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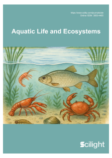

*Article*

# Optimal Stocking Densities and High-Density Stress Response Mechanisms in Two Size Classes of Juvenile Largemouth Bass (*Micropterus salmoides*)

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**Abstract:** To clarify the optimal stocking density and density stress mechanism of *Micropterus salmoides* juveniles of different sizes, two size classes of *M. salmoides* (size 1: 8.0 ± 0.5 g, size 2: 50 ± 3.2 g) were selected as the research objects. Five stocking density gradients (0.25, 0.75, 1.25, 1.75 and 2.25 $kg \cdot m^{-3}$) were set up. After 30 days of culture, the growth performance, feeding and swimming behaviors, oxidative stress, and water quality indices were determined. The results showed that the survival rates of 0.25, 0.75 and 1.25 $kg \cdot m^{-3}$ groups had no significant difference and were significantly higher than those of high-density groups. The optimal stocking density of size 1 *M. salmoides* was 0.75–1.25 $kg \cdot m^{-3}$, which could balance optimal growth and high survival; beyond this range, the growth performance decreased significantly. High stocking density led to intensified feeding competition, increased stress-induced swimming activity, aggravated oxidative damage and water quality deterioration. In addition, size 2 *M. salmoides* was more sensitive to high-density stress, with a significantly narrower optimal density window, and its optimal stocking density was identified as 0.75 $kg \cdot m^{-3}$. Density stress exerted its effects through the pathway of spatial competition → behavioral abnormalities → oxidative damage → growth inhibition, and formed a negative feedback loop with water quality deterioration. This study provides a scientific basis for the precision regulation of stocking density in intensive culture of *M. salmoides*.

**Keywords:** *Micropterus salmoides*; stocking density; growth performance; oxidative stress; behavioral response

## 1. Introduction

### *1.1. Research Background and Significance*

Largemouth bass (*Micropterus salmoides*), an eurythermal freshwater carnivorous species, with fast-growing rate and higher-quality flesh are highly sought-after. As an essential high-valued aquaculture product in China, it has gradually expanded its industrial scale and economic benefits [1–3]. In order to improve the effectiveness of fishery production, intensively managed farms are popular because they can increase output and reduce water consumption per unit area significantly [4–6]. However, excessive stocking density, as a persistent environmental stressor, leads to overcrowding and deterioration of water quality [7,8], intensifies competition [9], and consequently triggers a range of adverse effects. These include abnormal feeding behavior [10], imbalance in energy metabolism [11], exacerbated physiological stress [12], and reduced growth performance [13,14]. Such impacts not only compromise individual health and survival rates but may also constrain the sustainable development of the industry.

The physiological metabolic rate, spatial requirements, behavior, and stress tolerance of fish vary significantly across different ontogenetic stages. During its juvenile period, Fish have a higher metabolism and sensitivity to environmental changes [15,16]. With an increase in size, due to a higher bioload per unit of water volume, there are stronger needs for space and dissolved oxygen. Under such circumstances, responses to density stress might be more complex [17]. Research at present mainly investigates stock densities for only one-size-class largemouth bass; thus, no direct comparisons have been made among various-sized stocks. It is difficult to reveal the size-dependent patterns of density effects [11,13,18]. Therefore, systematically compare the responses of different-sized largemouth bass to stocking density and elucidate its physiological and ecological mechanism have both theoretical value and practical guide for establishing size-based density regulation strategy and achieving an effective healthy management in intensive aquaculture.

### *1.2. Advances in Domestic and International Research*

Extensive research has been conducted domestically and internationally on the effects of stocking density on fish, primarily focusing on growth performance [19], behavior [20], physiological stress [21], and water quality changes [22]. Growth performance is used directly to evaluate the impact of density effects. In addition to the optimally dense state, there is a negative correlation between WGR and SGR at higher densities; meanwhile, FCR shows an increase trend [23–25]. Behaviorally, at higher densities, intense food stress will occur among individuals, increasing the frequency of aggressive feeding behaviour and injury risks [26,27]. At the physiological level, an index of environmental stress commonly refers to oxidative stress. Long-term stress has an accumulation effect on Reactive Oxygen Species (ROS), it leads to lipid peroxidation through ROS promotion, and finally initiates the antioxidant defense system [28,29]. Antioxidant capacity imbalance can be one of the main reasons for tissue injury and immune suppression [30,31]. Regarding water quality: high density cultivation accelerates the formation of ammonia nitrogen and nitrite. A vicious cycle has been formed because density stress causes a decline in water quality, which then intensifies further [7,8].

Regarding largemouth bass, existing studies have confirmed that high stocking density suppresses its growth and impairs nutritional quality [7,8,10]. However, these investigations are often limited to a single size class or isolated physiological parameters. They lack a systematic integrative analysis across five key aspects: growth performance, feeding behavior, swimming activity, oxidative stress, and water quality feedback. Furthermore, few studies have compared the differential responses of various size classes to density gradients.

### *1.3. Research Objectives and Scope*

This study aims to conduct a culture experiment using two size classes of largemouth bass (8.0 ± 0.5 g and 50 ± 3.2 g). Establish a comprehensive assessment index system to investigate its effect on changes in growth, physiology and ecology at different levels across several sizes of fish under various stocking densities. Then select the most effective one from them. Specifically, the following research aims have been set out: (1) Evaluate the effects of five stocking densities at 0.25, 0.75, 1.25, 1.75, and 2.25 kg/m$^3$ on growth performance, feed consumption, swimming behaviour, oxidative stress indicators, water quality parameters, etc., to determine each size class’s most ideal stocking density; (2) Compare whether there is a difference in the response mode of two sizes when subjected to density stress by analyzing behavioural and physiological changes under different stocking densities, thereby discovering the size-dependency rule for density impact from multi-level views.

## 2. Materials and Methods

### *2.1. Experimental Materials*

Experimental largemouth bass came from Changxin Fishery Co., Ltd. in Alaer City, Xinjiang, China. Before the experiment, a strict screening process was carried out. The fish have an even body shape, without any external wounds, and their body weight is between (8.0 ± 0.5 g) and (50 ± 3.2 g). All experimental fish were acclimatized in an indoor recirculating aquaculture system for 14 days to adapt to the experimental environment and resume normal feeding. During the acclimation period, one-third of the water was replaced each day. Water temperature was set within (26 ± 1) °C and continuously circulated. At the same time, continuous aeration was provided to maintain dissolved oxygen levels above 6.5 mg/L regularly. Fish were fed two times per day, morning and afternoon, using commercial largemouth bass formulated feed (crude protein > 45%, crude fat > 8%), until the full amount.

## *2.2. Experimental Design*

The experiment was conducted indoors at the aquaculture base of Changxin Fishery Co., Ltd. in Alaer City, China. For size class 1 (8.0 ± 0.5 g), circular tanks with a volume of 700 L were selected as experimental units. These tanks, known as nursery tanks for juvenile largemouth bass in the industry, have no water flow dead areas to reduce accumulation of residual food and feces. For Size Class 2 (50 ± 3.2 g), square tanks with a capacity of 2040 L serve as the experiment unitie, standard rearing tanks for medium-sized largemouth bass in industrial-intensive aquaculture. This large volume can meet the space demand of bigger individuals. The selection of tank type is fully in line with the actual production scenario of largemouth bass culture. To reduce the impact of tank shape and size on the experiment's data, all environmental conditions were standardized. Each tank was equipped with a drainage valve at its centre position and had been connected to an air stone continuously for aeration so that enough dissolved oxygen could be generated in each tank. The culture time of each group was 30 days long, with the same light intensity, water temperature regulation, feeding habits and changes, and daily work routines among all tanks. The pretest revealed that, on average, the basic feeding and swimming behaviours of largemouth bass at different sizes within a specific range were similar across circular and square tanks with equal water volumes.

Five stocking density gradients were established, each with three replicates. The densities were determined based on the conventional range for largemouth bass culture, as follows: 0.25, 0.75, 1.25, 1.75, and 2.25 $kg \cdot m^{-3}$. The corresponding stocking numbers were:0.25 $kg \cdot m^{-3}$: 22 fish per tank for Size Class 1 and 10 fish per tank for Size Class 2; 0.75 $kg \cdot m^{-3}$: 66 fish per tank for Size Class 1 and 30 fish per tank for Size Class 2; 1.25 $kg \cdot m^{-3}$: 110 fish per tank for Size Class 1 and 50 fish per tank for Size Class 2; 1.75 $kg \cdot m^{-3}$: 153 fish per tank for Size Class 1 and 71 fish per tank for Size Class 2; 2.25 $kg \cdot m^{-3}$: 197 fish per tank for Size Class 1 and 92 fish per tank for Size Class 2.

During the experimental period, the photoperiod was set to 12 h of light followed by 12 h of darkness (12L:12D). The initial daily feeding rate was set at 3–5% of the fish body weight and was dynamically adjusted based on the actual feed consumption observed 30 min after each feeding. Uneaten feed and feces were promptly removed daily. A differentiated water exchange protocol was implemented according to the density groups. For the 2.25 $kg \cdot m^{-3}$ and 1.75 $kg \cdot m^{-3}$ density groups, characterized by higher feeding inputs and metabolic loads, water was exchanged twice daily. For all other groups, water was exchanged once daily, with approximately one-third of the total volume replaced each time.

