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Effects of dietary protein levels and rearing density on growth performance and stress response of Nile tilapia, *oreochromis niloticus* (L.)

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Abstract

This study was based on a 3 × 2 factorial design with three levels of dietary protein (25%, 35%, or 45%) and two rearing densities ($D1 = 150$ and $D2 = 300$ fish/m^3). In this study, Nile tilapia, *Oreochromis niloticus* (L.), (1.8 to 2.5 g) was distributed into the aquaria at a rate of 150 vs. 300 fish/m^3. Fish of each density were fed on a diet containing 25%, 35%, or 45% crude protein (CP). Fish were fed on one of the experimental diets till satiation twice daily, 6 days a week, for 10 weeks. At the end of the feeding trial, blood samples were taken to determine the different physiological variables. The growth parameters were positively affected by protein level and inversely affected by stocking density, but there was no effect of their interaction. Final body weight of tilapia, fed with different levels of protein diets, varied at *D*1 from 7.1 to 10.1 g and at *D*2 from 6.4 to 9.1 g. The best feed conversion ratio was obtained with 45% and 35% CP diets at lower density with insignificant difference. The highest values of protein efficiency ratio and protein productive value were obtained with 25% CP at stock densities of 150 and 300 fish/m^3. Moisture and CP contents in the whole-fish body were insignificantly affected by both factors, while ash content was significantly affected by protein level and rearing density. Total lipid content was affected by protein level alone. All physiological variables including activities of aspartate aminotransferase, alanine aminotransferase, total protein, total lipids, and glucose in plasma were significantly affected by dietary protein levels and/or rearing density. The overall results presented here indicate that the best growth performance of Nile tilapia was obtained when the fish fed on the 45% CP diet and were reared at a stocking density of 150 fish/m^3.

Keywords: Nile tilapia, Dietary protein, Rearing density, Growth performance, Blood chemistry

Background

Nile tilapia, *Oreochromis niloticus* (L.), is one of the most important fish species for aquaculture worldwide. It represents the species of choice due to its high growth rate, significant tolerance to environmental stress, ease of reproduction, and high market demand (El-Sayed 2006). For fish intensification, feed quality and rearing density should be considered to optimize their culture condition.

Rearing density is an important aspect for fish culture, and it is necessary to find a balance between the maximum profit and the minimum incidence of physiological and behavioral disorders (Ashley 2007; Ayyat et al. 2011). It has been demonstrated that rearing fish at inappropriate stocking densities may impair the growth and reduce

immune competence due to factors such as clustering stress and the deterioration of water quality, which can affect both the feed intake and conversion efficiency of the fish (Ellis et al. 2002). However, it is not clear whether the performance of fish is influenced by the stress caused by suboptimal water quality parameters associated with high densities (e.g., low oxygen level, elevated ammonia or carbon dioxide levels) or by the crowding experienced due to high density, which could cause aggressive behavior.

Stress in farmed fish is of considerable significance to both welfare and productivity as it has been linked to reduction in growth, abnormal behavior, and immunodepression (Ashley 2007; Wedemeyer 1997). Particular attention has been paid to stocking density as one of the key factors to influence the perceived level of stress in fish (Ellis et al. 2002; Turnbull et al. 2005).

Understanding the protein requirement of Nile tilapia in relation to its rearing density may lead to the coping with the stress caused and enhancing their growth and production. Moreover, assessing the effect of dietary protein levels on the metabolism of this species at different densities is of particular interest. Therefore, the present study was conducted to investigate the effect of dietary protein levels, rearing density, and their interaction on growth characteristics, feed utilization, and body composition of Nile tilapia, as well as their effect on the physiological status of the fish.

Methods

Diet preparation

Three experimental diets were formulated to contain 25%, 35%, or 45% crude protein (CP) (Table 1). The ingredients of each diet were blended together for 40 min to make a paste, which was separately passed through a grinder, and pelleted (1 mm in diameter) with a paste extruder. The diets were dried in a forced-air dryer at room temperature for 24 h and stored in plastic bags at −2°C for further use.

