

DIGESTIVE ENZYMES, IMMUNITY AND OXIDATIVE STATUS OF NILE TILAPIA (*Oreochromis niloticus*) REARED IN INTENSIVE CONDITIONS

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Abstract: High stocking density is significantly disturbing the growth and productivity of aquatic animals. Digestive enzymes, immunity and oxidative status of Nile tilapia were investigated in case of culturing in several densities. Fish (14.3 ± 0.03 g) were stocked in 12 aquaria (60 L) at four densities of 10 (SD10), 20 (SD20), 30 (SD30) and 40 (SD40) fish per aquarium for 30 days. Fish growth, feed efficiency ratio, digestive enzyme activity and dissolved water oxygen significantly ($P < 0.05$) decreased, while the total ammonia increased with increasing stocking density. Immunoglobulin and NBT levels decreased significantly ($P < 0.05$) in SD40 set compared to SD20 set without no differences with the other two groups. Lysozyme activity reported the highest significant ($P < 0.05$) values in SD10 and SD20 groups over the high stocking density group (SD40) without no difference with SD30 group. Bactericidal, phagocytic activities and phagocytic index reported significantly ($P < 0.05$) lower values in fish reared in SD30 and SD40 groups than fish reared in SD10 and SD20 groups. Peroxidase activity also showed significantly ($P < 0.05$) low values in SD40 and SD30 groups with the weakest activity in SD40 group. Total serum protein lowered relatively in SD30 and SD40 groups without no differences with the other groups. Furthermore, fish reared at high stocking densities resulted in significantly ($P < 0.05$) decreased superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities as well as increased malonaldehyde (MDA) activity in blood of tilapia suggesting suppressed antioxidant response. In conclusion, intensive conditions depressed the growth, digestive enzyme activity, immunity and oxidative status of Nile tilapia.

Key words: digestive enzyme activity; growth; immunity; Nile tilapia; oxidative status; stocking density

Introduction

Intensive aquaculture conditions can affect the water quality negatively; thus, can markedly threaten the fish health and productivity. It has

been found that increased stocking led to high ammonia accumulation, hydromineral balance and mortality in different fish species including, tambaqui, winter flounder, Nile tilapia and Japanese flounder (1-4).

The low growth performance is concretely affected by the intensive conditions due to decreased feed intake. The feed utilization is related to the activity of microbiota and digestive enzyme activity in the gastrointestinal tract (GIT), which increases feed digestibility and utilization and ultimately improves the growth and health status of fish (5). Protease, lipase and amylase are the major digestive enzymes, which play the main roles in feed digestion and absorption. If the activity of these enzymes increases, overall body metabolism may increase (6). The activities of digestive enzyme were depressed in Nile tilapia and Japanese flounder when reared in intensive conditions (3,4). Additionally, oxidative stress and immunosuppression are the direct features occurring during the intensive conditions, which either caused by the physiological stress (7) or water quality deterioration, such as a decrease in dissolved oxygen and an increase in ammonia levels (8). Due to intensive rearing conditions, the fish respiration rate increases leading to hydromineral imbalance (9, 10). Although not studied, high stocking densities may suppress immune responses of the fish, because physiological stress (11) and low water quality (12) are sources of immunosuppression. As a result, fish become more susceptible to infectious diseases.

Nile tilapia (*Oreochromis niloticus*) is one of the most important cultured fresh water species in the world. Tilapia is usually farmed in Egypt using intensive culture system which often caused stressful circumstances and depressingly disturb their growth and wellbeing (13). Low growth performance was observed in tilapia reared in intensive conditions as reported by Liu et al. (3) and Wu et al. (14). For Nile tilapia, there are enough information about the growth performance and feed utilization of fish reared under intensive conditions. However, there is little data on the effect of stocking density on the digestive enzyme activity, immunity and oxidative status of Nile tilapia. Moreover,

available information on the relationship between physiological changes with the growth of Nile tilapia stocked at different densities was limited. Therefore, this study aims to investigate the effect of intensive conditions on growth performance, digestive enzyme activity, immunity and oxidative status of Nile tilapia.