## *2.3. Measurements and Methods*

### 2.3.1. Growth Performance Indicators

Before and after each experiment, all the fish were not fed for 24 h and then counted and weighed. These are the chosen values.

$$\text{Weight Gain Rate (WGR, \%)} = [(\text{Final mean body weight} - \text{Initial mean body weight})/\text{Initial mean body weight}] \quad (1)$$

$$\text{Specific Growth Rate (SGR, \%/d)} = [(\ln \text{Final body weight} - \ln \text{Initial body weight})/\text{Culture days}] \quad (2)$$

$$\text{Feed Conversion Ratio (FCR)} = \text{Total dry feed weight fed}/(\text{Final total biomass} + \text{Biomass of dead fish} - \text{Initial total biomass}) \quad (3)$$

$$\text{Survival Rate (SR, \%)} = (\text{Final number of fish}/\text{Initial number of fish}) \quad (4)$$

### 2.3.2. Feeding and Swimming Behavior Indicators

Feeding behaviour: On Day 25 of the experiment, recording the feeding process in each tank during the morning feed time. The following analysis was carried out as follows: (1) Feeding response latency(s): The period of reaction to feed entering water and the first appearance of active eating behaviour; (2) Number of aggressive feeding actions in the first three minutes: refers to whether there are different kinds of aggressive behaviours caused by food stealing among test fish within 3 min after feeding. Includes two kinds of correct behaviours, and each behaviour is counted separately as one aggressive act: (1) Chasing behaviour: The test fish continues to chase another individual over a distance longer than five body lengths while accelerating rapidly; It has a clear intention to steal food or drive away other individuals; (2) Contact aggressive behaviour: Directly ramming into the body, fin or head of another individual's mouth in contact with it without any fear from injuring others.

Swimming behaviour: On day 20 of the experiment, at a non-feeding time point on that day, five-minute videos were captured from each tank via a fixed-camera setup. Using videos recorded in EthoVisionXT for analysis revealed: (1) mean swimming velocity; (2) number of swims per $\text{min}^{-1}$.

Injury Rate: Count the number of individuals that have visible external injuries at the end of the experiment for each group. Injury rate (%): Number of injured fish/Total number of fishes × 100 per cent.

### 2.3.3. Oxidative Stress Indicators

Six randomly selected fishes per experimental group at the conclusion of the experiment. Blood was drawn from the caudal vein, and then after clotting in a 24-h period at room temperature. After that, the serum was recovered through centrifugation under 4 °C and a speed of 3500 r/min for 15 min; it has been frozen at −80 °Cfor further examination. In accordance with the manufacturer's specifications for the assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), serum levels of malondialdehyde (MDA) were detected using the thiobarbituric acid method; SOD activity was tested hydroxylamine method, and total antioxidant capacity (T-AOC) was estimated through the ABTS method.

### 2.3.4. Water Quality Indicators

During the experimental period, the dissolved oxygen (DO, $\text{mg·L}^{-1}$), ammonium nitrogen ($NH_4^+$-N, $\text{mg·L}^{-1}$), and nitrite nitrogen ($NO_2^-$-N, $\text{mg·L}^{-1}$) concentrations in each tank were measured daily at a fixed time of 08:00 a.m., strictly prior to the morning feeding and daily water exchange operation, using a YSI ProPlus water quality analyzer prior to feeding.

## *2.4. Statistical Analysis*

The experimental data are presented in terms of the mean ± Standard Deviation. Analysis of statistical data using SPSS version 26.0. The data were first subjected to normality tests using the Shapiro-Wilk method and homogeneous-variance tests with Levene's test.

A two-way analysis of variance (two-way ANOVA) was first performed to evaluate the main effects of stocking density (5 levels), fish size (2 size classes), and their stocking density × fish size interaction on all core experimental indicators. The F values, degrees of freedom (df) and exact p-values are listed for all effects.

Then, using one-way ANOVA to test whether the stocking densities differed significantly at each level of five individuals' sizes for every sample point. ANOVA showed that there were significant differences among groups at $p < 0.05$; then, Duncan's multiple comparison test was conducted to identify which pairs of density levels differed significantly in performance. Groups with significant differences ($p < 0.05$) have lower case letter markings next to the bar charts:

Using Pearson correlation analysis to examine the degree of linkage among functionally related indices to reveal their intrinsic pathway by which density stress influences the development performance of *M. salmoides*.

Growth performance indicators (WGR and SGR) meet the requirements of one-way analysis of variance; These indices are overall growth rates calculated from the initial and final body weights of single-independent bioreplicates that can be regarded as a set of independent and identically distributed samples satisfying parametric conditions. A common method for assessing the effect of farm management practice changes on fish growth rate change during aquaculture research [19,32]. Given that the growth indicators were only evaluated at the beginning and end of the experiment without taking multiple readings under varying conditions over time, repeated-measures ANOVA is not suitable for analysing the data structure in this research.

All the figures were created with Origin 2023.

## 3. Results and Analysis

Two-way Analysis of Variance was used to examine the primary influence factors among stocking density at 5 levels, fish Size in two Classes; their combined effects on all core experimental outcomes. All the aggregated statistics are provided by Table 1. The stocking density showed a highly significant main effect on all the growth, behavioural, oxidative stress and water quality indices ($p < 0.001$). The size of the fish significantly affected all parameters, but not feeding response latencies ($p = 0.152$). A distinct interaction existed among the stocking densities of various groups for each specific core indicator ($p < 0.05$). Thus, we can determine that there is a considerable size-dependent response in *M. salmoides* under high stocking-density conditions at present.

**Table 1.** Effects of different stocking densities on the growth performance of *Micropterus salmoides*.

| **Indicator** | **Main Effect of Stocking Density F Value (df = 4, 20)** | ***p* Value** | **Main Effect of Fish Size F Value (df = 1, 20)** | ***p* Value** | **Stocking Density × Fish Size Interaction Effect F Value (df = 4, 20)** | ***p* Value** |
|---|---|---|---|---|---|---|
| Weight Gain Rate (WGR, %) | 78.34 | *** | 42.67 | *** | 6.89 | ** |
| Specific Growth Rate (SGR, %/d) | 82.17 | *** | 39.52 | *** | 7.24 | ** |
| Feed Conversion Ratio (FCR) | 96.48 | *** | 51.33 | *** | 8.16 | ** |
| Survival Rate (SR, %) | 67.29 | *** | 22.47 | *** | 5.93 | ** |
| Feeding response latency (s) | 38.62 | *** | 2.18 | Ns | 12.75 | *** |
| Number of aggressive feeding acts within 3 min | 108.53 | *** | 47.81 | *** | 9.37 | *** |
| Average swimming speed (cm·s$^{-1}$) | 72.46 | *** | 33.59 | *** | 6.12 | ** |
| Swimming frequency (acts·min$^{-1}$) | 65.38 | *** | 28.74 | *** | 5.47 | * |
| Injury rate (%) | 124.71 | *** | 59.26 | *** | 10.82 | *** |
| Malondialdehyde (MDA, nmol·mL$^{-1}$) | 112.64 | *** | 63.49 | *** | 11.53 | *** |
| Superoxide Dismutase (SOD, U·mL$^{-1}$) | 89.37 | *** | 37.22 | *** | 7.85 | ** |
| Total Antioxidant Capacity (T-AOC, U·mL$^{-1}$) | 76.55 | *** | 31.48 | *** | 6.92 | ** |
| Peak ammonium nitrogen (Peak $NH_4^+$-N, mg·L$^{-1}$) | 137.82 | *** | 78.63 | *** | 8.49 | ** |
| Peak nitrite nitrogen (Peak $NO_2^-$-N, mg·L$^{-1}$) | 129.46 | *** | 69.71 | *** | 7.63 | ** |
| Minimum dissolved oxygen (Minimum DO, mg·L$^{-1}$) | 92.15 | *** | 45.38 | *** | 5.71 | * |

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; Ns = Not significant ($p > 0.05$); df = Degrees of Freedom.

## *3.1. Effects of Different Stocking Densities on Size Class 1 Largemouth Bass*

### 3.1.1. Effects of Different Densities on Growth Performance

Two-way ANOVA showed that the stocking density significantly influenced all growth performance indices in *M. salmoides* at $p < 0.001$; There was also a notable interactive effect of stocking density and fish body weight on WGR, SGR, FCR and SR ($p < 0.006$). According to Table 2, there was not a statistically significant difference in the initial body weight of largemouth bass across different densities at the beginning of the experiment ($p > 0.872$). Thirty days after culture, the growth performance of size 1 fish exhibited a "medium optimum, high and low inhibition" nonlinear characteristic at different stocking densities. The final average body weight, weight gain rate (Figure 1a), SGR (Figure 1b). The trend was that 0.75 kg/m$^3$ group ≈ 1.25 kg/m$^3$ group > 1.75 kg/m$^3$ group ≈ 0.25 kg/m$^3$ group > 2.25 kg/m$^3$ group. Among which, the groups of 0.75 kg/m$^3$ and 1.25 kg/m$^3$ had the best growth effect. The 2.25 kg/m$^3$ group showed pronounced growth inhibitory effects; The final means of body weights were: (11.85 ± 0.93) g, and the highest FCR: FCR was 46.7%, $p = 0.032$. Although the growth index of the 0.25 kg/m$^3$ group was relatively higher compared with that of the other groups; However, it was significantly lower than that of the other three groups ($p < 0.05$). Based on correlation analysis, it was found that FCR had a highly negative correlation with SGR ($R^2 = 0.96$, $p < 0.001$); That is to say, as density increased, feed utilisation decreased; Therefore, growth inhibition may be directly caused by this decrease (Table 1). In terms of Survival Rate, There was no significant difference between Groups 0.25 kg/m$^3$, 0.75 kg/m$^3$ and 1.25 kg/m$^3$. All these were significantly better than 1.75 kg/m$^3$ and 2.25 kg/m$^3$ ($p = 0.003$). These results indicate that, for juvenile fish around 8.0 g, a stocking density of 0.75–1.25 kg/m$^3$ is most conducive to both optimal growth and high survival, and 0.25 kg/m$^3$ can also maintain high survival but with inferior growth performance.