Fish and feeding regime

This experiment was based on a 3×2 factorial design with three levels of dietary protein (25%, 35%, and 45%) and two rearing densities ($D1 = 150$ and $D2 = 300$ fish/m^3). Three aquaria (100 L each) were assigned for each treatment. Healthy Nile tilapia, *O. niloticus* (L.), was obtained from Abbassa fish hatchery, Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad, Sharqia, Egypt. Fish were acclimatized in indoor fiberglass tanks for 2 weeks by feeding on a commercial diet containing 20% CP. Fish (1.8 to 2.5 g) were distributed into the aquaria at stock densities of 150 and 300 fish/m^3. Fish of each density were fed on a diet containing 25%, 35%, or 45% CP. Fish were fed on the experimental diets till satiation twice daily, 6 days a week, for 10 weeks. Each aquarium was supplied with compressed air via air stones from air pumps. Well-aerated tap water was provided from a storage fiberglass tank. The temperature was adjusted at 27 ± 1°C using thermostatically controlled heaters. Three-fourths of the aquarium's water with fish excreta was siphoned every day before feeding and replaced by an equal volume of well-aerated tap water. Dead fish were removed and recorded daily. Fish in each aquarium was group-weighed every 2 weeks and at the end of the experiment to calculate fish growth and feed utilization.

Table 1 Ingredients and chemical composition of the experimental diets (in percent; on a dry-matter basis)

Experimental diets		Dietary protein levels		
		25%	35%	45%
Ingredients (g/100 g)	Fish meal	15.6	20.3	31.0
	Soybean meal	20.0	40.0	50.0
	Wheat bran	5.0	5.0	5.0
	Ground corn	52.63	28.42	9.44
	Fish oil + corn oil (1:1)	2.0	2.0	2.0
	Vitamins and minerals premix[b]	1.5	1.5	1.5
	Ascorbic acid	0.06	0.06	0.06
	Starch	2.21	1.72	0.0
	Carboxymethyl cellulose	1.0	1.0	1.0
	Total	100	100	100
Chemical analysis (%)[a]	Dry matter	92.48 ± 0.7	92.69 ± 0.6	93.09 ± 0.6
	Crude protein	25.32 ± 0.24	35.41 ± 0.33	45.56 ± 0.46
	Crude fat	5.87 ± 0.15	5.67 ± 0.25	5.99 ± 0.20
	Ash	5.51 ± 0.23	6.31 ± 0.36	7.31 ± 0.37
	Fiber	6.68 ± 0.15	5.50 ± 0.12	5.76 ± 0.13
	NFE[c]	56.62	47.11	35.38
	GE (Kcal/g)[d]	439.14	446.85	458.92

[a]Means of five replicates. [b]Vitamins and minerals premix: each 2.5 kg contains vitamins A (12 MIU), D_3 (2 MIU), E (10 g), K (2 g), B_1 (1 g), B_2 (4 g), B_6 (1.5 g), and B_{12} (10 mg); pantothenic acid (10 g); nicotinic acid (20 g); folic acid (1 g); biotin (50 mg); choline chloride (500 mg); copper (10 g); iodine (1 g); iron (30 g); manganese (55 g); zinc (55 g); and selenium (0.1 g). [c]Nitrogen free extract (NFE) = 100 – (protein percentage + lipid percentage + ash percentage + fiber percentage). [d]Gross energy (GE): calculated after National Research Council (NRC) Committee on Animal Nutrition (1993) as 5.64, 9.44, and 4.11 Kcal/g for protein, lipid, and NFE, respectively.

Parameters of fish growth and feed utilization

Growth performance of Nile tilapia was determined, and feed utilization was calculated as follows: Specific growth rate $(\mathrm{SGR})(\text{in percent per day}) = \dfrac{100\,(\mathrm{Ln}W_2 - \mathrm{Ln}W_1)}{T}$, where W_1 and W_2 are the initial and the final weights, respectively, and T is the number of days of the experiment;

$$\text{Feed conversion ratio } (\mathrm{FCR}) = \frac{\text{feed intake(in grams)}}{\text{weight gain(in grams)}};$$

$$\text{Protein efficiency ratio } (\mathrm{PER}) = \frac{\text{weight gain (in grams)}}{\text{protein intake (in grams)}};$$

$$\text{Protein productive value } (\mathrm{PPV})(\text{in percent}) = 100 \times \left[\frac{\text{protein gain (in grams)}}{\text{protein intake (in grams)}}\right];$$

$$\text{Fulton condition factor } (K) = 100 \times \left[\frac{\text{fish weight (in grams)}}{\text{fish length (in cubic centimeters)}}\right].$$

