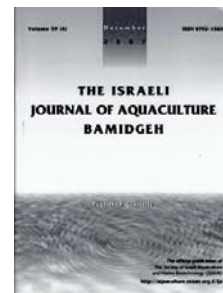


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## Effects of Stocking Density on Growth, Nonspecific Immune Response, and Antioxidant Status in African Catfish (*Clarias gariepinus*)

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### Abstract

African catfish were stocked at densities of 35, 65, 95, and 125 kg/m<sup>3</sup> for 60 days. The effects of the stocking density on growth, non-specific immune response, and antioxidant status were investigated on days 30 and 60. Serum cortisol levels were assayed on day 60. Increased stocking density led to decreased growth. Most non-specific immune indices (total number of leukocytes, differential leukocyte count in percent, respiratory burst activity, C3 and C4 complements, spleen and head kidney mass indices) were unaffected by the stocking density ( $p>0.05$ ). There were significant differences between the 35 and 125 kg/m<sup>3</sup> treatments in lysozyme activity on day 60 ( $p<0.05$ ) but no significant effects of stocking density on malondialdehyde (MDA) level on either day 30 or day 60 ( $p>0.05$ ). On day 30, only glutathione peroxidase (GSH-Px) activity was stimulated by increased stocking density while, on day 60, increased stocking density resulted in increased glutathione (GSH), GSH-Px activity, and superoxide dismutase (SOD) activity. The serum cortisol level on day 60 was unaffected by stocking density ( $p>0.05$ ). Findings suggest that high physiological welfare standards were maintained in African catfish reared in high stocking densities.

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## Introduction

Raising fish at relatively high stocking densities maximizes the use of culture area and is an effective measure for improving farm profitability. However, growth performance, health status, and disease susceptibility are related to stress conditions caused by high density. High stocking density can have positive or negative effects on fish growth and this interaction seems to be species-specific (Jørgensen et al., 1993; Hosfeld et al., 2009; Salas-Leiton et al., 2010; Tolussi et al., 2010). Increased stocking density can alter immunological responses, as well as physiological and biochemical processes related to metabolism and behavior (Barcellos et al., 2004; Santos et al., 2010).

The African catfish (*Clarias gariepinus*) is one of the most important cultured fish in China because of its fast growth, high disease resistance, and hypoxia tolerance. Increased stocking density may make fish more susceptible to stress and disease, followed by severe losses of stock. The purpose of this study was to investigate the effects of stocking density on the growth, cortisol level, non-specific immune response, and oxidative status of African catfish during nursing.

## Materials and Methods

*Fish and experimental design.* Juvenile African catfish (10-12 g) were obtained from Deren aquaculture center, Tianjin, China. Prior to the start of the experiment, fish were acclimated to laboratory conditions in 250-l aerated holding tanks at 5 kg fish/m<sup>3</sup> with 100% water exchange, optimal temperature of 27±1°C, and a photoperiod of 12L:12D. After acclimation, fish (30.71±0.89 g) were randomly divided into four treatment groups with three replicates of each density: 35 kg/m<sup>3</sup>, n = 136 fish; 65 kg/m<sup>3</sup>, n = 262 fish; 95 kg/m<sup>3</sup>, n = 370 fish; and 125 kg/m<sup>3</sup>, n = 481 fish. Our treatments included a lower density and two higher densities than those used in aquatic farms in Tianjin, China. Each tank (70 × 50 × 50 cm) contained 120 l aerated water, renewed daily at a rate of 1.5 times the volume. Throughout the experiment, fish were fed a commercial dry floating pelleted catfish feed (35% min crude protein, 5% min crude fat, 10% max crude fiber, 10% max ash; Tianxiang Feed Co. Ltd., Tianjin, China) at 2.0% of their wet body weight twice daily at 08:00 and 16:00.

*Sampling procedures.* Growth was assessed by mean fish weight, determined by dividing the total weight of the fish by the number of fish in each tank. On days 30 and 60, eight randomly selected fish per tank were anesthetized with 200 mg/l MS222. One ml blood was collected from the caudal vein and 3 mg Na<sub>2</sub>EDTA was added as an anticoagulant. These blood samples were used to determine respiratory burst activity, total number of leukocytes, and differential leukocyte count (%). An additional four fish were randomly selected from each tank and anesthetized with 200 mg/L MS222. Blood samples were collected from the caudal veins and left at room temperature for 1 h, then at 4°C for 4 h, after which the serum was harvested by centrifuging at 4000 rpm at 4°C for 12 min, then stored at 4°C until complement C3 and C4 analysis which was done within 12 h. Excised head kidneys were weighed and stored at -80°C for lysozyme activity analysis. Posterior kidneys were dissected and stored at -80°C for oxidative stress analysis.