Material and methods

Fish, diet and experimental protocol

Tilapia fingerlings were obtained from a private farm located in Kafrelsheikh, Egypt, and transported to Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, Egypt. After 2 weeks acclimation, 300 tilapia (14.3 ± 0.03 g) were put into 12 glass aquaria (60 L) and distributed in four stocking densities; at 10 (SD10), 20 (SD20), 30 (SD30) and 40 (SD40) fish per aquarium. Each aquarium was provided with an air stone for aeration. Feeding rate was fixed at 2 to 3 % of body weight per day with two feeding times 8:00 and 15:30 hr for 30 days. Fish fed diets prepared as described by Dawood et al., (13). The nutritional profile for each diet was confirmed by AOAC (15). The leftover feed was siphoned out after 3 h and 50 % of water was replaced daily with fresh, dechlorinated water of similar temperature. Lighting in the culture unit was set at 12:12 light: dark cycle throughout the study.

Water quality parameters were monitored regularly throughout the experimental period. Water temperature, pH and dissolved oxygen (DO) were measured using thermometer, portable digital pH meter (Martini Instruments Model 201/digital) and Waterproof Portable Dissolved Oxygen (model Hanna waterproof IP67). Total ammonia-nitrogen was measured calorimetrically.

Sampling schedule

All fish were fasted for 24 hr prior to final sampling. Fish were individually measured for final body-weight. Then, the intestine sampled for digestive enzymes analysis from 9 fish per experimental group. Fish were randomly caught and euthanized by "diluted tricaine methanesulfonate (MS-222; 400 ppm ratio; Sigma-Aldrich, Egypt)". Intestine aseptically

taken, washed with PBS (pH 7.5; 1 g per 10 mL), homogenized and centrifuged for 5 min at 8000 rpm. The supernatant was then kept at 4°C.

The total protein content was measured using diluted homogenates following the method of Lowry et al. (16) using bovine serum albumin as a standard. Protease activity was evaluated according to Anson (17) using Folin phenol reagent, and amylase activity was measured according to Jiang (18) and Worthington (19) using iodine solution to reveal non-hydrolysed starch. Protease and amylase activities were both expressed as “specific activity” (units per mg of protein).

Specific activity of lipase was assessed based on the protocol described by Borlongan (20) and Jin (21) with olive oil as a substrate. Fatty acid, derived from enzymatic hydrolysis of triglyceride on stable emulsion of olive oil, was titrated with NaOH. One unit of specific activity of lipase was determined as the volume of NaOH 0.05 N needed to neutralize fatty acid released after 6 hours-long incubation with substrate. Lipase activity was expressed as “units per gram” of intestine content.

Blood collection and immunological assays

Blood was collected from the caudal vein of 9 anaesthetized fish per group and quickly put into 1 ml EDTA coated vials for whole blood and non-coated vials for serum collection. The blood samples were left for 30 minutes till blood clotting then serum separation by centrifugation at 3000 rpm for 10 minutes. Serum samples were stored at -20 °C until further analysis. Blood total serum protein was carried out by RA-50 chemistry analyzer (Bayer) using readymade chemicals (kits) supplied by Spinreact Co. Spain, following manufacturer's guidelines. Immunoglobulin M (IgM) was measured by an ELISA assay using a commercial kit (Cusabio; Wuhan, Hubei, China). The result of IgM was expressed as mg per dl.

Respiratory burst activity of the whole blood was quantified by the nitro-blue-tetrazolium (NBT) assay according to Secombes (22). The NBT reduction was measured using the micro-

plate reader (Optica, Mikura Ltd, UK) at 630 nm.

Lysozyme activity was measured following Parry et al. (23). The result was expressed as “a reduction in absorbency of 0.001/min”. Serum bactericidal activity against *Aeromonas hydrophila* was detected by following Rainger and Rowley (24). The results were recorded as survival index (SI). Values were calculated as follows: “SI = CFU at end / CFU at start x100”.

