrated a similar trend within the SD1, SD2, and SD3 populations with a significant increase in day 60 and a significant decrease in day 120 (Fig.4B). Crude fat decreased significantly in day 60 and remained stable within each density population (Fig.4C). Crude protein in SD1 increased significantly in day 300, whereas crude protein in SD2 and SD3 decreased significantly in day 300 (Fig.4D).

3.7 Plasma Cortisol Among and Within Densities

In sampling times of day 120, 240 and 300, SD3 showed a significantly higher cortisol response levels compared to those in SD1 (Fig.5A). Plasma cortisol firstly displayed a significant decrease and then, in day 180, increased significantly within each density population. In day 300, cortisol levels showed significant down- regulation again in each density (Fig.5B).

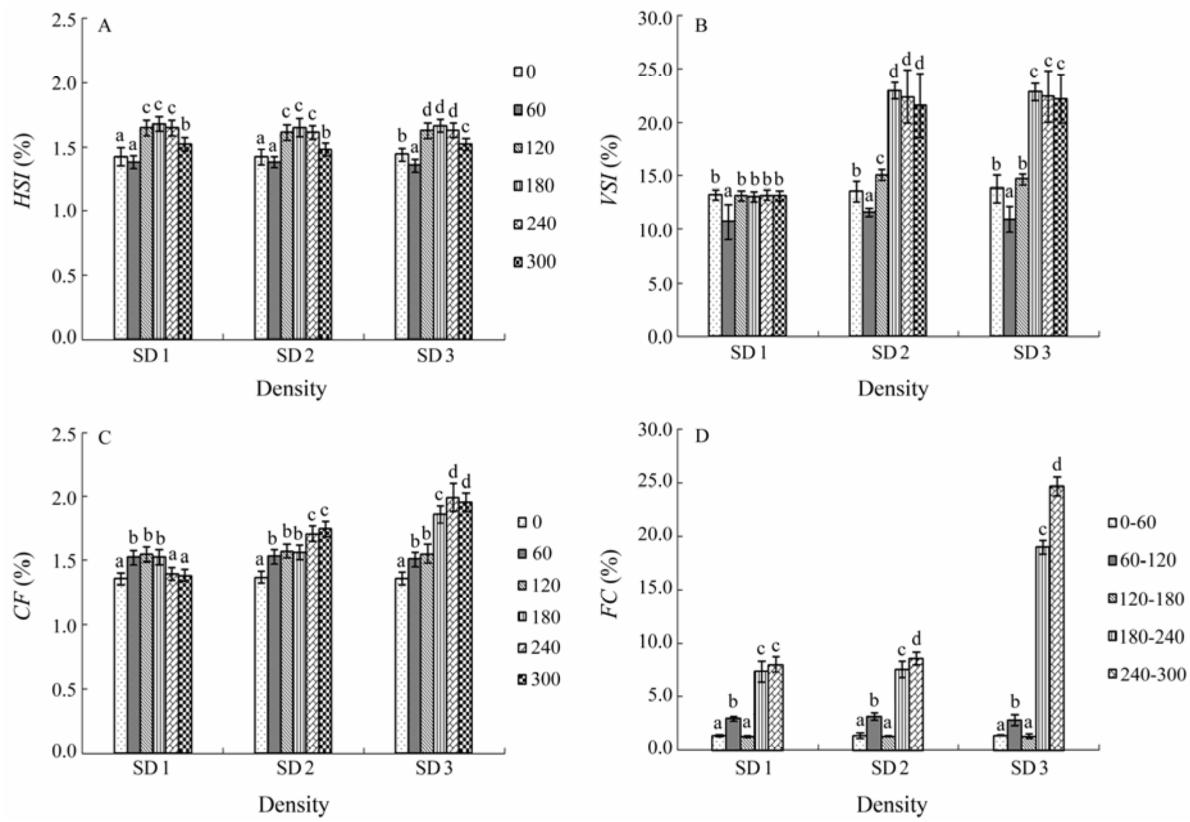

Fig.2 Changes of HSI (hepatosomatic index, A), VSI (viscerosomatic index, B), CF (condition factor, C), FC (food coefficient, D) of rainbow trout in SD1, SD2 and SD3. Data are presented as mean ± S.D. $n = 12$. Different superscripts within each density indicate significant difference.