Water quality measurements

Water samples were collected every 2 weeks at a depth of 15 cm from each aquarium. Dissolved oxygen was measured *in situ* with an oxygen meter (YSI model 58, Yellow Spring Instrument Co., Yellow Springs, OH, USA), unionized ammonia, using DREL/2

HACH kits (HACH Co., Loveland, CO, USA), and pH, with a pH meter (Digital Mini-pH Meter, model 55, Fisher Scientific, Denver, CO, USA). In all treatments, dissolved oxygen concentrations ranged from 6.2 to 6.7 mg/L, and the pH ranged from 7.5 to 7.8. The measured water quality parameters were within the acceptable ranges for fish growth (Boyd 1984).

Proximate chemical analysis of diets and fish

For proximate analysis, 50 fish were sampled at the start of the experiment, and 5 fish from each aquarium were sacrificed at the end of the experiment. Before analysis, samples were oven-dried and homogenized by grinding the sample in a Waring blender (Torrington, CT, USA). The proximate composition of experimentally tested diets and the whole-fish body from each treatment was analyzed according to the standard methods of AOAC (Helrich 1990) for moisture, CP, total lipids, and total ash. Moisture content was estimated by drying the samples to a constant weight at 85°C in a drying oven (GCA, model 18EM, Precision Scientific Group, Chicago, IL, USA), and nitrogen content, using a micro-Kjeldahl apparatus (Labconco Corporation, Kansas, MO, USA). Crude protein was estimated by multiplying the nitrogen content by 6.25. Lipid content was determined by ether extraction in a multiunit Soxhlet extraction apparatus (Lab-Line Instruments, Inc., Melrose Park, IL, USA) for 16 h. Ash was determined by combusting dry samples in a muffle furnace (Thermolyne Corporation, Dubuque, IA, USA) at 550°C for 6 h.

Physiological measurements

At the end of the feeding trial (10th week), fish were not fed during the 24 h immediately prior to blood sampling. Five fish from each aquarium were anesthetized with buffered tricaine methanesulfonate (20 mg/L), and blood was collected from the caudal vasculature. The collected blood was divided into two sets of Eppendorf tubes. One set contained 500 U of sodium heparinate/mL, used as an anticoagulant, for hematology (hemoglobin (Hb) (in grams per liter), hematocrit (Ht) (in percent), and red blood cell (RBC) ($\times 10^6$ per microliter) counting). The second set, without an anticoagulant, was left to clot at 4°C and centrifuged at $5{,}000 \times g$ for 15 min at room temperature. The collected sera were stored at −20°C for further assays. RBCs were counted under a light microscope using a Neubauer hemocytometer (Reichert, Inc., Depew, NY, USA) after blood dilution with phosphate-buffered saline (pH 7.2). Hemoglobin level was determined colorimetrically by measuring the formation of cyanomethemoglobin according to Van Kampen and Zijlstra (1961). Hematocrit values were immediately determined after sampling by placing fresh blood in glass capillary tubes and centrifuging for 5 min in a microhematocrit centrifuge. Glucose (in milligrams per liter) was determined colorimetrically according to Trinder (1969). Total protein (in grams per liter) and total lipid (in grams per liter) contents in plasma were determined colorimetrically according to Henry (1964) and Joseph et al. (1972), respectively. Activities of aspartate aminotransferase (AST) (in international units per liter) and alanine aminotransferase (ALT) (in international units per liter) in serum were determined colorimetrically according to Reitman and Frankel (1957). Serum cortisol levels (in milligrams per liter) were determined by an electrochemiluminometric assay using the Elecsys and Cobas e 411 Immunoassay Analyzer (Roche Diagnostics, Indianapolis, IN, USA). The test kit was prepared in accordance with the method described by Chiu et al. (2003).

