fishes

MDPI

Article

Effects of Subacute Ammonia Nitrogen Stress on the Growth, Antioxidant Capability, and Immunity of Blunt Snout Bream (*Megalobrama amblycephala*) Juveniles

Xiangyang Yuan, Qian Wang, Mengyang Dai, Xinyu Xiong, Hengjie Wang and Canli Wang *

School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an 223003, China; xiangyangyuan007@163.com (X.Y.)
* Correspondence: clwang5@163.com

Abstract: To investigate the effects of subacute ammonia nitrogen stress on the growth, antioxidant capacity, and innate immunity of juvenile blunt snout bream (*Megalobrama ambrycyphala*), a sample of fish (average body weight 6.55 ± 0.24 g) was divided into three groups: a control group (G1), a 10% LC_{50} ammonia nitrogen stress group (G2), and a 20% LC_{50} ammonia nitrogen stress group (G3). The fish in G1 were reared in tap water with aeration. The fish in G2 were reared in water with 6.82 mg/L ammonium chloride at 10% LC_{50}. The fish in G3 were reared in water with 13.64 mg/L ammonium chloride at 20% LC_{50}. The feeding period was 4 weeks. Then, the growth, antioxidant, and immune response parameters of the fish were analyzed. The results showed that the median lethal concentration (LC_{50}) of the ammonia nitrogen solution for blunt snout bream juveniles at 96 h was 68.18 mg/L. The final body weight, weight gain rate, specific growth rate (SGR), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), albumin, globulin, acid phosphatase (ACP), and alkaline phosphatase (AKP) of fish in the stress groups were significantly lower than in the control group ($p < 0.05$); however, the MDA content of fish in the control group was significantly lower than that of those in the stress groups ($p < 0.05$). Furthermore, the relative expression levels of the *GHRa*, *GHRb*, *IGF1*, and *IGF2* genes in the muscles of fish in the stress groups were significantly downregulated compared to the control group ($p < 0.05$). The relative expression levels of the *SOD*, *CAT*, *Leap1*, and *Leap2* genes in the liver of fish in the stress groups were significantly lower than those in fish in the control group ($p < 0.05$). In conclusion, subacute ammonia nitrogen stress inhibited growth performance and decreased the antioxidant capacity and immunity of blunt snout bream.

Keywords: subacute ammonia nitrogen stress; growth; antioxidant capacity; immunity; *Megalobrama amblycephala*

Key Contribution: Subacute ammonia nitrogen stress decreased the growth, antioxidant capacity, and immunity of fish.

check for updates

Citation: Yuan, X.; Wang, Q.; Dai, M.; Xiong, X.; Wang, H.; Wang, C. Effects of Subacute Ammonia Nitrogen Stress on the Growth, Antioxidant Capability, and Immunity of Blunt Snout Bream (*Megalobrama amblycephala*) Juveniles. *Fishes* **2024**, *9*, 502. https://doi.org/10.3390/fishes9120502

Academic Editor: Lluís Tort

Received: 19 October 2024
Revised: 28 November 2024
Accepted: 3 December 2024
Published: 8 December 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Given that fish are lower aquatic vertebrates, the growth and excretions of fish are influenced by the water environment, such as ammonia nitrogen, heavy metals, dissolved oxygen, temperature, and other environmental factors. Ammonia nitrogen, which is one of the main stress factors in the water environment, is a decomposition product of organic matter, including excrement, farm animal bait, and dead bodies, and is present in two forms: non-ionic (NH_3) and ionized ammonia (NH_4^+) [1]. The increasing concentration of non-ionic ammonia in water can inhibit the excretion of ammonia nitrogen in the body and lead to increasing ammonia in the blood and other tissues of fish, consequently disrupting the normal metabolism of blood. Furthermore, non-ionic ammonia without electric charge has liposolubility, easily fuses with the phospholipid bilayer of cells, and produces toxic

effects [2]. Many aquatic organisms, including fish, are very sensitive to concentrations of ammonia nitrogen [3]. Some previous studies have pointed out that ammonia nitrogen not only leads to damage to the gills and brain, leading to cell enlargement, nuclear deviation, dissolution, and vacuolization in various species of fish, such as gilthead seabream (*Sparus aurata*), mud crab (*Scylla paramamosain*), and rainbow trout (*Salmo gairdneri*) [4–6], but also results in decreased antioxidant enzyme activities in fish such as loach (*Paramisgurnus dabryanus*), mullet (*Mugil cephalus*), and black carp (*Mylopharyngodon piceus*) [7–9], as well as causes a series of inflammatory reactions in species such as loach and turbot (*Scophthalmus maximus*) [7,10]. However, studies on the alteration of antioxidation and immunity in fish exposed to ammonia nitrogen stress are still limited.

