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Stocking Densities and Chronic Zero Culture Water Exchange Stress' Effects on Biological Performances, Hematological and Serum Biochemical Indices of GIFT Tilapia Juveniles (*Oreochromis niloticus*)

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Abstract

Stocking densities and long term zero culture water exchange rate effects, on biological performances, hematological and serum biological indices of Genetically Improved Farm Tilapia (GIFT) strain cultured in tanks were investigated. The trial was divided into four groups with three replicates each, conducted under natural photoperiods for 30 days; data were analysed using one way ANOVA. Results showed that, high stocking densities and zero water exchange rate negatively affected the biological performances, hematological and serum biochemical indices of GIFT tilapia juveniles. Feeding efficiency, specifc growth and survival rates were significantly decreased. Hematoogical indices: red blood cells, white blood cells, Hemoglobin, Hematocrit and platelet decreased as stocking density increased. Indices of liver function (Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities) were significant increased. Serum glucose levels, an indicator of cortisol released in blood were increased as stocking density increased. Meanwhile, total protein, triglyceride, cholesterol, triiodothyroxine, thyroxine levels in the blood serum showed downward regulation under the same experimental conditions. This paper would provide useful scientific knowledge on biological performances and physiological responses of GIFT strain tilapia to stressors like high stocking density and zero water exchange. Research with different fish sizes and water exchange rates should be further conducted.

Keywords: GIFT-tilapia; Varied densities; Long term zero water exchange; Impacts

Introduction

Chronic stress results from a state of ongoing physiological arousal; which occurs when the body experiences stressors with such frequency or intensity that the autonomic nervous system does not have an adequate chance to activate the relaxation response on a regular basis; which means that the body remains in a constant state of physiological arousal, which affects virtually every system in the body, either directly or indirectly [1]. Stocking density is a crucial factor affecting fish wellbeing in the aquaculture industry, especially where high densities in confined environments are aimed at high productivity; this informs both the significance of species differences and the existence of a multifaceted array of factors which come to play; especially in chronic conditions which impact fish negatively [1-5]. Higher stocking and poor water quality are chronic stressors that are commonly encountered by fish [2], and have deleterious effects on their physiology and endocrinology. Stocking density as

a production parameter, can be use to ascertain the profitability and economic sustainability of a fish farm [3,4]. Commercial fish farmers often increase rearing density to boost their farm yield; meanwhile, even with supplementary feeding the scope of increasing stocking density and fish yield is limited; it increases to an optimum level and then starts decreasing. In intensive culture system, suboptimal conditions may result into chronic stress condition that can affect the wellbeing of fish [5]. Stocking density and water exchange rate have a close link with fish physiological responses [6] and their susceptibility to infectious diseases [7] Stocking density as stressor, have been studied in many bony fishes [8-10], it has a direct relation with feeding behavior [5,9]. Ammonia is a colorless pungent gas which is highly soluble in water; it accumulates in culture environment under high stocking density and poor water exchange rate. This colorless pungent gas is permeable to most biological membranes [10,11] and can cause physiological stress in fish. Chronic stress can alter hormone levels [12,13], enzyme activities [14], hematological indices [15,16] and growth performances of fish [17]. Changes in hormone levels, blood hematological and biochemical parameters can be use for optimizing culture conditions for fish [18-21]; especially when tanks are used for their culture. Tank-based aquaculture systems have become popular in many countries including the United States [22]. The negative impacts of high stocking densities can be intensified in undrainable tanks with accumulated waste products from fish population. Meanwhile, efficient removal of such metabolites by proper aeration of the tank water will enhance stocking rate, thereby boosting production.

This present study investigated the chronic effects of stocking densities and long term zero culture water exchange rate on the biological performances, hematological and serum biochemical indices of GIFT strain tilapia cultured in tanks. This paper provides information on the impacts of chronic stressors such as stocking density and poor water quality (zero exchange rate) on the biological performances and

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wellbeing of GIFT strain tilapia cultured in tanks with inadequate water supply or exchange.