Statistical analysis

The obtained data were subjected to two-way analysis of variance (ANOVA) to test the effect of dietary protein levels and rearing density as the two factors. Duncan's multiple range test was used as a *post hoc* test to compare between means at $P \leq 0.05$. The SPSS version 10 software (SPSS, Richmond, VA, USA) was used as described by Dytham (1999).

Results

Fish growth and survival

Fish growth represented by the final body weight and SGR of Nile tilapia were significantly affected by protein level and rearing density ($P < 0.05$), but not their interaction (Table 2). However, fish growth was positively affected by protein level and inversely affected by rearing density. The maximum growth rate was obtained at the fish group fed on a diet containing 45% CP and reared at *D*1. The growth of Nile tilapia cultivated at *D*2 and fed on the same protein diet (45% CP) was higher than that of the fish reared at *D*1. Condition factor and fish survival in different experimental groups were not significantly affected by protein levels, rearing density, or their interaction.

Feed utilization and protein turnover

Feed intake, FCR, PER, and PPV were significantly affected by protein level and rearing density ($P < 0.05$), but not their interaction (Table 3). Feed intake increased and FCR

Table 2 Growth parameters and survival of Nile tilapia affected by protein levels and rearing density

Variables/treatment means[a]	Protein level (%)	Final body weight (g)[d]	SGR (%/day)	*K* factor	Survival (%)
Fish density[b]					
*D*1	25	7.0	1.720	1.563	93.3
	35	8.9	2.063	1.497	93.3
	45	10.1	2.344	1.497	96.7
*D*2	25	6.4	1.591	1.617	93.3
	35	7.6	1.837	1.560	95.6
	45	9.1	2.094	1.530	96.7
Pooled SE		0.098	0.023	0.018	1.118
Means of main effects[c]					
*D*1		8.7 q	2.042 q	1.519	94.4
*D*2		7.7 r	1.841 r	1.569	95.2
	25	6.7 z	1.656 z	1.590	93.3
	35	8.3 y	1.950 y	1.529	94.5
	45	9.6 x	2.219 x	1.514	96.7
Two-way ANOVA (*P* values)					
Protein level		0.001	0.0001	0.232	0.473
Fish density		0.001	0.001	0.195	0.738
Protein level × fish density		0.371	0.543	0.942	0.890

[a]Treatment means represent the average values of three aquaria per treatment. Duncan's multiple range test was conducted for treatment means only if there was a significant interaction (ANOVA, $P < 0.05$). Means followed by the same letter are not significantly different. [b]*D*1 and *D*2 = 150 and 300 fish/m^3, respectively. [c]Main effect means followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test; x, y, and z for protein level and q and r for fish density. [d]Initial body weight was 2.1 g. SGR, specific growth rate; *K* factor, Fulton condition factor; SE, standard error; ANOVA, analysis of variance.

Table 3 Feed utilization and UA of Nile tilapia affected by protein levels and rearing density

Variables/treatment means[a]	Protein level (%)	Feed intake (g feed/fish)	FCR	PER	PPV (%)	UA (mg/L)
Fish density[b]						
*D*1	25	10.8	2.25	1.913	30.54	1.48
	35	12.7	1.81	1.623	24.82	1.94
	45	14.9	1.72	1.340	20.88	2.14
*D*2	25	10.7	2.46	1.720	26.64	2.13
	35	11.6	2.07	1.467	22.25	2.36
	45	13.4	1.89	1.250	19.82	2.84
Pooled SE		0.147	0.024	0.018	0.288	0.026
Means of main effects[c]						
*D*1		12.8 q	1.93 r	1.625 q	25.41 q	1.85 r
*D*2		11.9 r	2.14 q	1.479 r	22.90 r	2.44 q
	25	10.8 z	2.36 x	1.817 x	28.59 x	1.81 z
	35	12.2 y	1.94 y	1.545 y	23.54 y	2.15 y
	45	14.2 x	1.81 z	1.295 z	20.35 z	2.49 x
Two-way ANOVA (*P* values)						
Protein level		0.001	0.001	0.001	0.001	0.001
Fish density		0.010	0.001	0.002	0.001	0.001
Protein level × fish density		0.176	0.753	0.533	0.174	0.101