**Table 2.** Effects of different stocking densities on the growth performance of *Micropterus salmoides* (Size Class 1).

| **Stocking Density** | **Initial Average Body Weight (g)** | **Final Average Body Weight (g)** | **Feed Conversion Ratio (FCR)** | **Survival Rate (SR, %)** |
|---|---|---|---|---|
| 0.25 kg·m$^{-3}$ | 8.06 ± 0.37 | 15.72 ± 1.34 $^b$ | 1.31 ± 0.05 $^b$ | 98.33 ± 1.67 $^c$ |
| 0.75 kg·m$^{-3}$ | 8.04 ± 0.46 | 17.25 ± 1.46 $^c$ | 1.21 ± 0.03 $^a$ | 97.50 ± 2.08 $^c$ |
| 1.25 kg·m$^{-3}$ | 7.92 ± 0.54 | 16.92 ± 1.31 $^c$ | 1.23 ± 0.04 $^a$ | 96.67 ± 2.31 $^c$ |
| 1.75 kg·m$^{-3}$ | 7.96 ± 0.39 | 13.56 ± 1.14 $^b$ | 1.65 ± 0.07 $^c$ | 90.00 ± 3.46 $^b$ |
| 2.25 kg·m$^{-3}$ | 8.08 ± 0.50 | 11.85 ± 0.93 $^a$ | 1.93 ± 0.09 $^d$ | 85.00 ± 4.08 $^a$ |

Note: Experimental subjects were 8.0 ± 0.5 g juvenile *Micropterus salmoides*, with a 30-day culture period and 3 biological replicates per group. Data are presented as mean ± standard deviation (mean ± SD). Different lowercase superscripts in the same column indicate significant differences among groups ($p < 0.05$, one-way ANOVA followed by Duncan's multiple range test).

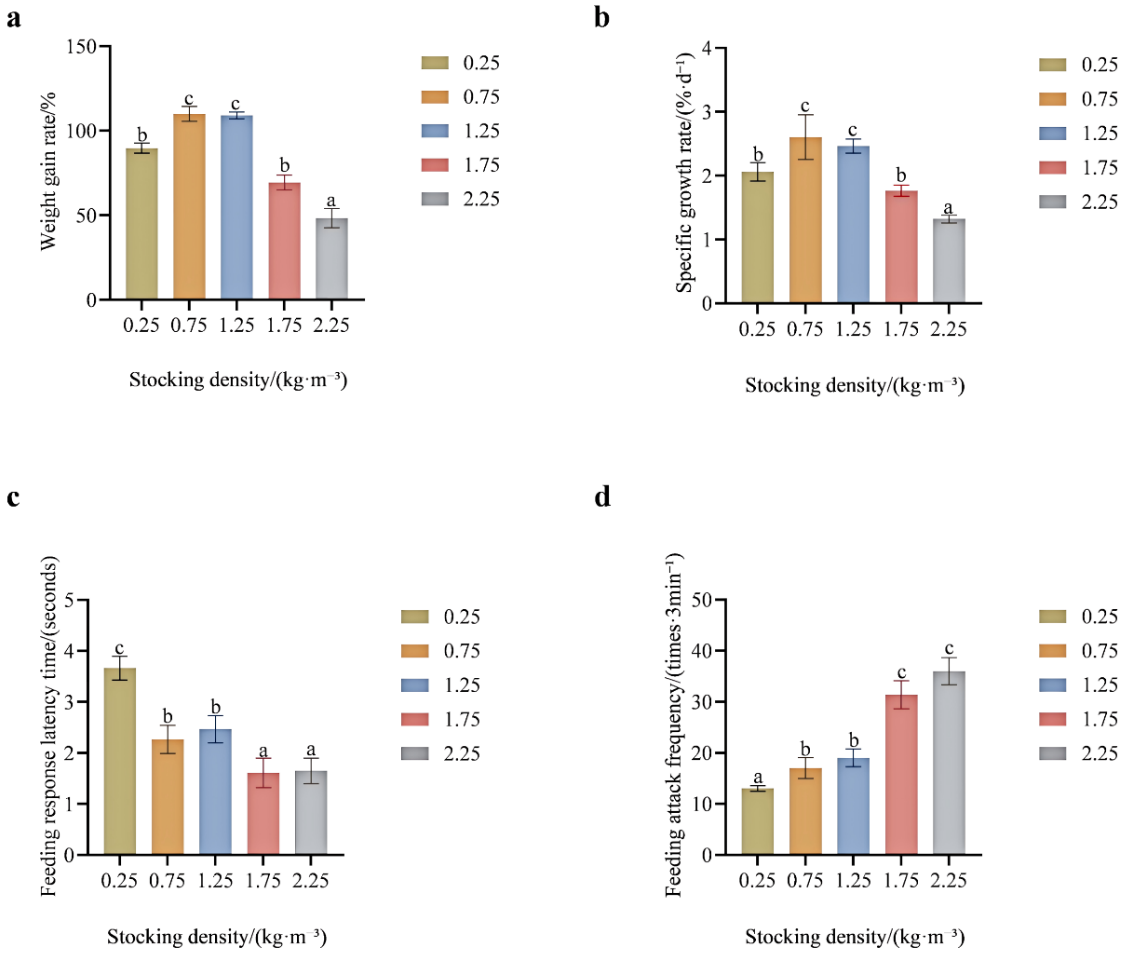

**Figure 1.** Effects of different stocking densities on the feeding and growth of *Micropterus salmoides*. Note: (**a**) Weight Gain Rate (WGR, %); (**b**) Specific Growth Rate (SGR, %/d); (**c**) Feeding response latency (s); (**d**) Number of aggressive feeding acts within the first 3 min (times·3 $\text{min}^{-1}$). Data are presented as mean ± SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among groups ($p < 0.05$, one-way ANOVA followed by Duncan's multiple range test).

### 3.1.2. Feeding Behavior: Changes in Response Latency and Aggressive Frequency

Two-way ANOVA revealed that the stocking density significantly influenced feeding response latency and the number of aggressive feeding behaviors (F = 38.62, df = 4, 20, $p < 0.001$) and there was also a substantial difference at this point due to variations in size (F = 12.75, df = 4, 20, $p < 0.001$). Observations of feeding behaviour (Figure 1) reveal the actual reasons for the differences in growth rates. Stocking density has both positive and negative effects on feeding response time (F = 22.47; df = 4.10, $p < 0.001$); The stocking densities of 1.75 kg/$\text{m}^3$ and 2.25 kg/$\text{m}^3$ were found to have the shortest latency (highest feeding tendency), whereas those of 0.25 kg/$\text{m}^3$ showed the longest delay (lowest feeding tendency) in relation to differences in animal body weight among different stocking densities.

At this time point, there was a notable increase in the number of aggressive feeding actions under high-density conditions; The result indicated that stocking density had an influence on aggressive feeding behavior (F = 108.53, $p < 0.001$). The number of aggressive acts in the 0.25 kg/$\text{m}^3$ group was 4.2 times that of the 0.75 kg/$\text{m}^3$ group, and correlation analysis showed that this indicator was significantly positively correlated with FCR ($R^2 = 0.93$, $p < 0.001$).

A moderate stocking density (0.75–1.25 kg/$\text{m}^3$), stimulating an active feeding behaviour but not causing intense inter-individual conflict. High stocking density causes fierce fighting among individuals, and a decrease in feed utilisation rate may be related to this problem.

### 3.1.3. Abnormal Swimming Behavior and Physical Injury

Two-way ANOVA confirmed that stocking density had an extremely significant main effect on swimming speed, swimming frequency and injury rate ($p < 0.001$), with a significant density × size interaction effect on all three indicators ($p = 0.002$). High-density farming induced significant abnormalities in swimming behavior (Figure 2).

The average swimming speed and frequency of the 0.25 kg/m$^3$ group were significantly higher than those of all other groups ($p$ < 0.01); it showed sustained stress-induced swimming activity; The 0.75 kg/m$^3$ group that achieved the best growth performance maintained a relatively stable swimming status. Correlation analysis showed that the number of aggressive feeding actions was significantly positively correlated with the average swimming speed ($R^2 = 0.92$, $p < 0.001$); therefore, intra-specific competition is the main cause of abnormal stress-induced swimming behavior in the present study.

With an increase in abnormal behaviour, the injury level of fish also increased accordingly at higher stocking densities ($p < 0.01$). The injury rate in the 0.25 kg/m$^3$ group was as high as 32.15%, which was sixfold higher than that of the 0.75 kg/m$^3$ group; there was a highly negative correlation between survival rates ($R^2 = 0.95$, $p < 0.001$).

High-density stress intensifies intraspecific conflict, leading to sustained stress-induced swimming and physical injury, which is the key reason for the decline of survival rate in high-density groups of size 1 fish.