A previous study reported that ammonia can inhibit the growth of fish [11]. For instance, the final weight of rabbitfish (*Siganus rivulatus*) decreased with increased ammonia concentration [12]. The growth hormone (GH)–insulin-like growth factor (IGF) axis plays an important role in the endocrine regulation of vertebrate growth [13]. Briefly, the GH released by the pituitary gland is first identified and combined with the growth hormone receptor (GHR) on the hepatic cell membrane. And then, the GHR binds to GH and transfers the biological signals to the hepatic IGF-synthesizing machinery. Finally, the liver synthesizes and secretes the IGF, which acts on particular cells to regulate the growth of fish. Recently, some researchers have found that external environment stressors could affect the growth function through interference with the GH/IGF axis [14]. Up to now, there has been poor information on ammonia stress interfering with the GH/IGF axis.

Fish are exposed to different stresses in the water environment during the feeding period. In particular, when body tissue suffers ammonia nitrogen stress, abnormal oxidizing reactions happen in the aerobic metabolic pathway by virtue of the sudden lack of oxygen, which leads to an intensive accumulation of oxygen in the body and an increase in stress to the antioxidant system [15]. Reactive oxygen species (ROS), which include superoxide anions ($\cdot O^{2-}$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2), are generally generated from aerobic organisms during normal oxidative metabolism [16]. The antioxidant defense system in fish consists of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione, and other scavengers [17]. This defense system plays an important role in scavenging ROS. If the balance between the generation and removal of ROS is broken, the increase in ROS causes oxidative stress, which will make fish more prone to diseases [18]. Generally, cultured fish exposed to ammonia nitrogen easily generate oxidative stress. Therefore, research regarding changes in antioxidant enzymes and innate immunity is necessary.

The blunt snout bream (*Megalobrama amblycephala*), which is mainly a cultivated species, is an economically important herbivorous fish in China [19]. It is estimated that the yield of blunt snout bream in 2023 in China was approximately 0.74 million tons (*China Fishery Statistical Yearbook*, Fishery Bureau, Ministry of Agriculture, China). To date, studies regarding ammonia nitrogen stress have focused on acute ammonia nitrogen stress and its recovery. However, information on ammonia nitrogen stress in aquaculture is still lacking. Therefore, taking juvenile blunt snout bream as the experimental objective and ammonia nitrogen as the stress source, the aim of this study was to assess the growth, antioxidant capacity, and innate immunity of blunt snout bream under subacute ammonia nitrogen stress. It is anticipated that our results might provide a scientific basis for the healthy culture of fish and the assessment of environmental pollutants.

2. Materials and Methods

2.1. Fish and Experiment Design

Experimental fish were obtained from the Longxi aquaculture farm in Huzhou (Zhejiang, China). Firstly, all fish were fed for seven days to acclimate them to the experimental condition before experiments. Our previous study found that the median lethal concentration (LC_{50}) of 96 h was 68.18 mg/L. Then, all experimental fish with initial average weights of 6.55 ± 0.24 g were selected and divided into three groups: a control group (G1), a 10% LC_{50}

ammonia nitrogen stress group (G2), and a 20% LC_{50} ammonia nitrogen stress group (G3). The fish in G1 were reared in tap water with aeration. The fish in G2 were reared in water with 6.82 mg/L ammonium chloride at 10% LC_{50}. The fish in G3 were reared in water with 13.64 mg/L ammonium chloride at 20% LC_{50}. In total, 120 fish were randomly sorted into 12 tanks (150 L), with 10 fish in each tank containing 100 L of water, and they were hand-fed a commercial diet (Tongwei Group: 32% crude protein, 6% crude lipid) to apparent satiation three times daily at 7:00 a.m., 11:30 a.m., and 5:30 p.m. for 4 weeks. The uneaten feed pellets and feces were cleaned in a timely manner. The daily feeding amount was from 3% to 5% of the body mass of the fish. The feeding amount was decided by the condition of the remaining feed pellets. Approximately 50 L of the water in the tanks was replaced daily by water. The ammonia-N concentration was reached by adding a concentrated NH_4Cl solution. The concentrations of ammonia-N were determined using the Nesslerization method. Un-ionized ammonia was calculated using the equation of El-Shafai et al. [20]. The water quality parameters were listed in Table 1.

Table 1. The water quality parameters during a 4-week stress trial.