[a]Treatment means represent the average values of three aquaria per treatment. Duncan's multiple range test was conducted for treatment means only if there was a significant interaction (ANOVA, $P < 0.05$). Means followed by the same letter are not significantly different. [b]*D*1 and *D*2 = 150 and 300 fish/m^3, respectively. [c]Main effect means followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test; x, y, and z for protein level and q and r for fish density. FCR, feed conversion ratio; PER, protein efficiency ratio; PPV, protein productive value; UA, unionized ammonia; SE, standard error; ANOVA, analysis of variance.

decreased significantly by increasing the dietary protein levels, but feed intake decreased and FCR increased significantly by increasing the rearing density ($P < 0.05$). Further, PER and PPV decreased significantly by increasing dietary protein levels and rearing density ($P < 0.05$). The best FCR (1.72) was obtained with a 45% CP diet at *D*1, but the poorest FCR (2.25 and 2.46, respectively) were obtained with a 25% CP diet at *D*1 and *D*2. The highest values of PER and PPV (1.913 and 30.54, respectively) were obtained with a 25% CP diet at *D*1. The lowest values of PER and PPV (1.25 and 19.82, respectively) were obtained with the diet containing 45% CP at *D*2. Unionized ammonia (UA) was significantly affected by protein levels and rearing density ($P < 0.05$), but not their interaction (Table 3). However, UA increased significantly by the increasing protein levels and stocking density of reared fish ($P < 0.05$).

Proximate chemical composition of the whole-fish body

Moisture and CP contents in the whole-fish body were not significantly affected by protein levels and rearing density ($P > 0.05$; Table 4). Total lipid content was significantly affected by protein level only ($P < 0.05$); meanwhile, total ash content was significantly affected by protein levels and rearing density ($P < 0.05$). Moreover, all chemical components (moisture, CP, total lipids, and total ash) were not significantly affected by protein-density interaction ($P > 0.05$).

Table 4 Proximate analysis of the body of Nile tilapia affected by protein levels and rearing density

Variables/treatment means[a]	Protein level (%)	Moisture[d]	Crude protein	Total lipid	Total ash
Fish density[b]					
*D*1	25	72.8	14.8	8.9	3.5
	35	73.5	15.0	7.6	3.9
	45	73.8	15.2	6.9	4.1
*D*2	25	72.9	14.9	8.4	3.8
	35	73.5	15.3	7.3	3.9
	45	73.4	15.4	6.7	4.5
Pooled SE		0.864	0.178	0.090	0.047
Means of main effects[c]					
*D*1		73.4	15.0	7.8	3.8 r
*D*2		73.3	15.2	7.5	4.1 q
	25	72.9	14.9	8.7 x	3.7 z
	35	73.5	15.2	7.5 y	3.9 y
	45	73.6	15.3	6.8 z	4.3 x
Two-way ANOVA (*P* values)					
Protein level		0.929	0.589	0.001	0.001
Fish density		0.955	0.585	0.090	0.028
Protein level × fish density		0.992	0.974	0.792	0.232

[a]Treatment means represent the average values of three aquaria per treatment. Duncan's multiple range test was conducted for treatment means only if there was a significant interaction (ANOVA, $P < 0.05$). Means followed by the same letter are not significantly different. [b]*D*1 and *D*2 = 150 and 300 fish/m^3, respectively. [c]Main effect means followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test; x, y, and z for protein level and q and r for fish density. [d]In percent; on a fresh-weight basis. SE, standard error; ANOVA, analysis of variance.

Physiological alterations

RBCs, Hb, plasma protein, plasma lipids, and plasma cortisol values were significantly affected by protein level and rearing density only; meanwhile, plasma glucose, AST, and ALT were significantly affected by protein level, rearing density, and their interaction ($P < 0.05$; Tables 5 and 6). In addition, Ht was significantly affected by protein level only ($P < 0.05$). It is noticed that glucose, cortisol, AST, and ALT increased significantly ($P < 0.05$), while plasma lipids decreased significantly by the increasing fish density of reared Nile tilapia ($P < 0.05$).