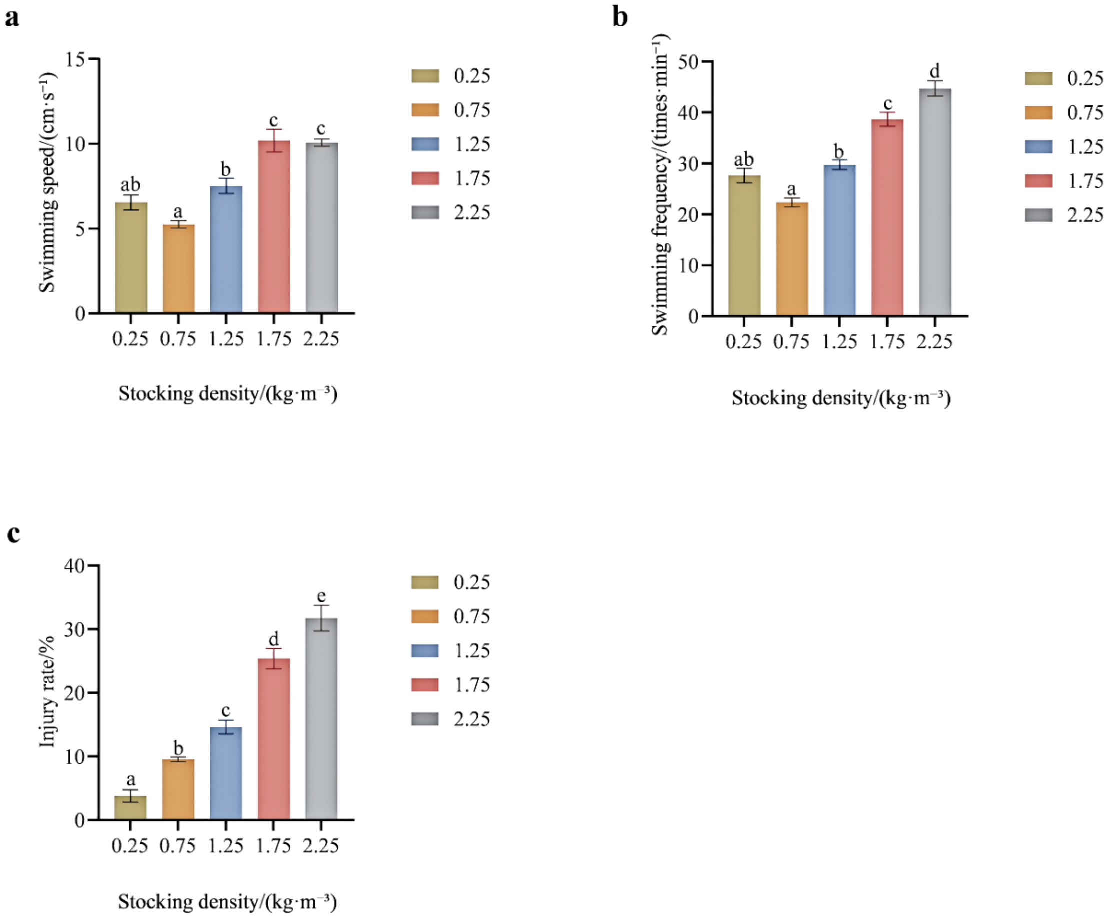

**Figure 2.** Effects of various stocking densities on the swimming behaviour of *Micropterus salmoides*. **(a)** Average swimming speed (cm/s); **(b)** Swimming frequency (acts/min); **(c)** Injury rate (%). Data are presented in the form of means ± S.D. ($n = 3$). Significance marking rules are the same as Figure 1.

### 3.1.4. Oxidative Stress Levels: Lipid Peroxidation and Antioxidant Defense

Two-way ANOVA revealed that there was a highly significant two-way main effect of stocking density on MDA levels, SOD activity and T-AOC ($p < 0.001$). However, the combination effects between size and stocking density were statistically non-significant for all parameters ($p = 0.003$); The stocking density changed significantly in the redox homeostasis of size 1 fish (Figure 3).

The MDA content, which reflects the severity of lipid hydroperoxyl damage, increased in parallel with stocking density ($p < 0.05$). The MDA value of the 2.25 kg/m$^3$ group was 2.1 times that of the other groups. Therefore, it showed the greatest extent of oxidation under a high-density condition.

Thus, the SOD activity and T-AOC showed an order as follows: 0.75 kg/m$^3$ group ≈ 1.25 kg/m$^3$ group > 0.25 kg/m$^3$ group > 1.75 kg/m$^3$ group ≈ 2.25 kg/m$^3$ group. The activities of SOD and T-AOC in the 1.75 and 2.25 kg/m$^3$ groups were lower compared to other levels; therefore, their antioxidant defense systems may be exhausted due to excessive consumption during prolonged high-load exercise.

Correlation analysis revealed a significant negative correlation between MDA contents and SGR ($R^2 = 0.91$, $p < 0.001$) as well as an increase in the coefficient of determination between MDA values and average swimming speeds ($R^2 = 0.89$, $p < 0.001$).

Long-term sustained high-density stress results in an overaccumulation of ROS and a weakened antioxidant defence system in size 1 fish; oxidative damage has become the main physiological mechanism connecting abnormal stress behaviour with growth suppression.

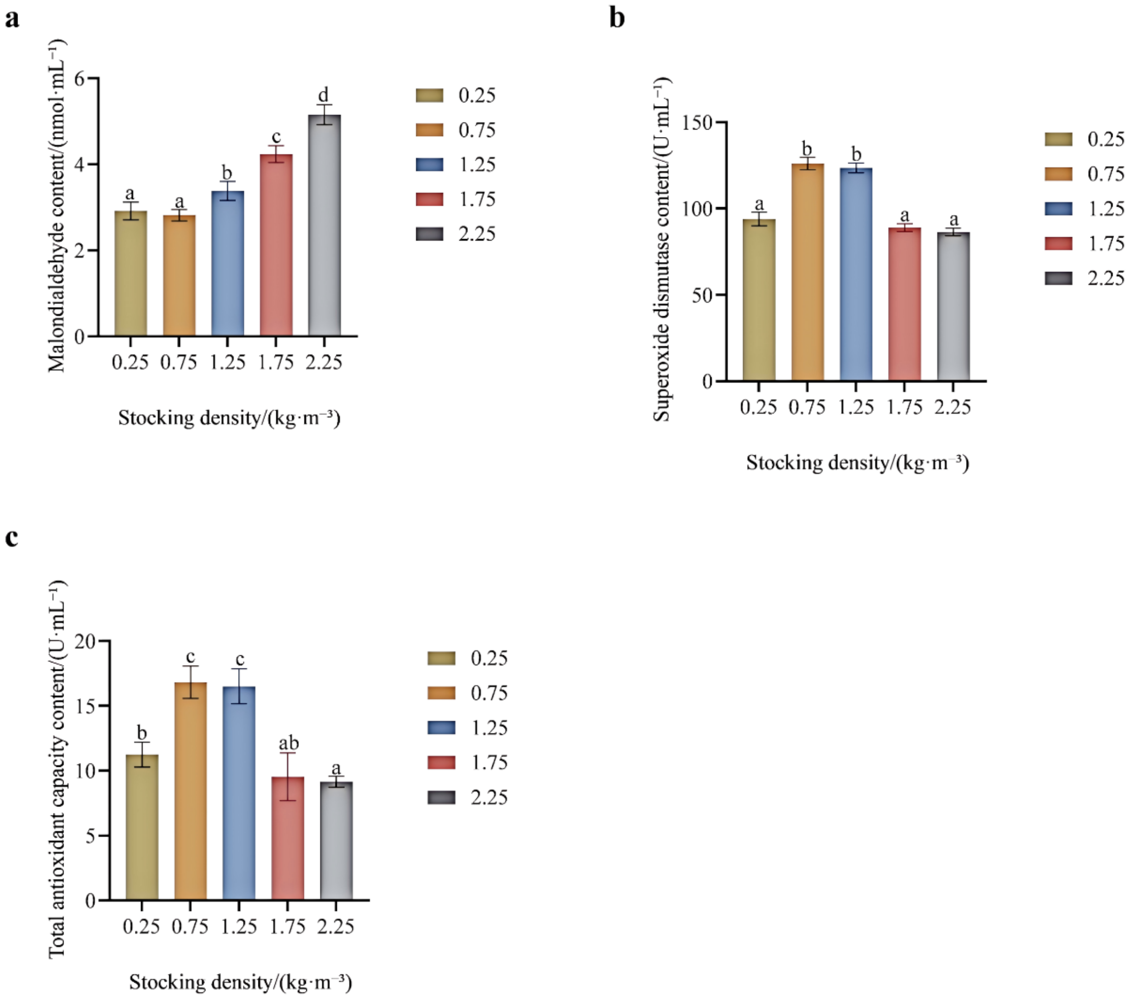

**Figure 3.** Effects of various stocking densities on the oxidative stress in *Micropterus salmoides*. **(a)** Malondialdehyde (MDA, nmol·mL$^{-1}$); **(b)** Superoxide dismutase (SOD, U·mL$^{-1}$); **(c)** Total antioxidant capacity (T-AOC, U·mL$^{-1}$). All indicator detection reagents were purchased from Nanjing Jiancheng Bioengineering Institute of China. The means ± SD of the data are presented ($n = 6$; 6 fish randomly chosen from each replicate): Significance marking rules are the same as Figure 1.

### 3.1.5. Dynamic Response of Aquaculture Water Quality Parameters

Two-way ANOVA showed that there was an extremely high probability of the effect of stocking density on all water quality indices ($p < 0.001$); A significant three-way density × size interaction occurred for peaks in $NH_4^+$-N, $NO_2^-$-N and minimum DO. Stocking Density had a significant impact on Water quality in cultured systems (Figure 4).

There was a significant increase in the peaks of $NH_4^+$-N and NO2-N at high stocking densities ($p < 0.001$). The maximum value of $NH_4^+$-N for the 2.25 kg/m$^3$ treatment was 3.4 times higher than that of the 0.75 kg/m$^3$ treatment. The lowest DO concentration reached almost at the limit of safety (6.5 mg·L$^{-1}$) established in this study.

The peak NH4$^+$-N concentration showed a high positive correlation with MDA contents. Therefore, it can be inferred that poor water quality has promoted physiological oxidation stress in fish due to increased pollutant accumulation.