The total peroxidase activity of serum was also assessed using the spectrophotometer at 540 nm as described by Quade and Roth (25) and partially modified by Sahoo et al. (26).

Phagocytic activity and phagocytic index were determined following Kawahara et al. (27). The number of phagocytized cells was counted in the phagocytic cells to calculate the phagocytic index according to the following equations: “Phagocytic activity (PA) = Macrophages containing yeast/Total number of Macrophages x100; Phagocytic index (PI) = Number of cells phagocytized/Number of phagocytic cells”.

Oxidative status

Superoxide dismutase (SOD), malonaldehyde (MDA), catalase (CAT), and glutathione peroxidase (GPX) in fish serum were measured using the diagnostic reagent kits following the manufacturer's (Cusabio Biotech Co., Ltd; China) procedure.

Growth performance calculations

During the final sampling, all fish per tank were weighed separately. Growth performance and feed utilization were evaluated using weight gain (WG), specific growth rate (SGR) and feed efficiency ratio (FER). Calculations were made using the following formulae: $WG (\%) = (FBW - IBW) \times 100 / IBW$; $SGR (\% BW/day) = 100((\ln FBW - \ln IBW) / T)$; $FER = WG / FI$. Where FBW = body weight final (g), IBW = body weight initial (g), T = duration of the trial in days, WG = wet weight gain (g) and FI = estimated feed intake (g).

Statistical analysis

Shapiro-Wilk and Levene tests confirmed normal distribution and variance homogeneity. All statistical differences were assessed by one-way ANOVA tests (SPSS version 22, SPSS Inc., IL, USA) with Duncan's as post-hoc test where differences in experimental groups occurred. The level of significance was accepted at $P < 0.05$. All data are presented as means \pm standard error (SE).

Results

Water quality values

Water physicochemical characteristics are shown in Table 1. Intensive conditions led to significant ($P < 0.05$) decrease in dissolved oxygen (DO) and increase in total ammonia levels of rearing water. The lowest DO and the highest total ammonia values were detected in SD30 and SD40 groups. No significant ($P > 0.05$) differences were detected among all the groups in terms of rearing water temperature and pH levels.

Growth and feed efficiency

Growth performance of fish (FBW, WG and SGR) decreased significantly in SD40 group than the other groups (Table 2). Also, feed efficiency ratio decreased significantly ($P < 0.05$) in SD40 compared to SD10, while no significant ($P > 0.05$) differences were reported among the other groups (Table 2). Survival rate lowered significantly ($P < 0.05$) in SD30 and SD40 groups than the low stocking density group (SD10) without no differences with SD20 group.

Digestion enzymes

Amylase, lipase and protease enzymes showed significantly ($P < 0.05$) higher activities in fish reared in low stocking densities (SD10 and SD20 groups) over the high stocking density (SD40) without no significant difference ($P > 0.05$) with SD30 group (Fig. 1).

Immune response

High stocking density led to significant immunosuppression in tilapia as declared by the

blood immunity in Figure 2. Immunoglobulin and NBT levels decreased significantly ($P < 0.05$) in SD40 group compared to SD20 group without no differences with the other two groups. Lysozyme activity reported the highest significant ($P < 0.05$) values in SD10 and SD20 groups over the high stocking density group (SD40) without no difference with SD30 group. Bactericidal, phagocytic activities and phagocytic index reported significantly ($P < 0.05$) lower values in fish reared in intensive conditions (SD30 and SD40 groups) than fish reared in low stocking density (SD10 and SD20 groups). Peroxidase showed significantly ($P < 0.05$) lower value in SD40 and SD30 groups compared to the other groups with the lowest level in SD40 group. Total serum protein lowered relatively in SD30 and SD40 groups without no differences with the other groups.