Items	G1	G2	G3
TA-N/mg L^{-1}	0.05 ± 0.01	3.57 ± 0.04	7.14 ± 0.04
Actual unionized NH_3/mg L^{-1}	0.00 ± 0.00	0.06 ± 0.04	0.12 ± 0.05
Water temperature/°C	27.0 ± 0.50	27.0 ± 0.50	27.0 ± 0.50
pH	7.44 ± 0.06	7.43 ± 0.04	7.42 ± 0.05
Dissolved oxygen/mg L^{-1}	7.51 ± 0.02	7.50 ± 0.01	7.51 ± 0.02

2.2. Sample

The fish were fasted for 24 h before sampling. Two fish from each tank were anesthetized using the MS-222 at the concentration of 100 mg/L (Sigma-Aldrich Shanghai Trading Co., Ltd. Shanghai, China). Firstly, the body weight of these selected fish was determined. Blood was collected from the caudal vessel of the fish using heparinized syringes and centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was reserved at –20 °C for immune enzyme activity analysis. Then, the liver was carefully removed and homogenized with nine volumes (v/w) of chilled saline using the homogenizer in an ice bath. The extract was later centrifuged at 2500 rpm for 10 min at 4 °C. The supernatant of the liver was reserved at –20 °C for antioxidant enzyme activity analysis. Other livers and muscles were collected for RT-qPCR (reverse transcription quantitative polymerase chain reaction) analysis.

2.3. Enzyme Activity Assay

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined following the methods described by Rietman and Frankel [21].

Total antioxidant capacity (T-AOC) activity was measured using the ABTS method. Superoxide dismutase (SOD) and catalase (CAT) activities were measured based on the methods of Marklund and Marklund [22] and Sinha [23]. Glutathione peroxidase (GPx) activity was determined using the method of Dabas et al. [24]. Malondialdehyde (MDA) content was determined using the thiobarbituric acid test [25].

Plasma immune parameters consist of total protein (TP), albumin, globulin, lysozyme, acid phosphatase (ACP), and alkaline phosphatase (AKP) contents. Plasma total protein and albumin content were measured by the Biuret and BCG dye binding method and the bromocresol green binding method [26]. Lysozyme activity was estimated using the turbidimetric assay according to Obach et al. [27]. ACP and AKP activities were analyzed according to a disodium phenyl phosphate method [28].

2.4. Total RNA Extract, cDNA Synthesis, and RT-qPCR

Total RNA in the muscle and liver was extracted using the Trizol Reagent (Accurate Biology, Changsha, China). Subsequently, the quality of the extracted RNA was evaluated

using electrophoresis. The first-strand cDNA was synthesized from RNA using a reverse transcription kit (AG11728, Accurate Biology, Changsha, China). The PCR primers (Table 2) were designed using the primer premier 5.0 program and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The real-time quantitative PCR analysis was performed according to the reagent instructions of SYBR Green Premix Pro Taq HS qPCR Kit II (AG11702, Accurate Biology, Changsha, China).

Table 2. Primer sequences used for real-time quantitative PCR.

Function	Genes	Sequence (5′-3′)	Genbank No.
Housekeeping gene	*EF-1α*	CTTCTCAGGCTGACTGTGC CCGCTAGCATTACCCTCC	×77,689.1
Antioxidant genes	*SOD*	AGTTGCCATGTGCACTTTTCT AGGTGCTAGTCGAGTGTTAGG	[29]
	CAT	ACCGAGGTGCTGAACGAAGC GAACGGCCATCAGGTTTTGC	XM_048158628
	NOX2	TCACTGGATGGGACCAGAGT CCAGTTCGGTCGGCCATAAT	[30]
	Bach1	TTACAGCAGCGAAGTGAGCA CGGGCTGCAATACGGTTTTT	[30]
Immunity genes	*Leap1*	CAGACCGCAGCCGTTCCCTT AGCAGTATCCACAGCCTTTG	JQ308841
	Leap2	GTGCCTACTGCCAGAACCAT GAACATTACCTATTGCCTCC	JQ344324
Growth genes	*GHRa*	AGCCTCCTCCTGAATCCT TTCCAGCAGTGAGAAGGTAT	JN896373.1
	GHRb	GCAAAGCGGCAGAGGAGA GCCACAGCACCAGTGAACA	JN896374.1
	IGF1	CCGATTTAAGGTCCGTATT GTGCAGCCGTAGTTCAGTT	JQ398496
	IGF2	TGTTTGCCATACCTGCTTG ACGCCGACTGTTCGACCTA	JQ398497.1

EF-1α, elongation factor-1 α; *SOD*, superoxide dismutase; *CAT*, catalase; *NOX2*, NADPH oxidase 2; *Bach1*, BTB and CNC homolog 1; *Leap1*, antimicrobial peptide 1; *Leap2*, antimicrobial peptide 2; *GHRa*, growth hormone receptor a; *GHRb*, growth hormone receptor b; *IGF1*, insulin-like growth factor 1; *IGF2*, insulin-like growth factor 2.