Materials and Methods

Fish and acclimatization process

Sixteenth generation GIFT tilapia juveniles, bred at the Yixing farm of Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences in China, were used as test object in this trial. Prior to the official experiment, they were accliamatized for 7 days in concrete tank at water temperature 26 (± 0.3) °C. During the acclimatization process, continuous aeration was ensured and floating commercial feed was fed to fish to nearsatiation, two times daily. The Fish subsisted under natural photoperiod of 12 hr: 12 hr (light: dark) with water pH 8.0 (± 0.2), as ammonia and nitrite levels were held below 0.01 mg/L.

Experimental design and feeding management

Twelve tanks of equal dimensions (1 m×1 m×1.5 m) were impound with water up to a depth of 1 m and stocked with GIFT tilapia juveniles; mean initial weight 51.13 ± 0.43 g and length 14.23 ± 1.12 cm, in triplicate densities of 8 fish/ k^1, 17 fish/m^3, 25 fish/m^3 and 33 fish/m^3, corresponding to S1, S2, S3 and S4 respectively. Fish were fed commercial diet, 3% of their total biomass, twice a day; 08:00 and 16:00. Fish were grouped weighed on a weekly basis, for readjustment of feeding amount. The total amount of feed consumed by each group was then subsequently calculated as summation of given feed during the course of the experiment. The commercial diet (crude protein 35%; Fiber 8.0%; Ash 18%; Moisture 12%; Calcium 1.0%; total phosphorus 0.5%; NaCl 3.0%; Lysine 1.7% per kg feed) was from Tian Bang Freshwater fish feed industry,Ningbo, China. Solid wastes and uneaten floated feed, 15 min after fish were served, were removed from the respective tanks using finely-meshed scoop net. The experiment was carried out under natural photoperiod of 12 hr:12 hr (light: dark) in Yixing; culture waters in all the tanks (same volume) were not refreshed i.e. zero exchange rate, as fish were cultured in them for 30 days. The culture water temperature in all the treatment tanks was subjected to daily changes in ambient environmental temperature and mean value was 28.02 ± 1.18°C.

Sampling procedure and management

At the end of the trial, fish were systematically captured (per treatment replicates, tank after tank) for blood sample collection; three fish from each replicate tank in the respective treatments were obtained. Prior to blood sample collection, fish were captured, transferred and retrieved unconscious from a bucket of water containing tricaine methanesulfonate (2% MS-222), to avoid changes in measurement parameters that could have been caused by fighting due to handling stress.

Measurement of fish biological performances

Fish were not fed in the morning, on the day the experiment was terminated; and five fish per tank (15 fish per treatment), were systematically captured and individually weighed, for computation of biological parameters. Biological performance parameters including Specific Growth Rate (SGR), Feeding Efficiency (FE) and survival rate were calculated as follows:

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

Feeding efficiency (FE)=$(W_2-W_1)/F$ and Survival rate (SR) (%)=$N_2/N_1\times 100$

Where, W1, W2 and N2, N1 were body weights (g) and total number of fish at starting (t1) and ending time (t2) respectively; ln and F are natural logarithm and total feed given to fish (g).

Measurement of hematological indices

After whole blood samples were collected from the caudal vein of a total of nine fish per treatment (three fish from each replicate tank), using heparinized medical syringe, 2-ml, Red Blood Cells (RBCs), White Blood Cells (WBCs), Hemoglobin (Hb), Hematocrit (Ht) and Platelets (Pt) were then measured using Auto Hematology Analyzer (BC-5300Vet, Mindray, P.R. China) and test kit purchased from Shenzhen Mindray Medical International Co. Ltd. P.R. China.