Oxidative status

Oxidative and antioxidative enzymes activities are shown in Figure 3. SOD and CAT decreased significantly ($P < 0.05$) in SD30 and SD40 groups compared to SD10 and SD20 groups. Similarly, GPX exhibited significantly ($P < 0.05$) lower activity in SD30 and SD40 groups than SD10 and SD20 groups with the highest level in SD10 group. However, MDA increased significantly ($P < 0.05$) in fish reared in intensive conditions (SD30 and SD40 groups) over fish reared in low stocking density (SD10 and SD20 groups).

Discussion

Aquaculture is based on the culture of fish in an optimal environmental and culture conditions. High stocking density is among the rearing strategies of aquatic animals. In this system the water components are utilized efficiently in order to get higher fish production per unit of rearing water. Nevertheless, over stocking density can be a risky stress which suppress the growth, survival, immune response and oxidative status (28, 29).

Table 1: Water quality parameters of Nile tilapia reared under different stocking densities

Item	Test group			
	SD10	SD20	SD30	SD40
Temperature (°C)	27.07±0.15	27.1±0.24	27.2±0.2	27.4±0.13
pH	7.2±0.06	7.23±0.12	7.17±0.09	7.23±0.07
Dissolved oxygen (mg L ⁻¹)	5.43±0.09 ^c	4.83±0.15 ^b	4.4±0.15 ^a	4.2±0.06 ^a
Total ammonia (mg L ⁻¹)	0.57±0.03 ^a	1.22±0.2 ^b	1.71±0.09 ^c	1.86±0.2 ^c

*Values expressed as means ± SE ($n = 3$). Different superscript letters indicate significant differences for each pairwise comparison between treatments

Table 2: Growth and feed efficiency of Nile tilapia reared under different stocking densities

Item	Test group			
	SD10	SD20	SD30	SD40
IBW	14.43±0.23	14.2±0.17	14.23±0.34	14.37±0.33
FBW	31.18±3 ^b	34.6±2.67 ^b	32.83±2.25 ^b	28.28±2.31 ^a
WG (%)	115.06±20.78 ^b	138.61±18.4 ^b	126.42±15.53 ^b	95.01±16 ^a
SGR	1.26±0.15 ^b	1.44±0.13 ^b	1.35±0.12 ^b	1.1±0.14 ^a
FER	0.85±0.03 ^b	0.79±0.07 ^{ab}	0.77±0.06 ^{ab}	0.73±0.02 ^a
Survival	100±0 ^b	96.67±1.67 ^{ab}	92.22±1.11 ^a	92.5±1.44 ^a

*Values expressed as means ± SE ($n = 3$). Different superscript letters indicate significant differences for each pairwise comparison between treatments

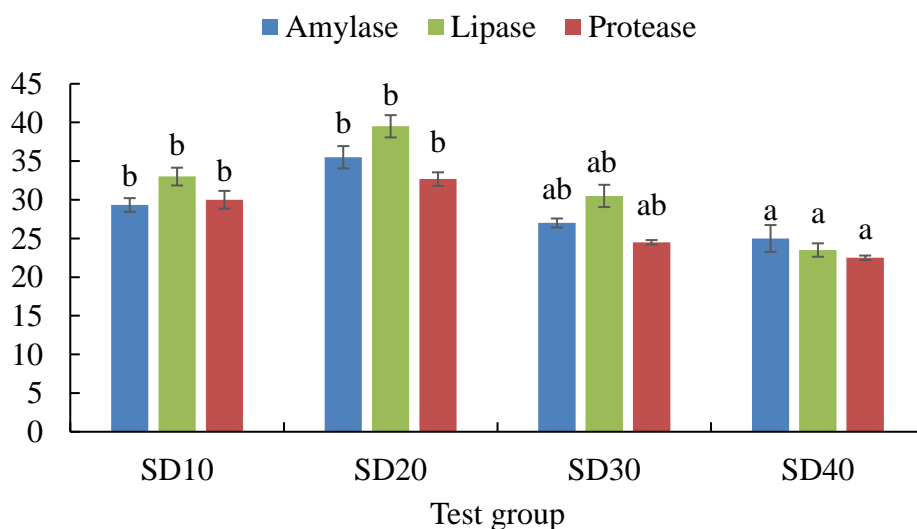


Figure 1: Digestive enzymes activities of Nile tilapia reared under different stocking densities. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group ($P < 0.05$)