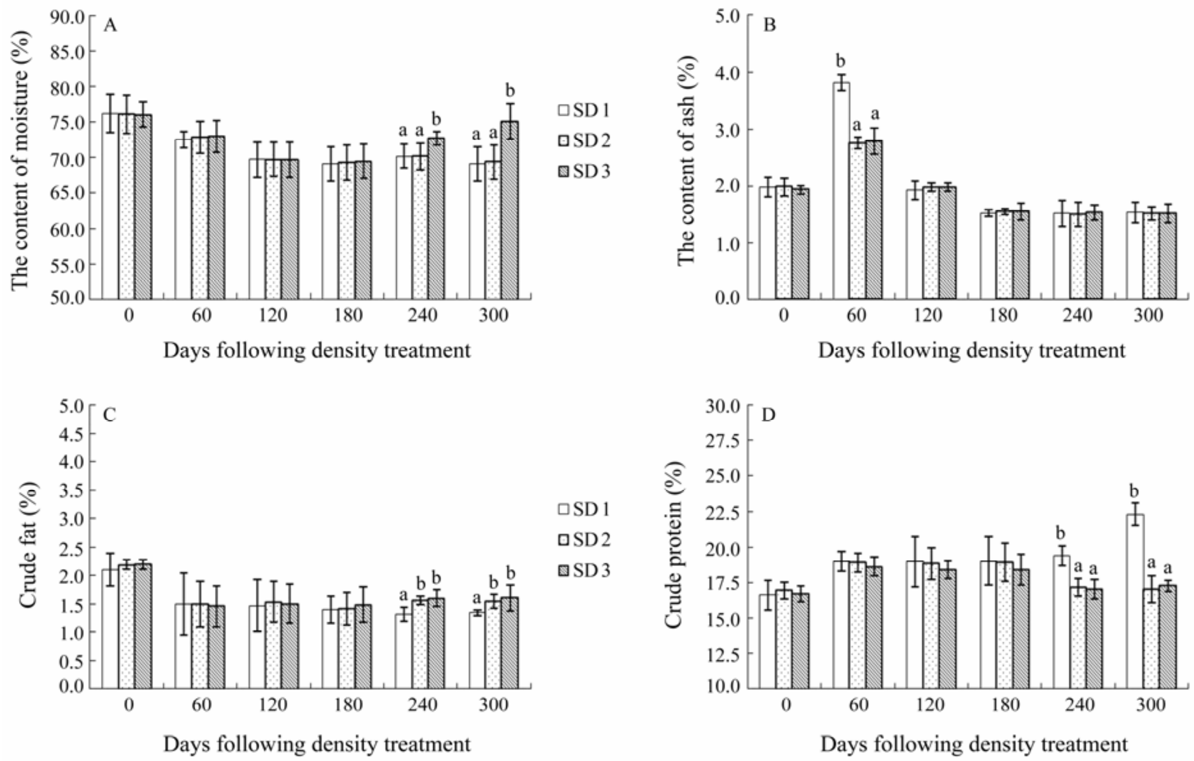

Fig.3 Changes in body composition parameters (moisture A, ash B, crude fat C and crude protein D) of rainbow trout in SD1, SD2 and SD3. Data are presented as mean ± S.D., $n = 12$ (moisture, ash), $n = 10$ (crude fat and crude protein). Different superscripts in same day following density treatment indicate significant difference.