Measurement of serum biochemical indices

After blood samples were collected from the caudal vein of another nine fish (three from each replicate tank), using 5-mL non heparinized medical syringe, they were placed in a 4°C refrigerator for 2 hours and centrifuged at 3000 r/min for 10min at controlled temperature of 4°C, to obtain the serum, which were then stored at -80°C pending analyses. Serum Glucose (GUL), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) activities, Cholesterol (CHO), Triglyceride (TG) and Total protein levels (TP), were measured by colorimetric method, using test kit from Mindray Bio Medical Co., Ltd and Mindray Auto Bio-chemical Analyzer (BS-400, Mindray, P.R. China). Serum Triiodothyronine (T3) and thyroxine (T4) were measured by Chemiluminescence immune competition method using Automated Chemiluminescence Immunoassay Machine (MAGLUMI 1000, SNIBE, P.R. China) and test kit purchased from Shenzhen New Industries Biomedical Engineering Co., Ltd., P.R. China.

Data analysis

Statistical Package for Social Sciences (SPSS) program for Windows (version 19, Chicago, IL, USA), was used for data analyses; data were subjected to one-way Analysis Of Variance (ANOVA) and difference among means was determined via Duncan's multiple-range comparison test, significant level was set at $P<0.05$. All data were analyzed using descriptive statistics and results are presented in tables.

Results

The results of biological performances, hematological and serum biochemical indices of GIFT tilapia juveniles stocked at varied densities and chronic zero culture water exchange rate are presented in Tables 1-4.

Biological performances of GIFT tilapia juveniles

Both Feeding Efficiency (FE) and Specific Growth Rate (SGR) dwindled with increased stocking density. There was a significant difference in FE and SGR between S1 and the other treatment groups ($P<0.05$). Meanwhile, there was a significant difference in SGR between S2 and S4 ($p<0.05$); no significant difference in SGR existed between S2 and S3, and between S3 and S4. Survival rate dwindled as stocking density increased. The highest survival rate was recorded in S1 followed by S2 and S3 respectively; S4 had the least survival rate. No mortality occurred in S1 unlike S2, S3 and S4 (Table 1).

Hematological indices

Hematological indices (Table 2) such as Red Blood Cells count (RBCs), White Blood Cells count (WBCs), Hemoglobin (Hb), Hematocrit (Ht) and Platelets count (Pt) of GIFT strain tilapia juveniles

Treatment	SGR (% day-1)	FE	SR(%)
S1	1.60 ± 0.06c	0.75 ± 0.03b	100.00
S2	0.43 ± 0.01b	0.18 ± 0.03a	64.71
S3	0.36 ± 0.05ab	0.13 ± 0.03a	50.66
S4	0.33 ± 0.06a	0.12 ± 0.04a	41.41

Data are represented as mean ± S.D, n=15. Means with the same letter in the same column for each parameter are not significantly different. Significant difference (P<0.05). SGR=Specific Growth Rate, FE=Feeding Efficiency, SR=Survival Rate

Table 1: Effect of different stocking densities and chronic zero water exchange rate on biological performances of GIFT tilapia.

Treatment	RBCs (x 10^{12} cells L^{-1})	WBCs (x 10^9 cells L^{-1})	Hb (gL^{-1})	Ht (%)	Pt (x 10^9 cellsL^{-1})
S1	2.15 ± 0.23c	202.67 ± 0.58d	68.13 ± 0.42d	42.13 ± 2.37b	74.33 ± 0.97d
S2	1.88 ± 0.13b	184.33 ± 1.53c	60.67 ± 2.08c	38.40 ± 2.74b	66.00 ± 1.00c
S3	1.69 ± 0.02ab	179.00 ± 1.00b	56.00 ± 1.00b	39.53 ± 3.66b	58.00 ± 1.00b
S4	1.54 ± 0.10a	175.67 ± 1.53a	53.00 ± 1.00a	30.80 ± 3.73a	49.00 ± 1.00a

Data are represented as mean ± S.D, with n=9. Means with the same letter in the same column for each parameter are not significantly different. Significant difference (P<0.05). RBCs=Red Blood Cells; WBCs=White Blood Cells; Hb=Hemoglobin; Ht=Hematocrit; Pt=platelet

Table 2: Effect of different stocking densities and chronic zero water exchange rate on hematological parameters of GIFT tilapia.