High-dense culture accelerates the formation of metabolic products, causing a deterioration in water quality; at this time, both overcrowding and stress caused by it occur simultaneously.

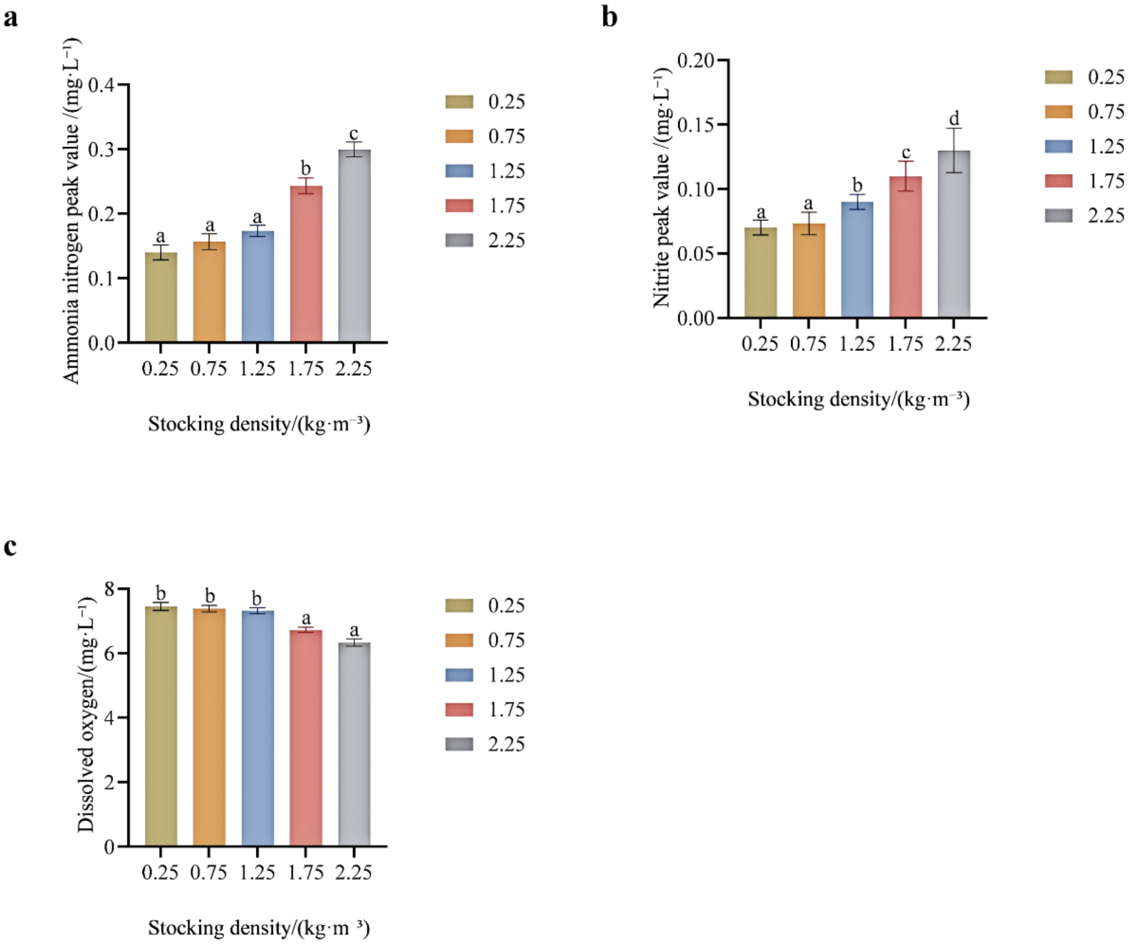

**Figure 4.** Effects of different stocking densities on the culture water quality of *Micropterus salmoides*. Note: (**a**) Peak ammonium nitrogen ($NH_4^+$-N, mg·$L^{-1}$); (**b**) Peak nitrite nitrogen ($NO_2^-$-N, mg·$L^{-1}$); (**c**) Minimum dissolved oxygen (DO, mg·$L^{-1}$). Data are presented as mean ± SD ($n = 3$). Significance marking rules are the same as Figure 1.

### *3.2. Effects of Different Stocking Densities on Size Class 2 (50 ± 3.2) g Largemouth Bass*

#### 3.2.1. Density-Gradient Inhibition Effects on Growth Performance

For size class 2, it is found that with increasing fish body weight, the adverse effect of stocking density on growth increases further. All growth performance indices, such as the final mean body weight (Table 3); Weight increase rate curve: (Figure 5a), specific growth rate (Figure 5b), and feed conversion ratio (Table 3) had an obvious descending trend under different stocking densities in this study ($p < 0.01$). Among them, the 0.75 kg/$m^3$ group had the highest weight increase rate and special development speed, as well as a low feed utilization efficiency ($p < 0.001$). This represents that it reached its maximum growth performance. At a Density of 1.25 kg·$m^{-3}$, there was a marked drop in growth performance ($p < 0.008$). Among them, there is a significant growth inhibition in the densest group at 2.25 kg·$m^{-3}$ and survival rates dropped markedly as density increased ($p < 0.01$). Similar to size class 1, the survival rate of this group was significantly higher than that of other groups. However, it had a final body-weight mean value, weight gain rate, specific growth rate were respectively smaller than those of the 0.75 kg/$m^3$ group at $p = 0.017$ indicated an excessively low density is not conducive to growth. In comparison with size 1, the growth performance of size 2 at 1.25 kg/$m^3$ was already somewhat weaker than that of the 0.75 kg/$m^3$ group ($p = 0.008$). It can be inferred from this that there is a weak correlation between size classes and high-density tolerance. The range of optimal density for growth differs between different groups. Collectively, these results demonstrate that as individual size increases, largemouth bass becomes more sensitive to stocking density, the density window suitable for growth narrows, and both excessively high and low densities constrain their growth performance.

**Table 3.** Effects of different stocking densities on the growth performance of *Micropterus salmoides* (Size Class 2).

| Stocking Density | Initial Average Body Weight (g) | Final Average Body Weight (g) | Feed Conversion Ratio (FCR) | Survival Rate (SR, %) |
|---|---|---|---|---|
| 0.25 $kg \cdot m^{-3}$ | 50.60 ± 1.23 | 84.21 ± 2.05 $^c$ | 1.38 ± 0.05 $^b$ | 96.67 ± 1.67 $^a$ |
| 0.75 $kg \cdot m^{-3}$ | 50.21 ± 2.76 | 105.62 ± 5.83 $^d$ | 1.29 ± 0.04 $^a$ | 95.00 ± 2.89 $^{a,b}$ |
| 1.25 $kg \cdot m^{-3}$ | 50.82 ± 2.17 | 86.86 ± 4.51 $^c$ | 1.42 ± 0.08 $^b$ | 90.00 ± 3.61 $^b$ |
| 1.75 $kg \cdot m^{-3}$ | 49.91 ± 2.18 | 78.53 ± 4.21 $^b$ | 2.01 ± 0.10 $^c$ | 83.33 ± 4.17 $^c$ |
| 2.25 $kg \cdot m^{-3}$ | 50.00 ± 3.03 | 69.38 ± 3.85 $^a$ | 2.35 ± 0.12 $^d$ | 76.67 ± 4.76 $^c$ |

Note: Experimental subjects were 50 ± 3.2 g juvenile *Micropterus salmoides*, with a 30-day culture period and 3 biological replicates per group. Data are presented as mean ± SD. Values within the same column with different lowercase superscripts (a–d) indicate statistically significant differences ($p < 0.05$), while values with the same superscript indicate no significant difference. Statistical analysis and significance marking rules are the same as Table 2.