Discussion

Growth performance and feed utilization

Fish growth and feed utilization were significantly retarded herein with increasing the rearing density irrespective of protein levels. It has been demonstrated that rearing fish at high density (*D*2) may reduce their growth due to factors such as social interaction and the deterioration of water quality, which can affect the feed utilization by fish (Ellis et al. 2002). In many cultivated fish species, growth and feed utilization are inversely related to rearing density, and this is mainly attributed to social interactions such as competition for food and/or space that can negatively affect fish growth (Canario et al. 1998; Irwin et al. 1999). Similar results for Nile tilapia were obtained by Huang and

Table 5 Changes in physiological parameters in Nile tilapia affected by protein levels and rearing density

Variables/ treatment means[a]	Protein level (%)	RBCs ($\times 10^6$/μL)	Hb (g/L)	Ht (%)	Protein (g/L)	Lipids (g/L)	AST (IU/L)	ALT (IU/L)
Fish density[b]								
*D*1	25	1.262	6.06	14.3	2.07	16.6	17.4 d	6.1 c
	35	1.298	6.57	15.6	2.64	18.9	26.7 c	6.3 c
	45	1.528	7.95	16.5	3.45	19.8	63.5 a	7.8 b
*D*2	25	1.314	6.69	13.8	2.33	14.7	23.6 c	6.1 c
	35	1.522	7.64	15.0	2.71	14.9	38.0 b	7.4 b
	45	1.608	8.40	15.8	3.58	15.7	65.9 a	8.8 a
Pooled SE		0.017	0.086	0.179	0.034	0.199	0.083	0.498
Means of main effects[c]								
D1		1.363 r	6.86 r	15.5	2.72 r	18.4 q	35.9	6.7
D2		1.481 q	7.58 q	14.9	2.87 q	15.1 r	42.5	7.4
	25	1.288 z	6.38 z	14.1 y	2.20 z	15.7 y	20.5	6.1
	35	1.410 y	7.11 y	15.3 x	2.68 y	16.9 x	32.4	6.9
	45	1.568 x	8.18 x	16.2 x	3.52 x	17.8 x	64.7	8.3
Two-way ANOVA (*P* values)								
Protein level		0.0001	0.001	0.002	0.001	0.003	0.001	0.001
Fish density		0.004	0.001	0.120	0.042	0.001	0.001	0.001
Protein level × fish density		0.123	0.347	0.974	0.517	0.074	0.026	0.011

[a]Treatment means represent the average values of three aquaria per treatment. Duncan's multiple range test was conducted for treatment means only if there was a significant interaction (ANOVA, $P < 0.05$). Means followed by the same letter are not significantly different. [b]*D*1 and *D*2 = 150 and 300 fish/m^3, respectively. [c]Main effect means followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test; x, y, and z for protein level and q and r for fish density. RBCs, red blood cells; Hb, hemoglobin; Ht, hematocrit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SE, standard error; ANOVA, analysis of variance.

Chiu (1997), El-Sayed (2002), and Ayyat et al. (2011) who found that the increase of stocking density inversely affected the growth of Nile tilapia. Ridha (2006) reported that a density of 200 fish/m^3 significantly decreased the growth performance of Nile tilapia compared with a density of 125 fish/m^3.

The condition factor and survival rates were not affected significantly by the rearing density, suggesting that there was no competition for space. Moreover, the good survival rate of Nile tilapia at high density indicates the amenability of this fish to the intensive culture practice. Huang and Chiu (1997) found that condition factor and survival of Nile tilapia had not been significantly affected by rearing density.

The optimum dietary protein level for Nile tilapia fry reared at *D*1 and *D*2 was 45%. Similarly, Abdel-Tawwab et al. (2010) found that the best growth of Nile tilapia fry was obtained at a high dietary protein level (45%) rather than 25% or 35% protein. Moreover, feed intake and FCR herein were significantly affected by protein levels and rearing density, but not their interaction. The optimum feed utilization for Nile tilapia was obtained at a 45% CP diet under lower density. Abdel-Tawwab et al. (2010) reported that feed intake increased and FCR decreased with increasing the dietary protein level. The affected feed utilization in the present study is much more expected; as the number of fish stocked in an aquarium increases, the amount of