Treatment	ALT(U/l)	AST (U/l)	T3 ($ngml^{-1}$)	T4 ($ngml^{-1}$)
S1	28.42 ± 0.93a	121.35 ± 1.46a	3.00 ± 0.19c	4.11 ± 0.09d
S2	33.57 ± 1.06b	126.57 ± 0.19b	2.73 ± 0.01b	3.84 ± 0.05c
S3	37.23 ± 1.62c	128.09 ± 0.93c	2.27 ± 0.03a	2.64 ± 0.06b
S4	38.52 ± 0.59d	132.69 ± 1.44d	2.09 ± 0.04a	2.21 ± 0.01a

Data are represented as mean ± S.D, n=9. Means with the same letter in the same column for each parameter are not significantly different. Significant difference (P<0.05). ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; T3=Triiodothyroxine; T4=Thyroxine

Table 3: Effect of different stocking densities and chronic zero water exchange rate on serum indicative parameters of liver and thyriod function in GIFT Tilapia juveniles.

Treatment	GLU (mmol/L)	CHO (mmol/L)	TG (mmol/L)	TP (g/L)
S1	7.73 ± 0.17a	3.23 ± 0.11c	1.29 ± 0.12d	33.02 ± 0.81d
S2	8.62 ± 0.32b	2.73 ± 0.19b	0.83 ± 0.02c	29.57 ± 0.74c
S3	9.31 ± 0.21c	2.54 ± 0.11a	0.63 ± 0.03b	27.46 ± 0.22b
S4	10.77 ± 0.35d	2.47 ± 0.02a	0.52 ± 0.01a	25.53 ± 0.35a

Data are represented as mean ± S.D, n=9. Means with the same letter in the same column for each parameter are not significantly different. Significant difference (P<0.05). GUL=Glucose; CHO=Cholesterol; TG=Triglyceride; TP=Total protein

Table 4: Effect of different stocking densities and chronic zero water exchange rate on serum glucose, cholesterol , triglyceride and total protein level in GIFT tilapia.

decreased significantly (P<0.05) under varied densities and zero water culture exchange rate.

Serum biochemical indices of GIFT strain tilapia

There were significant increases in mean levels of ALT, AST and glucose under varied stocking and zero water exchange rate (P< 0.05). ALT and AST activities of S1 were lower than S2, S3 and S4 respectively. Highest AST, ALT and serum glucose levels respectively, were observed in S4; meanwhile, serum total protein, triglyceride and cholesterol levels were found to decrease under varied densities and long term zero culture water exchange rate, no significant difference in cholesterol level existed between S3 and S4 (P>0.05). High levels of serum protein, triglyceride and cholesterol (Table 4) were observed in the lowest density group (S1), as opposed to AST, ALT (Table 3) and glucose levels.

Triiodothyroxine (T3) levels decreased with increased stocking density; meanwhile, contrary to S1 and S2; there were significant differences amongst S1, S3 and S4, which was also true for S2 (P<0.05). Thyroxine (T4) level decreased under increased stocking densities and chronic zero culture water exchange rate (P<0.05); the highest level of thyroxine (T4) was recorded in S1 whiles the least was recorded in S4 (Table 4).