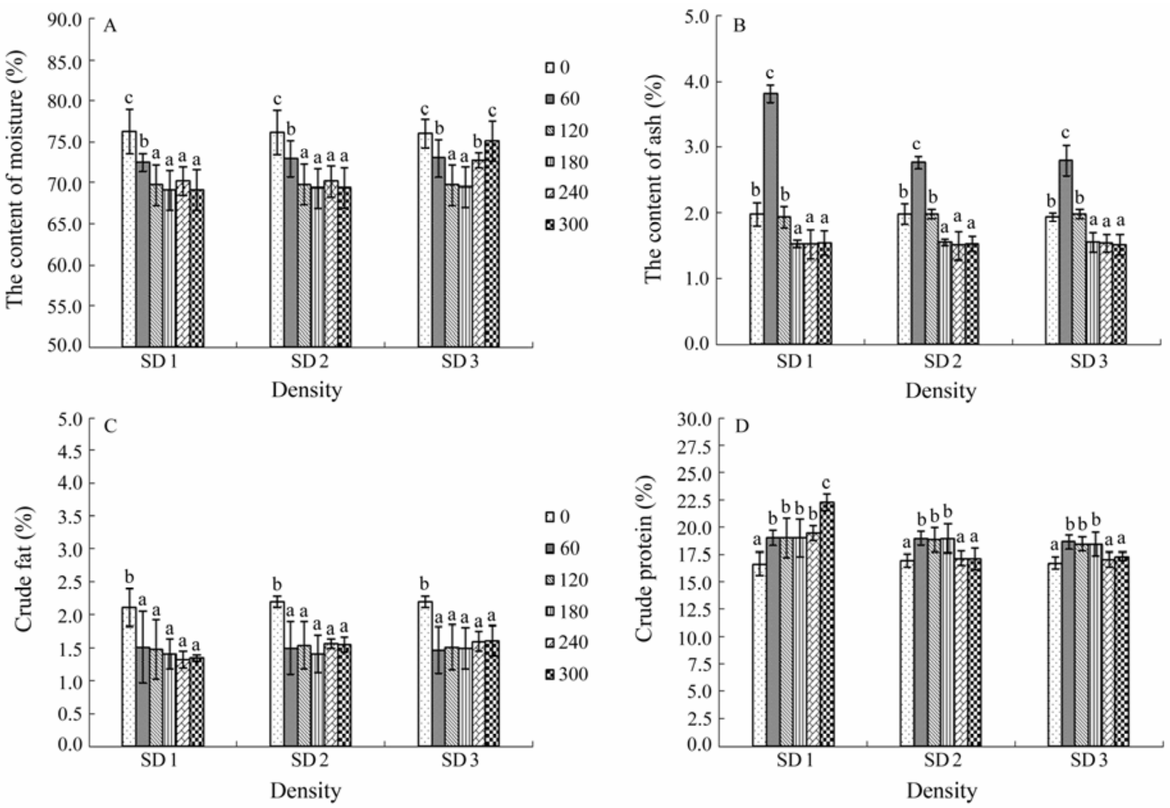

Fig.4 Changes in body composition parameters (moisture A, ash B, crude fat C, and crude protein D) of rainbow trout in SD1, SD2 and SD3. Data are presented as mean ± S.D. $n = 12$ (moisture, ash), $n = 10$ (crude fat and crude protein). Different superscripts within each density indicate significant difference.

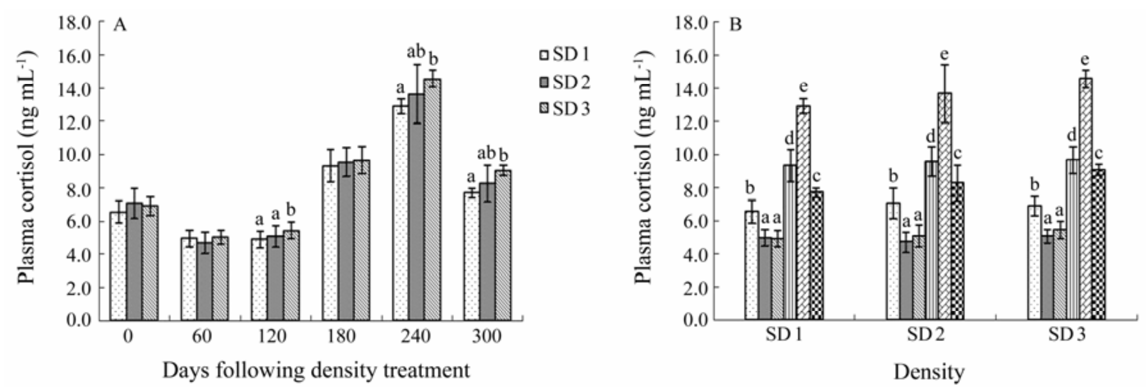

Fig.5 Changes in plasma cortisol of rainbow trout in SD1, SD2 and SD3 in density treatment (A) and within each density (B). Data are presented as mean ± S.D. $n = 12$. Different superscripts indicate significant difference.

4 Discussion

Several factors are known to suppress growth in high stocking densities, which included foe example reducing food consumption (Oppedal *et al*., 2011), inhibiting social interactions (Sloman *et al*., 2000), and worsening water quality (Fivelstad *et al*., 1995, 1998). In this experiment, population density clearly affected water quality and with the increase of density, high COD, NH_4^+-N and low DO appeared. Water quality significantly changed after day 180 when the density exceeded 40.6 kg m^{-3} among different densities, and the differences in NH_4^+-N, COD and DO may have been responsible for the significant differences of fish mortality.