2.5. Calculation

The growth indicators in this study were analyzed as follows:

Weight gain rate (WGR, %) = 100 × (final body weight − initial body weight)/initial body weight.

Specific growth rate (SGR, %/d) = (ln(final body weight) − ln(initial body weight)) × 100/days.

Survival rate (SR, %) = (initial number of fish − final number of fish) × 100/initial number of fish.

Feed conversion ratio (FCR) = feed consumption/fish weight gain.

2.6. Statistical Analysis

The experiment data were analyzed by a one-way ANOVA using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) after the normality distribution and homogeneity analysis of data. All data are presented as average ± S.E.M (standard error of the mean). Differences between groups were significant when the F value was significant at the level of probability of 0.05.

3. Results

3.1. Effect of Subacute Ammonia Nitrogen Stress on the Growth of Blunt Snout Bream

As can be seen from Table 3, subacute ammonia nitrogen stress significantly affected the growth of the blunt snout bream. The growth performance of the blunt snout bream

decreased with the increased ammonia nitrogen concentration. The final body weight, weight gain rate, and specific growth rate of fish in the stress groups were significantly lower than fish in the control group ($p < 0.05$). However, the feed conversion ratio of fish in the stress groups was significantly higher than fish in the control group ($p < 0.05$). The survival rate of fish in G3 was significantly lower than fish in the control group ($p < 0.05$).

Table 3. Effect of subacute ammonia nitrogen stress on the growth of blunt snout bream.

Items	G1	G2	G3
Initial body weight/g	6.52 ± 0.10	6.50 ± 0.02	6.51 ± 0.09
Final body weight/g	14.33 ± 2.55 ^{a}	13.03 ± 0.34 ^{b}	10.30 ± 1.39 ^{c}
Weight gain rate/%	120.19 ± 40.36 ^{a}	100.39 ± 3.32 ^{b}	55.49 ± 2.16 ^{c}
Specific growth rate/% d^{-1}	2.61 ± 0.07 ^{a}	2.28 ± 0.09 ^{b}	1.48 ± 0.07 ^{c}
Feed conversion ratio	1.93 ± 0.09 ^{a}	2.46 ± 0.08 ^{b}	2.47 ± 0.07 ^{b}
Survival rate/%	91.00 ± 2.00 ^{a}	86.00 ± 3.00 ^{ab}	81.00 ± 2.00 ^{b}

Data are the average ± S.E.M of four replicates. Means in the same line with different superscript letters are significantly different ($p < 0.05$).

3.2. Effect of Subacute Ammonia Nitrogen Stress on the Metabolic Enzyme Activities of Blunt Snout Bream

The effects of subacute ammonia nitrogen stress on the metabolic enzyme activity of fish are shown in Figure 1. The AST and ALT activities in the livers of fish in the stress groups were significantly lower than in the control group ($p < 0.05$). However, subacute ammonia nitrogen stress had no effects on the AST and ALT contents in the plasma of those fish ($p > 0.05$) compared to the control group.