After the first sampling, the water volume in the tanks was reduced to maintain the required stocking density. For the serum cortisol analysis on day 60, ten fish per tank were caught and anesthetized with 400 mg/l MS222 before other fish were sampled as above. Blood samples were collected from the caudal vein with a syringe. The catching, anesthetizing, and blood collection for all fish was completed within 5 min. Blood samples were left at 4°C overnight, and the serum was harvested by centrifuging at 4000 rpm for 12 min, then stored at 4°C until cortisol analysis, done within 24 h.

*Serum cortisol analysis.* Serum cortisol, a measure of stress in fish, was measured with an assay kit (Coated Tube Cortisol 125I RIA Kit, Beijing North Institute of Biological Technology, Beijing, China) developed for the quantitative measurement of cortisol by radio-immunoassay.

*Monitoring non-specific immune responses.* Ten mm<sup>3</sup> blood was diluted with 2 ml reagent (0.012 g crystal violet, 0.006 g bismarck brown R, 1.7 g NaCl, 200 ml distilled

water). For fixation, 10 mm<sup>3</sup> formaldehyde was added to the dilution which was then loaded onto a hemocytometer covered with a cover slide to be counted under a microscope. The leukocyte count was determined using the following formula: leukocyte count (cell/mm<sup>3</sup>) =  $N/0.4 \times 200$ , where N represents the total number of leukocytes in the four large corner squares of the hemocytometer. To count the leukocytes, blood smears were air dried, fixed with methanol, and stained with Wright's and Giemsa stains. The percentage of each type of leukocytes was determined microscopically on randomly selected fields of stained smears based on cell morphology (Ellis., 1977).

Head kidney samples were homogenized in ice-cold physiological saline at a ratio of 1:9, and the homogenates were centrifuged at 10,000 rpm for 11 min at 4°C. The final supernatants were used for lysozyme activity analysis. Lysozyme acts as a potent non-specific immune factor against parasitic and bacterial infections and its activity was measured using a modified turbidimetric method according to Ellis (1999) with a kit (Jiancheng Biotech Co., Ltd., Nanjing, China). Briefly, a *Micrococcus lysodeikticus* suspension at a concentration of 0.1 mg/ml was added to the head kidney supernatant at a 10:1 ratio, and the decrease of absorbance was read at 0.5 and 2.5 min intervals at 530 nm in a spectrophotometer (UV-752N, Shanghai Precision Instrument Co., Ltd., China). One unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min. The total protein content of the supernatant was assayed according to Bradford (1976) using bovine albumin as the standard.

The oxidative radical production by phagocytes during respiratory bursts was assayed by the reduction of nitroblue tetrazolium (NBT) to formazan (Eo and Lee, 2008). Briefly, blood was mixed with 0.2% NBT (Sigma, China) in equal proportions (1:1) and incubated for 30 min at 28°C, then 100 µl of the mixture was removed to a new tube, 2 ml dimethyl formamide was added to dissolve the reduced formazan product and it was centrifuged at 2000 g for 5 min. The supernatant was collected and NBT was measured at an optical density of 540 nm with dimethyl formamide as the blank.

Serum complements C3 and C4, which play an important role in disease resistance and inflammatory response, were measured by immunoturbidimetry with kits (Elikang Biological Technology Co., Ltd., Zhejiang, China). The spleen and head kidney mass indices were calculated using the following formula: spleen mass index = 100(spleen wt/body wt) and head kidney mass index = 100(head kidney wt/body wt).

*Monitoring antioxidant status.* Approximately 0.5 g refrigerated posterior kidney samples were homogenized in 4.5 ml ice-cold physiological saline and centrifuged at 2500 rpm for 10 min at 4°C. The final supernatants were divided into subsamples to determine malondialdehyde (MDA) level, glutathione (GSH) content, and glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities using assay kits (Jiancheng Biotech Co., Ltd., Nanjing, China). MDA level was quantified by the thiobarbituric acid (TBA) method (Dogru et al., 2008). GSH-Px activity was measured by the method of Zhang et al. (2004). One unit of GSH-Px activity was defined as the amount of enzyme that oxidizes 1 mmol/l of GSH per min in 1 mg protein. GSH content was determined by the DTNB method (Ji et al., 2006). SOD activity was measured spectrophotometrically by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals (Trenzado et al., 2006). Total protein content of the supernatant was assayed according to Bradford (1976) using bovine albumin as the standard.

*Statistical analysis.* Data were expressed as means±SD and subjected to one-way analysis of variance (ANOVA, SPSS version 10.0) to determine significant differences between groups. These differences ( $p < 0.05$ ) were reanalyzed by least significant difference multiple-range test.