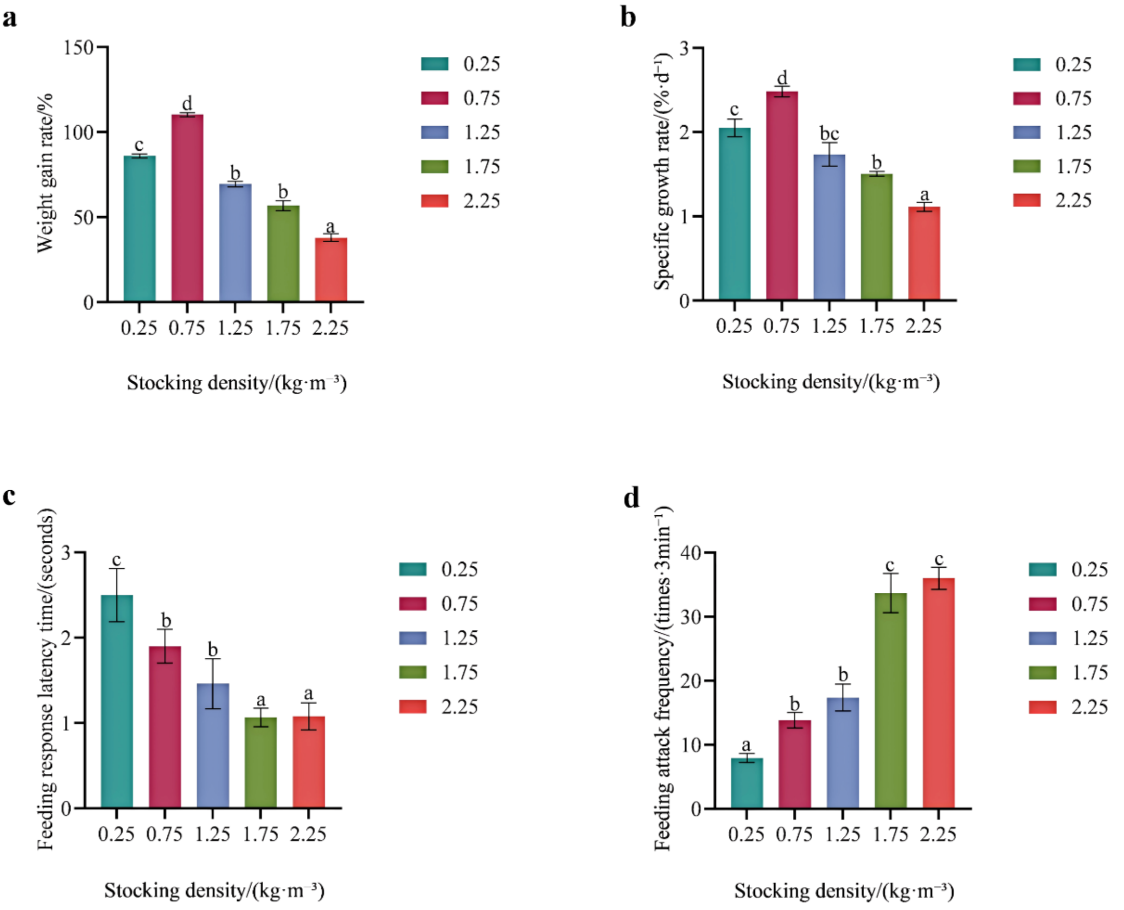

**Figure 5.** Effects of different stocking densities on the feeding and growth of *Micropterus salmoides*. Note: (**a**) Weight Gain Rate (WGR, %); (**b**) Specific Growth Rate (SGR, %/d); (**c**) Feeding response latency (s); (**d**) Number of aggressive feeding acts within the first 3 min (times·3 $min^{-1}$). Data are presented as mean ± SD ($n = 3$). Significance marking rules are the same as Figure 1.

### 3.2.2. Intensified Feeding Competition and Bipolarization of Feed Intake

Two-way analysis of variance indicated a significant density × size interaction effect on the feeding response latency and number of aggressive feeding acts ($p < 0.001$); The feeding behaviour response to density stress differed significantly between the two size groups. When fish size and stocking densities increased, feeding behaviour became more disorderly (Figure 5).

The time it took for pigs to eat in kilograms after being fed varied significantly between groups 1.75 $kg/m^3$, 2.25 $kg/m^3$ and others ($p < 0.001$) (Figure 5c). The frequency of aggressive feeding behavior during the first three minutes was significantly greater for the 1.75 $kg/m^3$ and 2.25 $kg/m^3$ groups than those in other groups ($p < 0.001$) (Figure 5d).

The larger individuals of *M. salmoides* exhibit stronger aggression during feeding at a higher population density, therefore, this increased level of aggressiveness causes greater feeding hierarchies disorder among these smaller-size fish species.

### 3.2.3. Stress-Induced Swimming Activity and Escalated Injury Severity

Two-way ANOVA showed a significant interaction effect of density × size on the swimming speed, swimming frequency and injury rates ($p = 0.002$). It was found that larger-size fishes were more responsive to density stress behaviourally (Figure 6). The average swimming speed and frequency of the 1.75 kg/m$^3$ group, as well as that of the 2.25 kg/m$^3$ group, were all greater than those of other groups. Therefore, they showed a high level of stress (Figure 6a,b). A sharp rise in the number of injuries occurred simultaneously (Figure 6c). Based on these results, we can determine that there are more behavioural conflict and stress-related physiological costs when the density of organisms in a community is relatively high.

Large people are more likely to be injured due to physiological energy loss from a stressed state during swimming at a high-density level. Therefore, their mortality risk has dropped significantly.

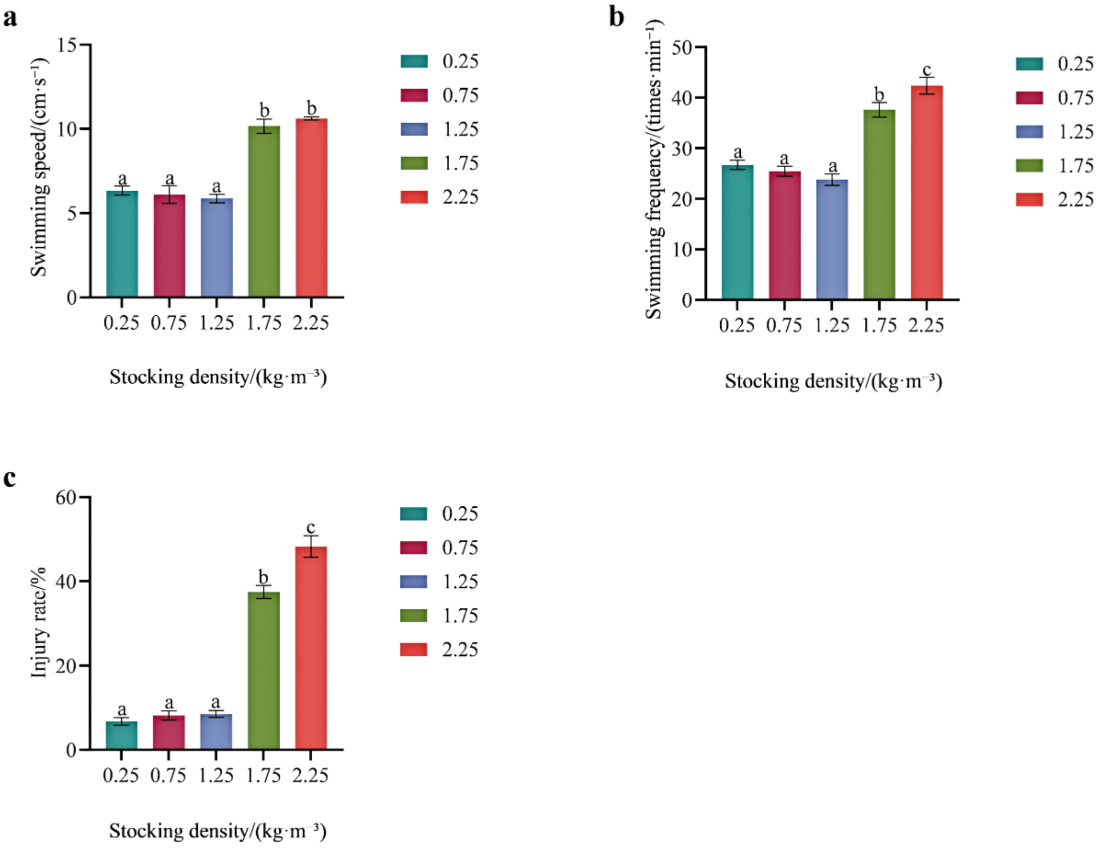

**Figure 6.** Effects of different stocking densities on the swimming behavior of *Micropterus salmoides.* Note: (**a**) Average swimming speed (cm·s$^{-1}$); (**b**) Swimming frequency (acts·min$^{-1}$); (**c**) Injury rate (%). Data are presented as mean ± SD ($n = 3$). Significance marking rules are the same as Figure 1.

### 3.2.4. Aggravated Oxidative Damage and Breakdown of the Antioxidant System

Two-way ANOVA revealed a significant density × size interaction effect on MDA content, SOD activity, and T-AOC ($p = 0.003$). Therefore, it was found that the extent of oxidative damage induced by increased concentration per unit volume in two groups was more severe than in one group (Figure 7). MDA contents in the 1.75 kg/m$^3$ and 2.25 kg/m$^3$ groups reached levels significantly higher than those of other groups ($p < 0.001$). Thus, there was a highly serious oxidative stress (Figure 7a). Concurrently, their antioxidant defense system was under greater pressure. Both SOD activity and T-AOC in the 1.75 kg·m$^{-3}$ and 2.25 kg·m$^{-3}$ groups were significantly downregulated compared to the other groups ($p < 0.001$) (Figure 7b,c).

Correlation analysis showed that MDA content was significantly negatively correlated with SGR ($R^2 = 0.94$, $p < 0.001$), with a higher correlation coefficient than that in size 1 fish.

Aggravated oxidation damage under high-density stress is the central physiological basis that leads to the greater sensitivity of large-sized *M. salmoides* in stocking density tests.