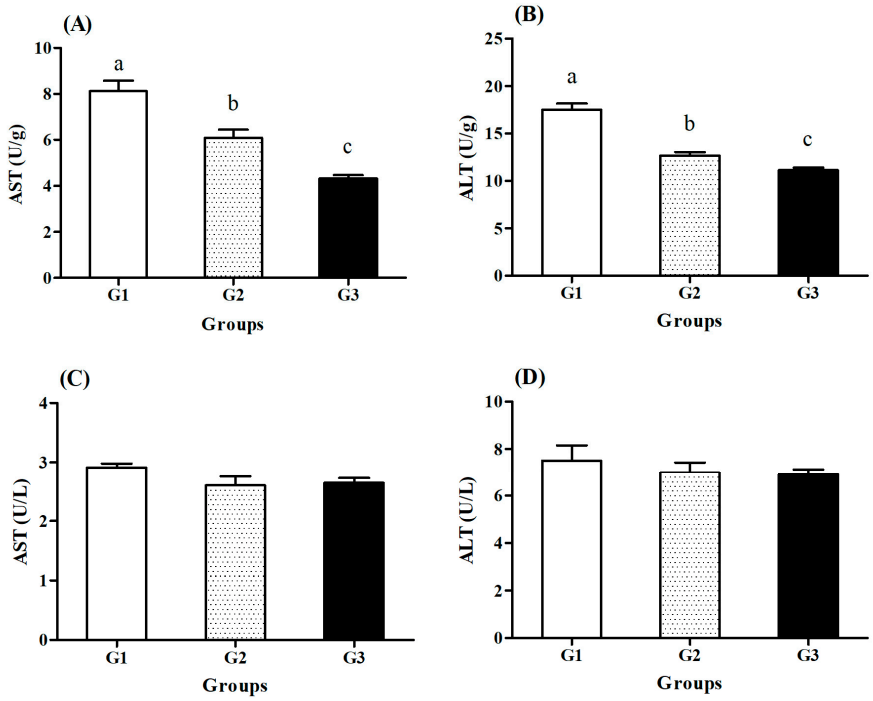

Figure 1. Effect of the subacute ammonia nitrogen on the metabolic enzyme activities in the liver (**A**,**B**) and plasma (**C**,**D**) of blunt snout bream. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant differences ($p < 0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase.

3.3. Effect of Subacute Ammonia Nitrogen Stress on the Antioxidant Capacity in the Liver of Blunt Snout Bream

The antioxidant enzyme levels in the livers of the fish are presented in Figure 2. The T-AOC, SOD, GPx, and CAT contents of fish in the stress groups were significantly lower

than those in the control group ($p < 0.05$). However, the MDA level of fish in the control group was significantly lower than that in the stress groups ($p < 0.05$).

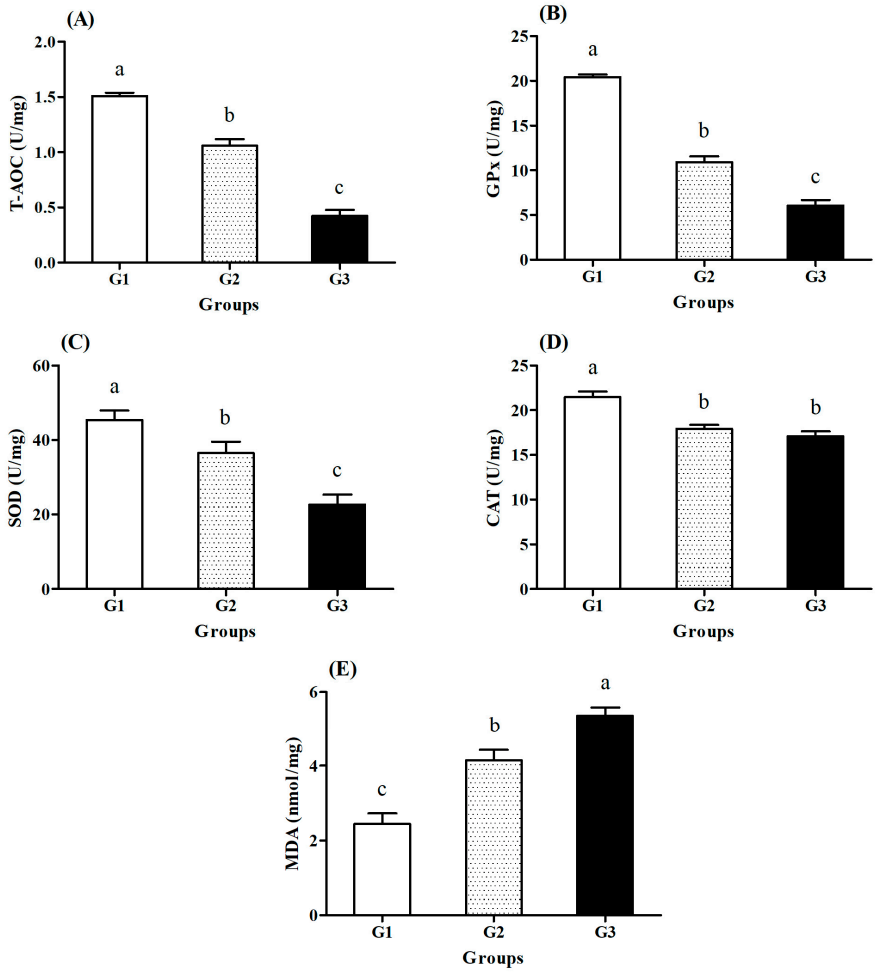

Figure 2. Effect of subacute ammonia nitrogen stress on the antioxidant enzyme activity in the liver of blunt snout bream. (**A**) T-AOC activity; (**B**) GPx activity; (**C**) SOD activity; (**D**) CAT activity; and (**E**) MDA activity. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant differences ($p < 0.05$). T-AOC, total antioxidant capacity; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