Discussion

Stress has a wide range of negative impacts on production characteristics of fish [23,24]. Higher stocking densities and poor water quality are chronic stressors commonly encountered by fish [2]. Oxygen consumption by fish is generally affected by elevated nitrite and ammonia levels [25-27] in culture system and can lead to physiological imbalances in fish [28,11]. Stressors of this sort can modify the regulation of endocrine growth axisincluding pituitary Growth Hormone (GH) secretion, hepatic Insulin-Like Growth Factors (IGFs) synthesis [29,30], thyroxine and triiodothyroxine. Mean specific growth rates is one way of quantifying the effect of stocking density on growth [31-33], which varies between and among species in relation to culture environmental conditions; including water quality and population size. Growth rates can be flexible in fish and naturally vary over short periods of time; meanwhile, stunted growth can occur in chronic stress situations [34]. Higher stocking densities have been reported to have aggravated stress that resulted in reduced feeding efficiency and specific growth rate [35,6,9]; furthermore, high stocking densities resulted to reduced specific growth rates in European sea bass; *Dicentrarchus labrax* [36], rainbow trout; *Oncorhynchus mykiss* [33], and Atlantic cod; *Gadus morhua* [37]. Varied stocking densities and long term zero culture water exchange rate in this trial, resulted to reduced feed intakes that yielded lower specific growth and survival rates respectively of

GIFT tilapia juveniles in the high density groups (Table 1). Fish hematological parameters, an important tool for monitoring fish health status [38], decreased in levels of as stocking density increased in this study (Table 2), and can be ascribed to reduced feed intake suffered by the fish, which gives credence to the explanations of d`Orbcastel et al. [32], Paspatis et al. [39], Lambert and Dutil [37]; that chronic stress from high stocking density can affect fish feeding behavior. Moreover, physiological responses to chronic stress conditions are mediated by stress hormones that may have caused activation of metabolic pathways that led to the reduction in hematological indices [40]. High density groups of fish may have suffered from weakened immunity that resulted to their death. Mehrim [15] did similar study correlating stocking density and dietary probiotic, and observed that; at optimal density, the probiotic improved fish immunity, meanwhile, when stocking density went beyond the optimal, the effect of probiotic was suppressed and led to reduced levels of hematological parameters. In our study, incessant aeration was ensured right through but it did not improved the immunity of the fish; chronic NH_3 and NO_2 could have affected oxygen intake by the fish, which led to reduced hemoglobin count.

Effect of stressors on fish has been correlated with reduced body lipid content [41]. In this trial, triglyceride and cholesterol levels were found to decrease with increasing stocking density (Table 4). Triglyceride and cholesterol are energy based substances that are basically derived from lipid absorption in the intestines and liver fatty acid metabolism [42]; their levels in blood serum have been associated with stress management [43,44]. Vijayan et al. [45] reported a reduction in triglyceride level when brook charr (*Salvelinus fontinalis*) was exposed to a stressful situation that triggered higher energy demand; and was further supported by Da Rocha et al. [46], who also reported significant change in the above parameter in matrinxã (*Brycon cephalus*) after handling and acute crowding stress. The animals could have utilized substantial amount of metabolizable energy in their response to the stressful condition. The decreased trends of triglyceride and cholesterol in our trial are similar to what was observed in Senegalese sole; *Solea senegalensis* [47]. According to Casillas et al. [48], serum total protein, AST and ALT activities can give clue to liver damages in fish. In this trial, increases in AST and ALT activities could have resulted from the long stay in chronic NH_3 and NO_2 [49] set in by both fish wastes and uneaten feed. Others studies have reported that, Nile tilapia exposed to chronic ammonia, had reduced growth rate [50], gill hyperplasia [51,52], increased brain glutamine [53] and high ATPase levels [54]. Stress tolerance in fish varies between species; for example, European sea bass (*Dicentrarchus labrax*) subjected to different stocking densities showed decreased levels in serum protein [41]. Ruane et al. [55] reported marked decrease in plasma total protein level in common carp (*Cyprinus carpio*) when held at high density in confinement; Biswas et al. [56] reported decreased plasma total protein in red sea bream after short term handling stress; the above findings are analogous to the current findings (Table 4). On the contrary, Caipang et al. [57] did not observe such change after exposing Atlantic cod (*Gadus morhua*) to short-term high stocking density stress. These discrepancies could have resulted from differences in experimental design, stress duration and the test objects. In our trial, GIFT strain tilapia was used as test object and was held under chronic stress conditions for thirty days. Chronic stress can disrupt normal physiology of vital organs such as liver and gills; and in turn hinders the normal metabolism of some food substances. ALT is an important enzyme in liver and is closely related to metabolism of protein, fats and carbohydrate; this enzyme will be released in blood when the liver is damaged [58]. AST, under normal condition can be found in soluble cytosol of liver cells, with relatively low activity, and may increase in blood serum when cells are damaged [59,60]. The high levels of ALT and AST in our trial informed the gravity to which fish liver cells could have being damaged. Unionized ammonia ranging from 0.1 to 0.42 mg/L can modulate the levels of biochemical parameters in fish [26,27]. High Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities basically are indicators of damaged or weakened liver functions. AST and ALT activity levels can be use to assess finfish response to toxins, malnutrition, disease and other stress related factors. In this study, serum AST and ALT activities (Table 3) were found to increase gradually with increased stocking density under zero water exchange rate, and conform to the findings of Chen et al. [61] and Zhang et al. [58]; who subjected Half-smooth tongue sole (*Cynoglossus semilaevis*) and common carp (*Cyprinus carpio*) to high density stressors respectively.