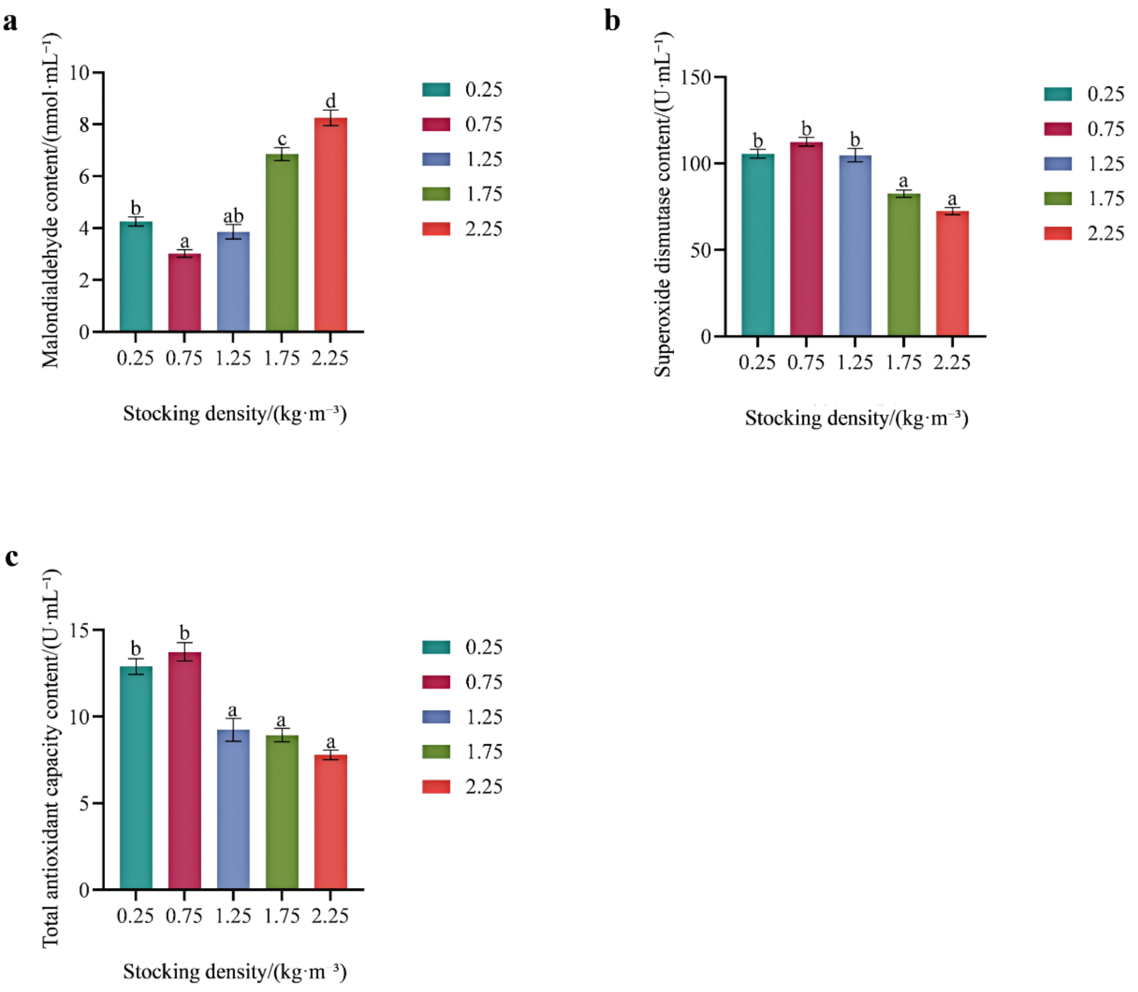

**Figure 7.** Effects of different stocking densities on the oxidative stress of *Micropterus salmoides*. Note: (**a**) Malondialdehyde (MDA, nmol·mL$^{-1}$); (**b**) Superoxide Dismutase (SOD, U·mL$^{-1}$); (**c**) Total Antioxidant Capacity (T-AOC, U·mL$^{-1}$). Data are presented as mean ± SD ($n = 6$, 6 fish randomly sampled per replicate). Significance marking rules are the same as Figure 1.

### 3.2.5. Multiplied Water Quality Pressure: Metabolic Load and Oxygen Consumption

Two-way ANOVA confirmed a significant density × size interaction effect on all water quality indicators ($p = 0.012$), indicating that under the same density gradient, the impact of size 2 fish culture on water quality was more significant than that of size 1 fish (Figure 8). Ammonium nitrogen and nitrite nitrogen at the maximum values in the 2.25 kg/m$^3$ group exceeded that of the same level in the first phase (Figure 8a,b). Due to an increase in the individual's oxygen consumption, the lowest concentration of dissolved oxygen reached below 6.5 mg/L in both the 1.75 kg/m$^3$ and 2.25 kg/m$^3$ groups (Figure 8c). Water quality in the other groups was still more stable. As the body mass of fish increases, the quantity and amount discharged at once also rise accordingly.

Correlation analysis revealed that the peak of $NH_4^+$-N concentration was highly positively correlated with MDA contents ($R^2 = 0.95$, $p < 0.001$). It can be inferred from this that greater worsening of water quality in large-scale fish farming has led to a stronger oxidative stress response.

The larger body size of people leads to greater oxygen expenditure during metabolism. When stocked at high densities under these conditions, the biological stress becomes more serious, thereby accelerating eutrophication.

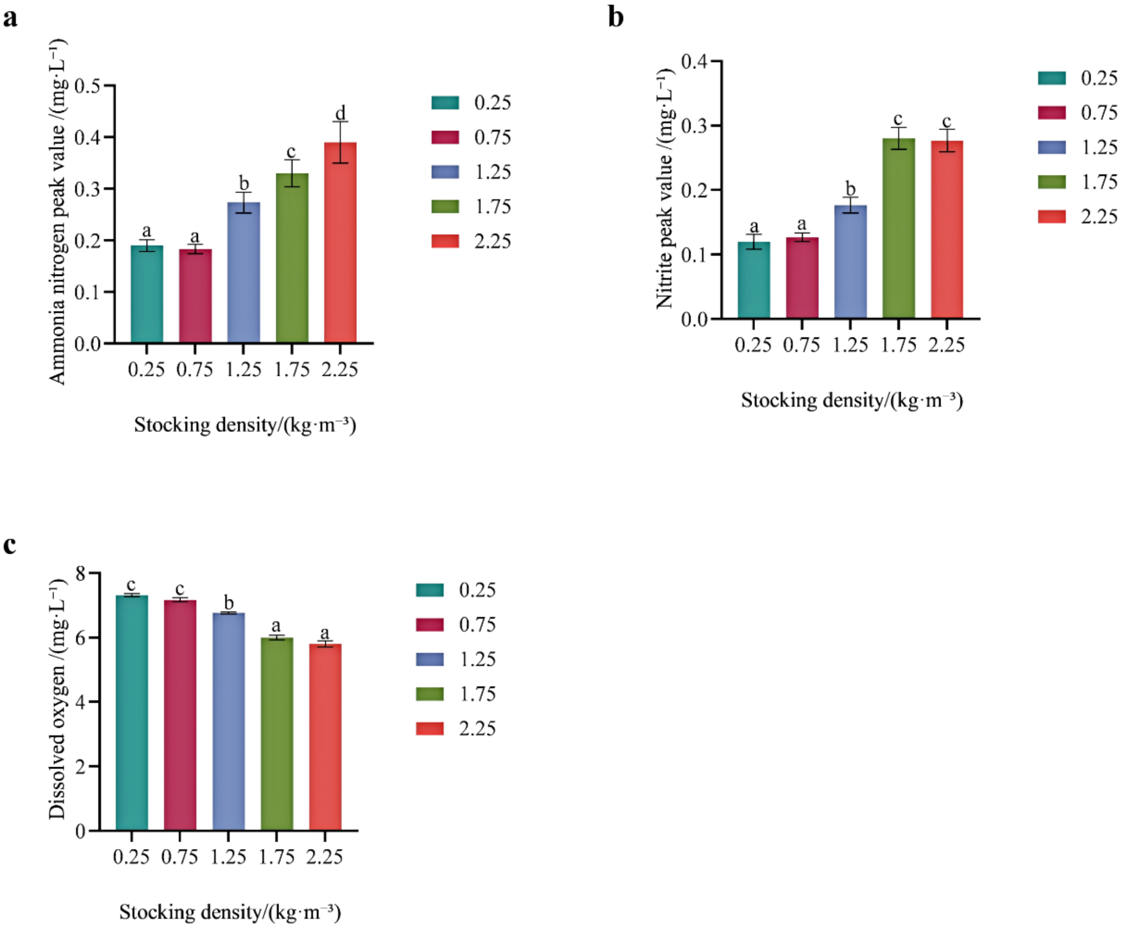

**Figure 8.** Effects of different stocking densities on the culture water quality of *Micropterus salmoides*. Note: (**a**) Peak ammonium nitrogen ($NH_4^+$-N, mg·L$^{-1}$); (**b**) Peak nitrite nitrogen ($NO_2^-$-N, mg·L$^{-1}$); (**c**) Minimum dissolved oxygen (DO, mg·L$^{-1}$). Data are presented as mean ± SD ($n = 3$). Significance marking rules are the same as Figure 1.

## 4. Discussion

Through a systematic evaluation of the impacts of stocking density on growth performance, behaviour expression, oxidation stress and aquaculture water quality for two-size groups of large-mouth bass during culture experiments. The core physio-ecological pathway for density stress is as follows: Spatial competition → behavioural disorders → Oxidative injury → Growth suppression; Confirming a substantial size dependence among the impacts described above.

### *4.1. Regulatory Mechanisms of Stocking Density on Growth Performance and Size-Dependent Differences*

Both the small and large size groups of this research show that at a moderate density, largemouth bass have achieved the best growth effect. Excessively high or low Densities will negatively impact growth [32,33]. Two-way ANOVA results further confirm a significant interaction between stocking density and fish size on all core growth indicators ($p < 0.01$), clarifies the significant size-dependent nature of density effects. For Size Class 1, densities of 0.75 kg·m$^{-3}$ and 1.25 kg·m$^{-3}$ were identified as suitable for (8.0 ± 0.5) g largemouth bass. The result is consistent with that of Ni Jinjin et al. (2020), showing that a moderate initial density has a positive effect on increasing the specific growth rate and weight gain rate for juvenile largemouth bass in pond-recirculating systems [34]. At appropriate density levels, there is moderate inter-fish competition for space, feeding distribution is relatively even, more resources are available for fish growth than stress response reactions. Thus effectively increasing feed utilization efficiency [35]. In contrast, the 0.25 kg·m$^{-3}$ group exhibited significantly lower growth indices than the 0.75 kg·m$^{-3}$ and 1.25 kg·m$^{-3}$ groups, primarily due to insufficient shoaling effect, prolonged feeding response latency, and reduced feeding motivation [36], which is further supported by the mildly elevated oxidative stress level in this group, indicating mild social deprivation stress. The high-density groups (1.75 kg/m$^3$ and 2.25 kg/m$^3$) exhibited significant growth inhibition due to the imbalanced distribution of energy under crowding stress, that is, more resources were allocated for coping with stress rather than promoting development [37,38].