Rainbow trout showed density-dependent growth performance in the present study as the highest body weight was observed in the lowest density and vice versa. It has been reported that rainbow trout growth is affected by high density (Trenzado *et al*., 2006) as high culture density can increase the demand for energy and metabolism. Additionally, chronic stress produces a series of defense mechanisms that can divert energy from being used for growth to other stress-responsive and energy-consuming processes (Elliott, 1976; Lupatsch *et al*., 2010). In the present study, higher stocking densities resulted in both significantly lower body weight and standard length in day 240 (36 kg m^{-3}), suggesting that 36 kg m^{-3} is a key point in which additional energy is used for body defense mechanisms, reducing the resources available for body growth.

Majority of energy from food is converted into net energy for maintenance (NE_M, including basic metabolism and adaptive processes for stressful situations) and net energy for production (NE_p, including factors such as growth performance, body fat, and reproduction) in teleost (Elliott, 1976; Fan, 2008). An intensive social interactions caused by dense rearing condition might contribute to increased basic metabolism demands, arrested growth, and low food intake in fish (Larsen *et al*., 2012; Liu *et al*., 2014). In the present study, FC of high density was significantly increased when density was 45 kg m^{-3}. This significant up-regulation of FC indicates that density stress reduce the ratio of growth energy from food energy. Moreover, CF and VSI also demonstrated significant increases within two higher density groups when density was over 45 kg m^{-3}. As CF and VSI can roughly indicate the body energy stores (Goede and Barton, 1990), these results suggest that the stress of higher density may lead to viscerosomatic energy storing and viscerosomatic mass growing processes, which may reduce the growth of trout.

Previous studies have found that stocking densities can affect the body composition of African catfish (*Clarias gariepinus*) and vundu catfish (*Heterobranchus longifilis*, Toko *et al*., 2007). In our study, high density clearly affected moisture content, crude fat and crude protein when density was over 45 kg m^{-3} in day 240. Rainbow trout effectively convert protein and fat, but not glucose, into energy for growth and adaptation to stressful situations (Fan, 2008). In teleost, net energy for production (NE_p) consists of growth performance, body fat store and reproduction (Elliott, 1976). Redundant energy within this category in a dense environment of poor growth performance facilitates its conversion into high body fat stores within the high density group. Moreover, significant up-regulations of VSI, CF and crude fat content within the high density group may also suggest that fat accumulated in viscerosomatic mass.

Cortisol is important during the response to stress. Its primary functions are to increase metabolism of carbohydrates, fats, and proteins, as well as to suppress the immune system. It is acknowledged that stress is unavoidable in fish aquaculture and can be harmful to fish. To cope with these negative influences, fish can display a series of responses that can be observed in the plasma cortisol levels (Mommsen *et al*., 1999). Previous studies suggest that the plasma cortisol levels in high stocking density is higher compared to fish living in low stocking density (Tolussi *et al*., 2010; Bolasina, 2011). It is noteworthy that in day 300, plasma cortisol levels were significantly lower than those of day 180 and day 240 within each density. As we know, stress has been associated with changes allostasis, and this concept is used to explain the adaptive progress for actively maintaining stability through change (McEwen and Wingfield, 2003). Cortisol usually can reflect the severity and duration of the stress response (Fevolden *et al*., 2002). In the present study, the severe changes of density appeared in day 180 within each density population. As a result, the severity of the density variations led to significant up-regulations of plasma cortisol. Moreover, in day 240, further increasing densities and its duration contributed to the maximum plasma cortisol levels. Responses under stress are considered to be adaptive, and their function is to help overcome the effects of the stress (Barton, 2002). Significant down-regulations of plasma cortisol levels in day 300 suggests that cortisol response of rainbow trout is more sensitive to severe density variations than persistent stress of high density. Previous studies have also indicated that high stocking density produces an initial elevation of plasma cortisol that would decrease subsequently (Mommsen *et al*., 1999).

In conclusion, when density was over 36 kg m^{-3} in day 240, significant differences in growth performance were observed among the different densities. At the same time, significantly lower crude protein and significantly higher crude fat were observed in highest density group. Water quality decreased significantly after day 180 when the density reached 40.6 kg m^{-3}. Because of the cumulative effects of density-related stress, changes in VSI, CF, FC, moisture, and crude protein were not unified with elapse of time under the different stocking densities. As changes of cortisol levels with density variation, we hypothesize that these variations of growth and body composition may be caused by the adaption process of trout. Based on our findings, we recommend that rainbow trout (114.44 g ± 6.21 g, 19.69 cm ± 0.31 cm) initially stocked in 6.6 or 8.6 kg m^{-3} (60 or 80 fishes m^{-3}) should be subdivided into lower densities (under 36 kg m^{-3}) after 240 days of dense farming.

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