Blood glucose levels can be used as an indirect method to detect cortisol release in the blood when animals are stressed; and can provide information about fish health status [55], which can be use to enhance management protocols that reduce stress in tank-based aquaculture system. The change in glucose level in our study (Table 4), can be explain in the sense that, it is an important source of energy for maintaining homeostasis in GIFT strain tilapia. During anaerobic glycolysis, the glycogen stored in fish liver and muscles could be used to produce ATP. Other studies have shown that, water quality problems including high ammonia level, induced oxidative stress in: brain and gills of mudskipper; *Boleophthalmus boddarti* [62] and liver of Nile tilapia; *Oreochromis niloticus* [53]. Thyroid hormones play important role in the growth and development of larvae and juvenile fish. Earlier studies have shown that, thyroid activity fluctuates in response to various environmental stimuli [63,6]. Decreased levels of Triiodothyroxine (T3) and Thyroxine (T4) (Table 3) as stocking density was increasing were observed in our trial, and can be attributed to the long term chronic stress that resulted from high stocking densities and zero water exchange rate. According to Silberman et al. [13], decrease in serum thyroid hormone levels due to chronic mild stress have been observed to negatively modulate T-cell response; this may have also impacted the expression of T3 and T4 levels in our trial. Besides, physiological responses to chronic stress conditions are mediated by stress hormones that could have affected their expression. Reduced food intake has been associated with reduced thyroid hormones concentrations in fish and other vertebrates; besides, thyroid hormones are generally associated with an increase in metabolic rate, and are usually reduced during periods of food deprivation as a means to conserve energy [64]. Poor growth [65-66] has often been observed in fish reared at high densities. It is therefore very imperative that chronic stressors such as stocking density and water exchange rate be monitored closely when GIFT tilapia juveniles are to be reared in confinement for longer durations. Fish wellbeing is an important issue for the aquaculture industry, not just for public perception, marketing and product acceptance, but also in terms of production efficiency, quality and quantity; to meet the growing human population and demand for food fish protein.

Conclusion

In conclusion, stocking densities and zero culture water exchange rate set in chronic stress conditions that resulted to reductions in levels of hematological parameters including RBCs, WBCs, Hb, Ht and Pt. Serum total protein, cholesterol, triglyceride, thyroxine and triiodothyroxine were decreased significantly while glucose levels, aspartate aminotransferase and alanine aminotransferase activities were elevated under stress conditions of chronic high stocking density and zero water exchange rate. Further research should be conducted using different fish sizes. Our findings will guide aqua culturists on the effects of stocking density and zero culture water exchange rate in relation to fish physiological responses, when limited water is available for their culture in tanks.

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