Table 6 Serum glucose and cortisol of Nile tilapia affected by dietary protein levels and rearing density

Variables/treatment means[a]	Protein level (%)	Glucose (mg/L)	Cortisol (mg/L)
Fish density[b]			
*D*1	25	64.9 d	31.8
	35	89.5 c	30.3
	45	124.2 a	28.9
*D*2	25	108.2 b	34.8
	35	119.4 a	32.4
	45	128.4 a	31.2
Pooled SE		1.274	0.373
Means of main effects[c]			
*D*1		92.9	30.3 r
*D*2		118.7	32.8 q
	25	86.6	33.3 y
	35	104.5	31.4 xy
	45	126.3	30.0 x
Two-way ANOVA (*P* values)			
Protein level		0.001	0.039
Fish density		0.001	0.002
Protein level x fish density		0.001	0.720

[a]Treatment means represent the average values of three aquaria per treatment. Duncan's multiple range test was conducted for treatment means only if there was a significant interaction (ANOVA, $P < 0.05$). Means followed by the same letter are not significantly different. [b]*D*1 and *D*2 = 150 and 300 fish/m^3, respectively. [c]Main effect means followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test; x, y, and z for protein level and q and r for fish density. SE, standard error; ANOVA, analysis of variance.

feed available to each fish decreases (Chang 1988). In addition, the reduced feed utilization at high rearing density had been attributed to changes in metabolism. This effect is based on the assumption that chronic stress, due to the high density, increases the fish's overall energy demand, which is then unavailable for growth (Wendelaar Bonga 1997). Likewise, high levels of locomotory activity have been shown to cause elevated metabolic rates as measured in rainbow trout (Cooke et al. 2000); however, the activity level of fish increased with increasing density, resulting in energy demands.

The protein utilization (PER and PPV) herein decreased with increasing dietary protein levels and fish density. The maximum PER and PPV values were obtained when fish at lower density were fed on the 25% CP diet. These results may have occurred because weight gain was related to the deposition of protein, and protein accretion is a balance between protein anabolism and catabolism. In this concern, Abdel-Tawwab et al. (2010) found that PER decreased with increasing dietary protein content. Moreover, fish density can affect the efficiency of feed utilization; as the number of fish stocked in a pond increases, the amount of feed available to each fish decreases (Chang 1988). Furthermore, UA was significantly affected by protein level and rearing density, but not their interaction. A direct relationship between protein intake and ammonia excretion has been found in fish (Li and Lowell 1992; Chakraborty and Chakraborty 1998). The increase in UA in the present study reflects an increase in protein

catabolism. It is known that UA is toxic to fish and may cause growth retardation (Boyd 1984; Lemarié et al. 2004).

Only the total ash content of Nile tilapia herein was significantly influenced by dietary protein level and rearing density; meanwhile, lipid content was significantly affected by rearing density alone. Nile tilapia fed on the 25% CP diet had a higher lipid content than those fed on the 35% or 45% CP diets. Nevertheless, proximate analyses indicated that lipid levels of the whole-fish body were slightly but consistently lower in the fish reared at the higher density. The low lipid content of high-density-reared fish finds confirmation with Montero et al. (1999), who found lower lipid levels in the liver of gilthead seabream, *Sparus aurata*, reared at high densities. Piccolo et al. (2008) evaluated the effect of feed quality and stocking density on Dover sole (*Solea solea*) and found that, except for the lipid content, which was higher in the low-density group, proximate composition of the sole's muscle was not influenced by treatments. Furthermore, changes in protein and lipid contents in fish body could be linked with changes in their synthesis and/or deposition rate in the muscle (Fauconneau 1984; Abdel-Tawwab et al. 2006). In contrast with our results, Gallagher (1999) did not find significant differences in moisture, protein, lipid, and ash contents in the whole body of sunshine bass fed with different protein levels.