In comparison to the size class 1 results, the sensibility of (50 ± 3.2) g large-mouth bass at a high-density level is higher than before. A density of 1.25 kg/m$^3$ already led to a considerable reduction in the weight gain rate. In line with the finding of Zhang Qi (2022) that stocking density exceeds a certain level has a negative impact on the growth of largemouth bass over 40 g in size, this also aligns with it. Mainly due to an increase in both space demand and metabolism of growing larger, there is a difference among sizes. Therefore, the pressure per unit volume of water increases accordingly, reducing its stress resistance. Second, large-sized individuals require more energy for breathing during their activities compared to small ones because they generate greater metabolic waste products such as $CO_2$ and $H_2O$ in a short period when crowdedness affects them. Together with crowding stress, these factors will be even stronger against growth [39]. Therefore, under the same density conditions, larger individuals have shown a stronger response to spatial competition.

### *4.2. Coordinated Changes in Feeding and Swimming Behaviors: From Behavioral Phenotypes to Growth Impact*

Behaviour can be seen as an immediate response of animals in the environment. Thus, it constitutes one end point for building up the core-density stress model we propose. The feeding and swimming behavior responses under density-induced stress were highly synchronized. The results of two-way ANOVA show that there is a significant density and Size interaction effect in all behavioural indices ($p < 0.001$). Feeding behaviour, swimming behaviour and injury rate are not directly associated with the growth performance indices in this study, rather, they serve as essential supporting factors for evaluating the behavioural state of fish and their welfare conditions. The two-way ANOVA shows that there is a significant density × size interaction effect on all behavioural indices ($p < 0.001$) and this is consistent with the scale-sensitive pattern in growth performance. At a suitable stocking density, the fish exhibited strong feeding drive due to sufficient space and few competitors. Therefore, they could gain better conditions for growth. Under high-density stress, the behavioural pattern of the fish changed systematically. On the one hand, there was an intensification of feeding competition, accompanied by a considerable amount of aggressive behaviour, resulting in disorderly establishment of feeding order and severe differences in feed intake among individuals. On the other hand, spatial crowding caused persistent stress-related swimming behaviour, which appeared as an abnormal increase in the amplitude of swimming speed and times.

Collectively leading to considerable waste of energy: intense feeding contests themselves consume energy resources, long-term continuous anxiety-inducing swimming activities increase the baseline metabolic cost [40–42]. Furthermore, behaviour-based conflicts may result in physical violence immediately. A higher injury rate exacerbates the problem of poor health and insufficient food intake. Thus, it forms a vicious circle “behavioral stress to energy dissipation to physical damage to growth restriction”. Therefore, the growth inhibition triggered by density stress is not simply because of lack of food. It is not due to these abnormal behaviours but rather because they reduce the available bioenergy resources for plant development at one time [41,43].

### *4.3. Oxidative Stress: The Pivotal Link Between Behavioral Stress and Physiological Damage*

The change of the oxidation and anti-oxidation balance system presents a direct physiological proof to verify this stress effects-chain mode: as an essential link among behaviours abnormality at higher levels triggering growth inhibition via oxidative-antioxidative balance systems within particular pathways. Two-way ANOVA results showed that stocking density, fish size, and their interactions had very high degrees of significance for all oxidative stress parameters ($p < 0.01$). This result is consistent with the coordinated change trend of behaviour and growth performance. A relatively higher level of MDA in the high-density farming group indicates lipid peroxidative injury and suggests that the cellular membrane systems of the fish have been attacked by ROS [44]. Furthermore, in response to particularly stressful environments, there is often a reduction in activities of the superoxide dismutase enzyme and overall antioxidants have decreased levels [45,46]. In this situation, there is a combination of enhanced oxidation and weakened antioxidants to demonstrate that under-stress conditions have exceeded the regulation range for fish’s physiological homeostasis.

A close connection exists among the generations of oxidant-stress, behaviour-related stress, and aquatic ecosystem pollution impacts. Firstly, prolonged behavioural stress triggers the activation of the neuroendocrine system to enhance the production of stress-related hormones (such as cortisol), thereby causing changes in metabolic pathways and excessively generating ROS [29,46]. Secondly, the degradation of water quality in conjunction with increased stocking density acts as an external reason to cause oxidative stress directly [35]. Therefore, oxidative stress results from the combined effects of behavioral abnormalities and water quality degradation, representing a core pathophysiological factor leading to tissue damage, immunosuppression, and ultimately reduced growth performance. The mildly elevated oxidative stress level observed in the 0.25 kg·m$^{-3}$ group suggests that the social deprivation caused by excessively low density may also act as a stressor, impairing physiological homeostasis. This aligns with studies reporting higher basal stress levels in certain fish species under isolated rearing conditions [36].

### *4.4. Interplay Among Density, Water Quality, and Physiology*

One of the primary managed influencing factors in changes across aquatic farm systems, sticking density. High-density directly increases the metabolism of a given water volume, accelerate the use rate of oxygen, exceed the self-purification ability and balanced state of dissolved oxygen in the water body quickly, causing poor water quality. Water quality degradation not only toxic to fish but also amplifies its synergistic effect with spatial crowding stress on behaviour and oxidative stress responses. According to our findings, there exists a significant positive correlation between the maximum value of ammonia-nitrogen and serum MDA levels (Size 1: $R^2 = 0.93$, $p < 0.001$; Size 2: $R^2 = 0.95$, $p < 0.001$). Thus we can confirm that this combined stress phenomenon does indeed exist. Water pollution causes an increase in oxidation stress mainly because of these two mechanisms: first, ammonia nitrogen and nitrite nitrogen, the main toxic metabolites in aquaculture water, can be absorbed into the fish through the gills, directly inducing the generation of ROS in tissues and inhibiting the activity of antioxidant enzymes; second, the decrease of dissolved oxygen in high-density groups leads to hypoxic stress, which not only impairs mitochondrial oxidative phosphorylation and increases electron leakage, but also activates the HPI axis and further promotes cortisol secretion, forming a synergistic effect with crowding stress.

To establish the following negative feedback loop: High density intensity leads to more intense behavioural competition, deteriorating water quality, subsequently causes oxidative stress and physiological damage. Therefore, stress resistance and metabolism are weakened. As a result, it becomes more sensitive to crowding and poor environmental conditions of water quality. Therefore, it needs to be kept within this range for maintaining system stability. In such cases, Fish exhibit their normal behaviours; there has been no change to a level that exceeds the environmental carrying capacity of water. Water quality can be maintained for a long time to support the normal functioning of fish in this condition.

## **5. Conclusions**

Based on a systematic analysis of stocking density’s overall impacts on growth, behaviour, physiology, and water quality in largemouth bass at two sizes through aquacultural experimentations. To explore its own underlying mechanism of density-stress and determine the most reasonable stocking densities under various size conditions. The following are the primary results:

1. The growth performance of largemouth bass exhibited a significant size-dependent density effect, and the corresponding suitable density parameters were identified. The survival rates of 0.25 and 0.75 $kg\cdot m^{-3}$ groups were not significantly different and were significantly higher than those of high-density groups. The impact of stocking density on its growth followed the pattern of “optimal at moderate levels, with both excessively high and low levels being inhibitory,” a trend that varied distinctly depending on fish size. For largemouth bass weighing 8.0 ± 0.5 g, the most suitable stocking density range was 0.75–1.25 $kg\cdot m^{-3}$, which could achieve a balance between optimal growth and high survival. For largemouth bass weighing 50 ± 3.2 g, a stocking density of 0.75 $kg\cdot m^{-3}$ is recommended, which could obtain the best growth performance while maintaining high survival, and exceeding this threshold leads to a significant decline in both growth and survival.
2. This study reveals the core physio-ecological pathway of density stress: spatial competition triggers behavioral abnormalities, which subsequently lead to oxidative damage and ultimately suppress growth. When stocking density exceeds the suitable range, compressed living space first intensifies feeding competition and stress-induced swimming, prompting a shift in energy allocation from growth to stress-related behaviors. The dysregulation at the behavioral level further induces physiological oxidative stress, ultimately resulting in bodily damage and growth limitation.
3. The study substantiates a negative feedback coupling between density stress and water quality deterioration. High-density farming directly increases metabolic load and depletes dissolved oxygen, leading to water quality degradation. Deteriorated water quality not only acts as an exogenous stressor directly inducing oxidative stress but also synergizes with crowding stress to exacerbate behavioral abnormalities and physiological damage, thereby forming a vicious cycle of “high density → behavioral and water quality deterioration → oxidative damage → reduced tolerance.” Therefore, maintaining density within an optimal range is a fundamental key to preserving system homeostasis and achieving healthy aquaculture.

## Author Contributions

Conceptualization, Z.N.; methodology, Y.X. and K.Z.; validation, J.W. and D.Z.; formal analysis, Y.R. and Z.H.; investigation, J.W.; resources, Z.H.; writing—original draft preparation, Y.X. and K.Z.; writing—review and editing, Z.N.; visualization, Y.R.; supervision, D.Z.; project administration, Z.H.; funding acquisition, Z.N. All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

All experimental procedures were approved by the Animal Research Ethics Committee of Tarim University (Approval No. PB20260408001) and complied with the guidelines for the use of experimental animals in China.

## Informed Consent Statement

Not applicable.

## Data Availability Statement

The raw data that support the findings of this study are available from the corresponding author upon reasonable request. No data are restricted by privacy, ethical or commercial proprietary concerns.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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