## Results

Final body weight decreased with increasing stocking density (Fig. 1a). Survival was higher in the 35 and 65 kg/m<sup>3</sup> treatments than in the 95 and 125 kg/m<sup>3</sup> treatments (Fig. 1b), while serum cortisol on day 60 was unaffected by stocking density (Fig. 1c). Leukocyte parameters did not significantly differ between treatments but GSH level, GSH-

Px activity, and SOD activity increased as the stocking density increased (Table 1). Lysosome activity significantly differed between the 35 and 125 kg/m<sup>3</sup> treatments on day 60 (Fig. 2).

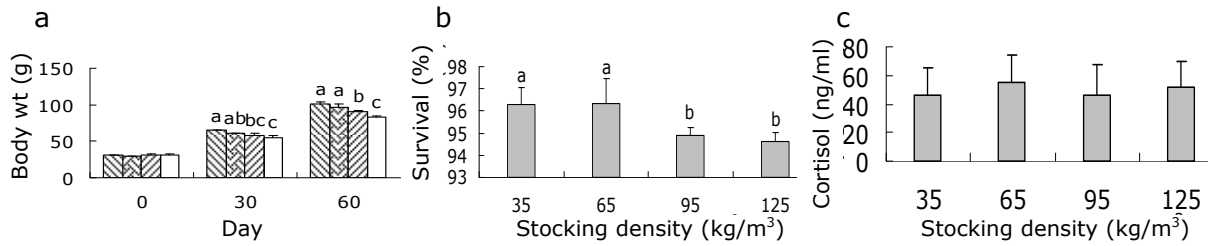


Fig. 1. (a) Weight, (b) survival, and (c) serum cortisol level in African catfish (*Clarias gariepinus*) reared in different stocking densities. Values with different letters significantly differ ( $p < 0.05$ ).

▨ = 35 kg/m<sup>3</sup>, ▩ = 65 kg/m<sup>3</sup>, ▪ = 95 kg/m<sup>3</sup>, □ = 125 kg/m<sup>3</sup>

Table 1. Effects of stocking density on leukocyte parameters and oxidative stress in the posterior kidney of African catfish (*Clarias gariepinus*).

	Day	Stocking density (kg/m <sup>3</sup> )			
		35	65	95	125
Leukocyte (10 <sup>3</sup> mm)	30	76.60±21.77	80.51±20.72	76.61±37.75	85.95±26.88
	60	66.34±20.94	57.37±15.65	66.21±17.93	57.32±15.05
Neutrophil (%)	30	25.94±12.08	26.38±10.94	27.67±5.46	24.19±6.54
	60	26.74±7.12	24.38±5.05	24.50±6.41	24.74±3.85
Lymphocyte (%)	30	49.22±15.21	46.81±17.09	47.43±9.11	53.38±10.18
	60	45.96±7.55	50.38±14.32	52.04±8.40	51.91±10.15
Monocyte (%)	30	25.11±6.32	26.81±10.25	24.90±8.14	22.48±7.64
	60	27.30±7.77	25.08±9.22	24.50±7.40	22.83±10.87
MDA (nmol/mg prot)	30	0.97±0.26	1.04±0.28	0.95±0.12	1.07±0.30
	60	0.84±0.21	0.92±0.11	0.96±0.12	0.84±0.16
GSH-Px (U/mg prot)	30	30.96±4.18 <sup>b</sup>	32.53±4.24 <sup>ab</sup>	34.34±3.10 <sup>a</sup>	33.53±3.21 <sup>ab</sup>
	60	25.17±2.91 <sup>b</sup>	29.00±4.28 <sup>a</sup>	29.68±2.75 <sup>a</sup>	28.80±5.99 <sup>a</sup>
GSH (mg/g prot)	30	6.42±1.79	7.28±2.31	7.51±1.79	7.17±1.72
	60	6.18±0.48 <sup>c</sup>	8.19±1.63 <sup>b</sup>	9.27±1.86 <sup>ab</sup>	10.19±1.70 <sup>a</sup>
SOD (U/mg prot)	30	3.73±0.77	3.23±0.74	3.65±0.42	3.65±0.44
	60	3.28±0.30 <sup>b</sup>	3.53±0.52 <sup>ab</sup>	3.48±0.57 <sup>ab</sup>	3.83±0.50 <sup>a</sup>

Values with different superscripts significantly differ ( $p < 0.05$ ).