3.4. Effect of Subacute Ammonia Nitrogen Stress on the Plasma Immune Enzyme Activity of Blunt Snout Bream

As is shown in Figure 3, subacute ammonia nitrogen stress affected the immune enzyme activity in the plasma of the blunt snout bream. Subacute ammonia nitrogen stress significantly decreased the albumin, globulin, ACP, and AKP activities compared with the control group ($p < 0.05$). Furthermore, the total protein and lysozyme activities of fish in G3 were significantly lower than those in the control group ($p < 0.05$); however, there was no significant difference compared to G2 ($p > 0.05$).

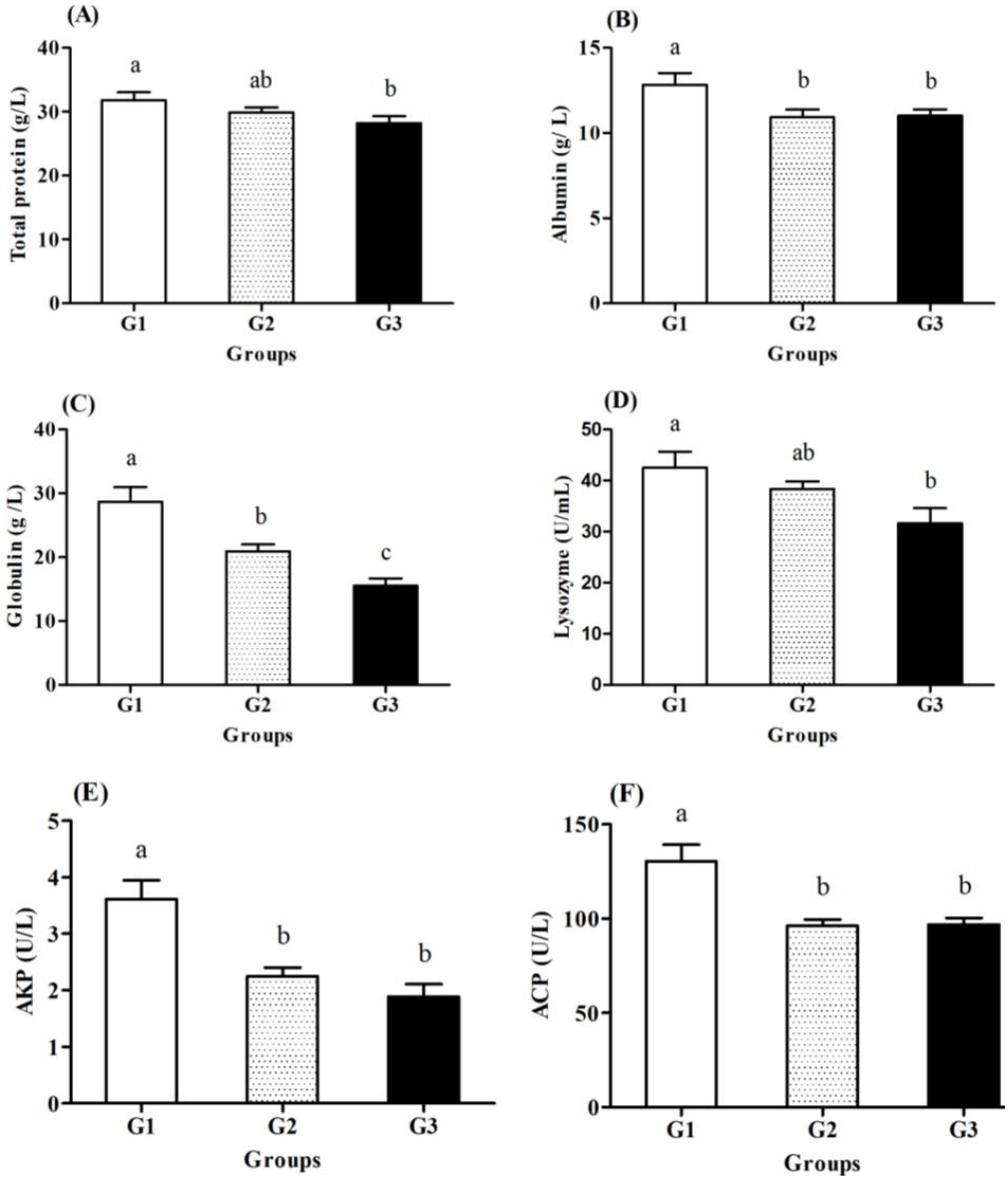

Figure 3. Effect of subacute ammonia nitrogen stress on the immune enzyme activity in the plasma of blunt snout bream. (**A**) Total protein content; (**B**) albumin content; (**C**) globulin content; (**D**) lysozyme activity; (**E**) AKP activity; and (**F**) ACP activity. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant differences ($p < 0.05$). AKP, alkaline phosphatase; ACP, acid phosphatase.