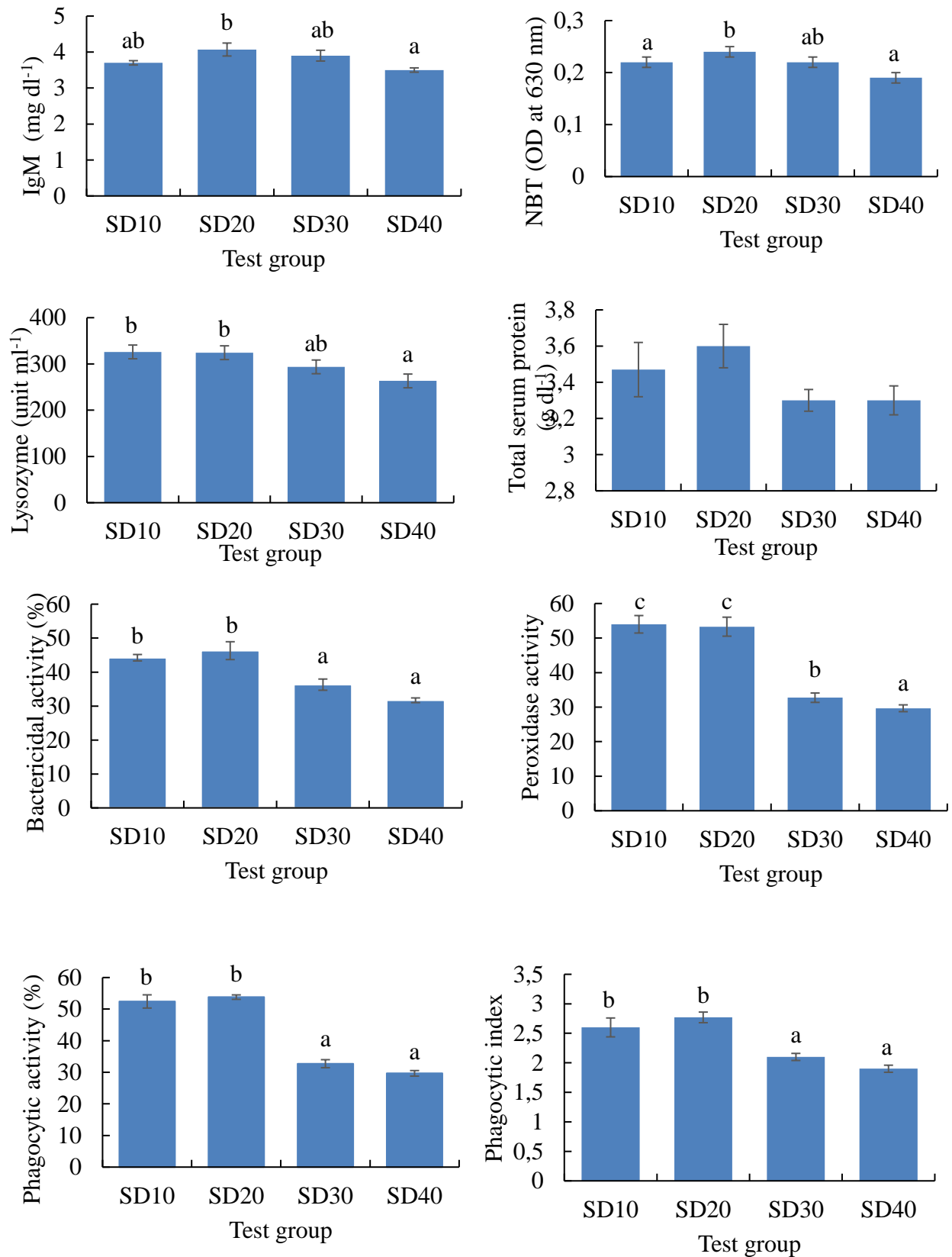


Figure 2: Activity of blood immune responses in Nile tilapia reared under different stocking densities. Values are expressed as mean \pm SE from triplicate groups. Bars with an asterisk are significantly different from those of control group ($P < 0.05$)