MDA = malondialdehyde, GSH-Px = glutathione peroxidase, GSH = glutathione, SOD = superoxide dismutase

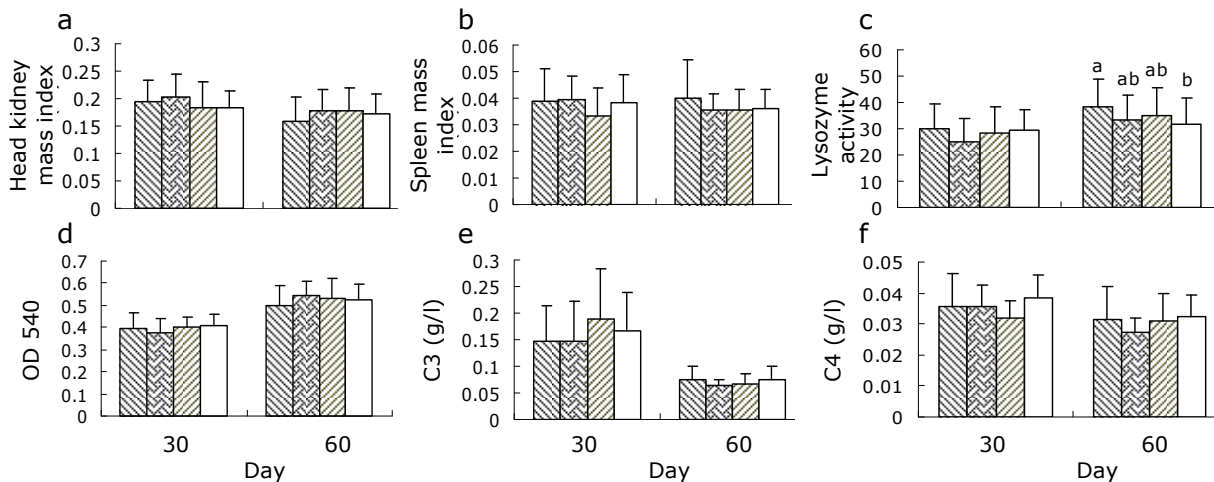


Fig. 2. (a) Head kidney and (b) spleen mass indices (%), (c) lysozyme activity in the head kidney (U/mg), (d) nitroblue tetrazolium (NBT) assay, and complements (e) C3 and (f) C4 in serum of African catfish (*Clarias gariepinus*) reared in different stocking densities. Values with different letters significantly differ ( $p > 0.05$ ). ▨ = 35 kg/m<sup>3</sup>, ▩ = 65 kg/m<sup>3</sup>, ▪ = 95 kg/m<sup>3</sup>, □ = 125 kg/m<sup>3</sup>

### Discussion

Increased stocking density is a chronic stressor that can lead to decreases or increases in the growth of African catfish (Kaiser et al., 1995; Hossain et al., 1998; van de Nieuwegiessen et al., 2008). In this study, growth of African catfish decreased as the stocking density increased. High stocking density can affect fish growth through the combined effects of crowding stress and water quality deterioration in recirculating systems (Santos et al., 2010). Here, 150% daily water exchange was insufficient to prevent deterioration of the water quality.

The hypothalamic-pituitary-interrenal (HPI) axis is an important component of the response of fish to most forms of environmental stress. Because of its rapid response to stressful conditions, serum cortisol is used as a measure of stress (Pickering and Pottinger, 1989). The energy-mobilizing properties of secreted cortisol are the expression of a fish's attempt to avoid or overcome immediate threats (Pickering and Pottinger, 1989). As a chronic stressor, crowding can induce changes in the level of cortisol (Rotllant et al., 1997; Wang et al., 2004), but we found that the cortisol level was not affected by stocking density.

Lysozyme is a bactericide that hydrolyses  $\beta$ -[1, 4]-linked glycoside bonds of cell wall peptidoglycans of bacteria, resulting in lysis (Magnadóttir, 2006). It is present in mucus, lymphoid tissue, plasma, and other body fluids of most fish species (Grinde et al., 1988; Lie et al., 1989). In our study, African catfish showed very little plasma lysozyme activity, in some cases it was below the detection limit of the kit. In *Carassius auratus*, resistance expressed by lysozyme activity was also depressed by crowding, however, it is difficult to conclude that the decreased lysozyme activity resulted in the reduced disease resistance since information concerning other immune indices is not given (Wang et al., 2004). Immunosuppressive effects of crowding were observed in other fish (Tort et al., 1996; Barcellos et al., 2004). However, in our study, except for lysozyme activity, other non-specific immune indices were unaffected by stocking density, suggesting that African catfish have a relatively high tolerance to high stocking densities as compared to other fish species.

Enhanced MDA is a vital sign of cellular oxidative damage. In this study, MDA content was not affected by stocking density, suggesting that there was no endogenous lipid peroxidation in the posterior kidney. GSH, GSH-Px, and SOD act as antioxidant defense factors against reactive oxygen species (ROS). High density lead to enhanced GSH, GSH-Px, and SOD on day 60, possibly as an adaptive response of the fish to chronic stress.

In this study, all the examined physiological indicators, except lysozyme activity, were not significantly affected by stocking density. Apparently the physiological response of African catfish is not sensitive to crowding stress. Integration with other welfare indicators, such as behavioral responses, is needed to assure that optimal stocking densities in cultures are adopted.

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