3.5. Effect of Subacute Ammonia Nitrogen Stress on the Growth-Related Genes of Blunt Snout Bream

As is shown in Figure 4, subacute ammonia nitrogen stress significantly decreased the relative expression levels of growth-related genes in the muscles of the blunt snout bream. The relative expression levels of the *GHRa*, *GHRb*, *IGF1*, and *IGF2* genes in the stress groups were significantly lower than those of the fish in the control group ($p < 0.05$).

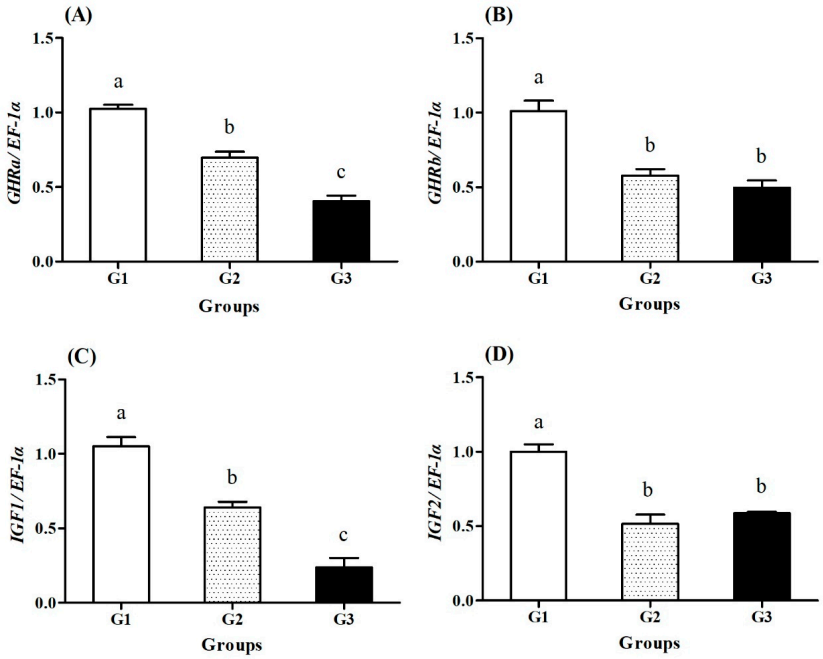

Figure 4. Effect of subacute ammonia nitrogen stress on the relative expression levels of growth-related genes of blunt snout bream. (**A**) *GHRa* gene; (**B**) *GHRb* gene; (**C**) *IGF1* gene; and (**D**) *IGF1* gene. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant difference ($p < 0.05$).

3.6. Effect of Subacute Ammonia Nitrogen Stress on the Antioxidant-Related Genes of Blunt Snout Bream

As is shown in Figure 5, subacute ammonia nitrogen stress had a significant influence on the relative expression levels of the antioxidant genes in the livers of the blunt snout bream. Subacute ammonia nitrogen stress significantly decreased the relative expression levels of *SOD* and *CAT* in the livers of the blunt snout bream compared to the control group ($p < 0.05$), but upregulated *NOX2* and *Bach1* mRNA levels in the livers of the fish ($p < 0.05$).