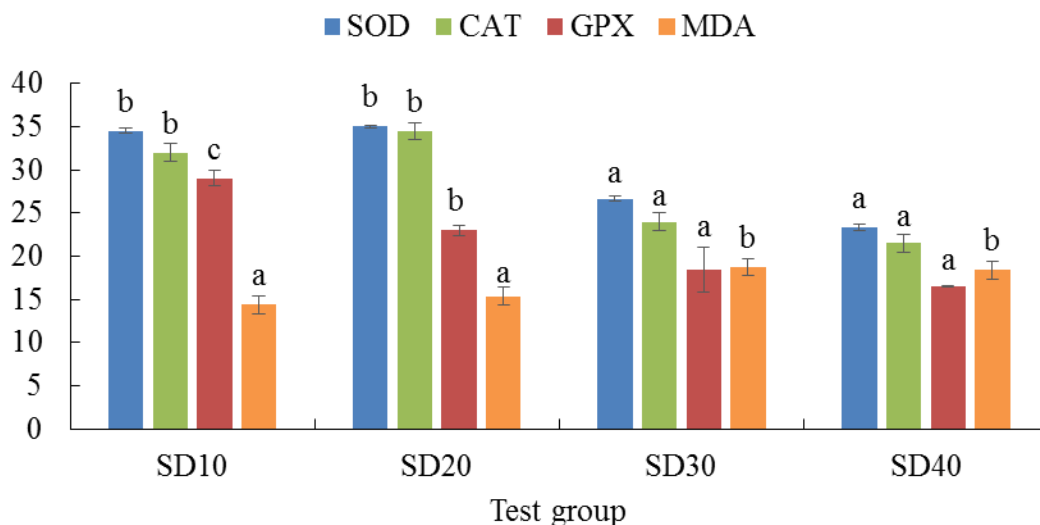


Figure 3: Oxidative status [SOD (IU L⁻¹), CAT (IU L⁻¹), GPX (IU L⁻¹) and MDA (nmol ml⁻¹)] of Nile tilapia reared under different stocking densities. Values are expressed as mean \pm SE from triplicate groups. Bars with an asterisk are significantly different from those of control group ($P < 0.05$)

The current study illustrated that the growth performance of tilapia reared in intensive conditions (SD40) was slower than the other densities indicating that growth was influenced by the unreasonable stocking density. Parallel results also were obtained in tilapia and other fish species (14, 28, 30). Wendelaar (31) concluded that the low growth of fish reared in intensive conditions is due to the high level of required energy level to deal with stress which resulted in low available energy for fish growth. Survival rate is a significant parameter to express the welfare and health status of fish (32). In agreement with our study, fish reared in intensive conditions showed depressed survival rates (14, 30, 33).

In this study, compared with the low stocking density (SD10), fish reared in intensive conditions (SD30 and SD40) showed lower feed efficiency ratio (FER). Low FER is related to energy metabolism rate (30, 32). Fish reared in intensive conditions are usually exposed to severe-stressor circumstances that increase the required energy (9, 29). The extra energy requirements were probably met by mobilizing body resources, resulting in lower growth and feed utilization (34).

The decreased growth and feed efficiency of Nile tilapia in this study might be related to

decreased activity of digestive enzymes. Major digestive enzymes produced by fish are protease, lipase and amylase to play its role in feed digestion and utilization. If these enzymes increase the overall body metabolism may increase (3,6). It is possible that intensive rearing conditions can weaken the digestion and utilization process of feed by affecting the activity of digestive enzymes. Further microbiome and proteomic studies are required to reveal the effect of intensive aquaculture conditions on the intestinal digestive enzymes and microbes.