Physiological variables

The physiological status of farmed fish is an integral part for evaluating their health status. However, physiological alterations might be used as indicators for unsuitable environmental conditions or the presence of stress factors such as toxic chemicals, excess organic compounds, and stressors encountered in intensive fish culture (Cnaani et al. 2004; Řehulka et al. 2004). Changes in RBCs and Hb were significantly affected by dietary protein levels and fish density, but not their interaction. The increase in RBC count may have occurred because of its release from the storage pool in the spleen (Vijayan and Leatherland 1989; Pulsford et al. 1994). Thus, it seems that splenic activity is affected by dietary protein level and rearing density.

Serum protein tended to increase with increased dietary protein level. Similar results were observed in European eel (Suárez et al. 1995), *Rhamdia quelen* (Melo et al. 2006), and Nile tilapia (Abdel-Tawwab et al. 2010). Although there was an increase in serum protein, the increased levels of ALT and AST suggest protein catabolism at high dietary protein levels. The amino acid surplus from protein-rich diets cannot be directly stored in fish, and they might be deaminated and converted into energetic compounds (Ballantyne 2001; Stone et al. 2003). In this study, the rise of serum protein with dietary protein could likely be due to the enhancement of digested protein (Lundstedt et al. 2002).

Serum lipids increased significantly due to the increase in protein level, and it may be because the muscle is a pivotal compartment directly linked to amino acid turnover. This involves protein synthesis or breakdown of those molecules as energetic substrates. On the other hand, serum lipid was inversely affected by fish density. This result may be due to the energy expenditure in high density; thus, lipids were used as an energy source.

Glucose, AST, and ALT in Nile tilapia herein were significantly affected by dietary protein, fish density, and their interaction; meanwhile, cortisol was significantly affected by both factors only. Increased glucose in the blood suggests gluconeogenesis as a consequence of increased dietary protein level. The expression of key enzymes of intermediary metabolism

is modulated by nutritional status in fish (Metón et al. 1999, 2003). The levels of amino acid-metabolizing enzymes and nitrogen excretion are reliable indicators of dietary protein availability. Metabolism of amino acids involves deamination and transamination reactions. The rise of ALT and AST activities observed in Nile tilapia fed on the 45% CP diet may reflect the use of excess hydrocarbons from amino acids to supply energy demands. Similar responses were observed in *Oncorhynchus mykiss* for ALT (Sánchez-Muros et al. 1998) and in *R. quelen* for AST and ALT (Melo et al. 2006). The rise in the hepatic activity of protein-metabolizing enzymes when fish were fed on the 45% CP diet may indicate the use of excess dietary amino acids for growth as well as substrate for gluconeogenesis, particularly for AST and ALT activities.

On the other hand, blood glucose and cortisol increased significantly with high fish density. This result suggests that high stocking density may cause stress. The primary response against stress involves the increases in plasma catecholamines and cortisol (Barton and Iwama 1991; Barton 2002). These hormones induce secondary stress responses, characterized by increased glucose levels, mobilizing glucose to tissues for homeostasis to cope with energy-demanding processes of restoration (Wendelaar Bonga 1997; Barton et al. 2002). Stress may thus lead to a high consumption of energy reserves, and this reallocation of metabolic energy negatively interferes with other physiological processes, *viz.* growth, reproduction, immunity, etc. (Wendelaar Bonga 1997; Barton and Iwama 1991; Pickering 1998; Mommsen et al. 1999).

Stress in fish is indicated by elevated blood cortisol levels, and indeed, a high stocking density typically leads to increased cortisol levels in a variety of fish (Wendelaar Bonga 1997; Barton et al. 2002) such as *Rhombosolea tapirina* (Barnett and Pankhurst 1998), *Pseudopleuronectes americanus* (Sulikowski et al. 2006), *Paralichthys olivaceus* (Bolasina et al. 2006), *Solea senegalensis* (Costas et al. 2007), wedge sole, *Dicologoglossa cuneata* (Herrera et al. 2009), and European sea bass, *Dicentrarchus labrax* (Lupatsch et al. 2010).

Conclusions

The overall results presented here indicate that Nile tilapia which fed on the 45% CP diet and reared at a stocking density of 150 fish/m^3 produced the best growth performance. However, the increase in fish density and/or the decrease of the dietary protein level would retard the fish growth. In addition, fish fed on the 45% CP diet could cope with the stress induced by increasing the rearing density.

Competing interests

The author declares that he has no competing interests.

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