Water quality is one of the main factors which affect the growth and feed utilization of fish (32, 35). The present study showed that high stocking density causes higher stress in Nile tilapia, leading to higher oxygen consumption and an increased ammonia level. There are many studies on the effects of stocking density on water quality with almost similar results. Similarly, increased stocking density led to lower DO and higher CO₂ levels (1, 14, 33). Randall and Tsui (36) reported that fish under stressful conditions excrete more ammonia and the present results suggest that the fish in high stocking densities experienced more severe stressed status. This is supported by the results of immune response and oxidative status.

It is well known that stress causes immunosuppression in fish (3). High stocking stress clearly declined soluble immune components in Nile tilapia with similar results in Senegalese sole and Rainbow trout (28). It has been proposed that, stressful conditions resulted in high produced corticosteroid levels which significantly inhibits the production of cytokines and immune responses (11). In this study, fish reared at high stocking densities exhibited suppressed immune responses “e.g. NBT, lysozyme activity, IgM, bactericidal activity and phagocytosis”. Immunoglobulins are “heterodimeric glycoproteins that play a vital role in recognizing natural antigens and exist in the skin, gill and gut mucus, bile as well as systemically found in the plasma of fish” (37). Low serum IgM was noticed in tilapia of SD40 group. The respiratory burst activity (NBT) is a reliable parameter used to detect oxidative radical production which reflect the immunity of cultured fish to show its ability to resist the infectious diseases and environmental stressors (22, 38).

The lysozyme activity can breakdown the polysaccharide walls of pathogenic bacteria and offers stronger innate immune defense in fish against stressors (32). The lysozyme activity depends on the leucocyte counts which produce lysozymes that catalyse the glycosidic bonds of pathogenic bacterial cell walls resulted in enhanced complement system and phagocytosis (39). In agreement with our results, high stocking density reduced lysozyme and peroxidase activities, suggesting some degree of immunosuppression in *Solea senegalensis*, Gilthead seabream, Rainbow trout and Nile tilapia reared in intensive conditions (29, 40, 41, 42).

Phagocytosis is one of the significant cellular immune system components in fish (43). Its role is to guarantees that fish can avoid pathogen attacks efficiently by recognize the pathogens and to bound their spread and progress (44). Our study demonstrated decreased bactericidal and phagocytosis, suggesting weakening immune response and tolerance against high stocking density. These results suggested that the resistance to the pathogenic bacteria could be weakened because of the intensive conditions.

The reactive oxygen species (ROS) is produced by animal cells in the presence of several antioxidant defense mechanisms. The oxidative stress normally happens when the production and removal of ROS is unbalanced, since the oxidative damage of cultured species is directly related to the quality of rearing environment (45). Among the antioxidant enzymatic defenses SOD, GPX and CAT enzymes (46, 47). In this study, fish reared in intensive conditions resulted in decreased SOD, CAT and GPX activities as well as increased MDA activity in blood of tilapia suggesting suppressed antioxidant response. The depression was a response to the continuous stresses of stocking density and might reflect the limited abilities for antioxidant systems in tilapia to wholly remove these harmful SOD, finally leading to oxidative damage (28, 40, 48). Usually, the antioxidant system is activated to control the ROS which resulted in oxidative stress (49). High level of lipid peroxidation is a result of excessive ROS production which ends by the production of MDA. High MDA levels finally leading to oxidative damage to DNA, protein and cytoplasm (50). The obtained results revealed increased MDA in fish at high stocking level indicated cell damage. Earlier reports also revealed decreased antioxidant enzyme (SOD, CAT and GPX) (40, 48) and increased oxidative enzyme (MDA) levels in fish reared in intensive conditions (28).

Conclusion

It can be concluded that, the intensive rearing conditions can impair the welfare of tilapia fingerlings and depress the growth, digestive enzyme activity, immune response and oxidative status.

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