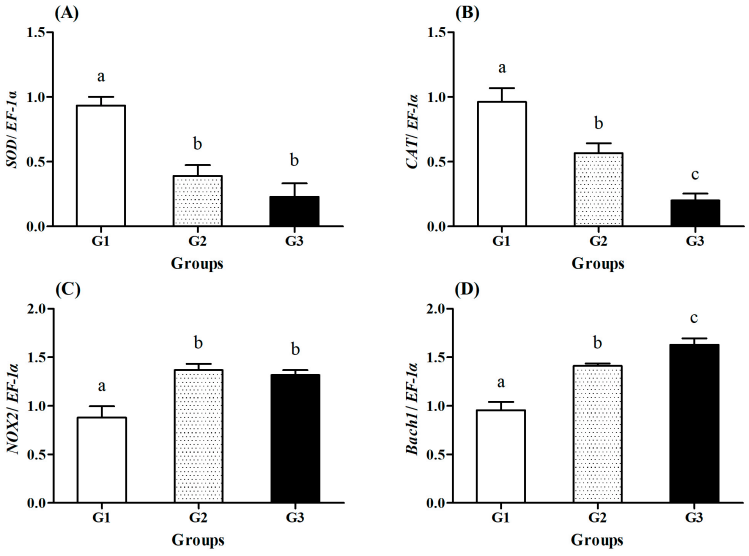

Figure 5. Effect of subacute ammonia nitrogen stress on the relative expression levels of the antioxidant-related genes in the liver of blunt snout bream. (**A**) *SOD* gene; (**B**) *CAT* gene; (**C**) *NOX2* gene; and (**D**) *Bach1* gene. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant differences ($p < 0.05$).

3.7. Effect of Subacute Ammonia Nitrogen Stress on the Immunity-Related Genes of Blunt Snout Bream

As is shown in Figure 6, subacute ammonia nitrogen stress had a significant effect on the relative expression levels of the immune genes in the livers of the blunt snout bream. The gene expression levels of *Leap 1* and *Leap 2* in the stress groups were significantly lower than that of fish in the control group ($p < 0.05$).

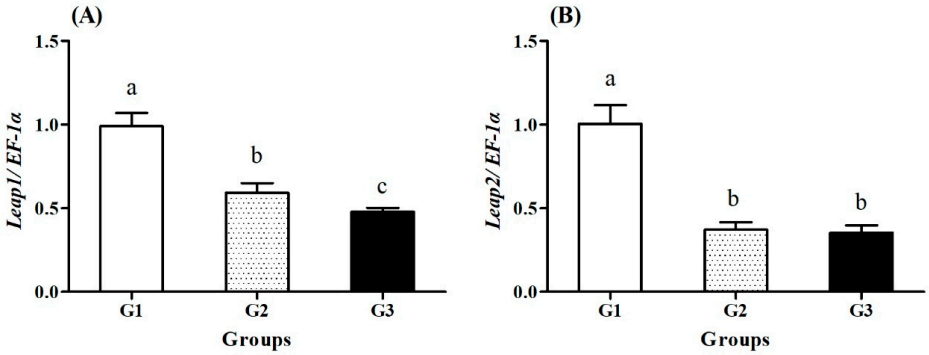

Figure 6. Effect of subacute ammonia nitrogen stress on the relative expression levels of immunity-related genes in the liver of blunt snout bream. (**A**) *Leap 1* gene and (**B**) *Leap 2* gene. Data are the average ± S.E.M of four replicates. Bars assigned with different letters present significant differences ($p < 0.05$).

4. Discussion

Ammonia nitrogen is an important index in evaluating the quality of water environments. A large number of studies have shown that the increased ammonia nitrogen concentration in water inhibits the growth of aquatic organisms. Paust et al. [31] reported that the growth rate of Atlantic flounder (*Hippoglossus hippoglossus*) was significantly inhibited after fish were cultured in water with an NH_3 mass concentration at 0.12 and 0.17 mg/L for 62 days. Similarly, Peng et al. [32] found that chronic ammonia nitrogen stress significantly decreased the survival rate, food intake, and growth of cuttlefish (*Sepia pharaonis*). In the present study, we also found that the weight gain rate and specific growth rate of fish in stress groups were significantly lower than that of fish in the control group. This was probably because ammonia nitrogen stress led to the decreased feeding rate of fish. Most of the energy in the bodies of the fish was used to resist the stress response caused by ammonia nitrogen; consequently, the growth of fish decreased. The result of the feed conversion ratio also revealed that ammonia stress decreased the feed intake of the fish in our study. Furthermore, ammonia nitrogen was harmful to the tissue structure, immune function, and blood biochemical indices of the fish, and therefore resulted in the decreased growth of fish [11]. In addition, subacute ammonia nitrogen stress significantly decreased the relative expression levels of *GHRa*, *GHRb*, *IGF1*, and *IGF2* in the muscle of the blunt snout bream. This result demonstrated that the ammonia nitrogen stress significantly decreased the growth performance at the genomic level again. Fish growth is a multifactorial result from complex molecular and genetic interactions in which the growth hormone plays an important function in all vertebrate fish [32]. The biological action of growth hormone is mediated by insulin-like growth factor I (IGF) [33], and on the target organs through its interaction with a specific cell surface receptor, the GH receptor (GHR). GHR synthesis regulates growth hormone stimulation, which is dependent on biological and environmental factors. This is why the ammonia nitrogen stress decreased the relative expression levels of growth-related genes of the blunt snout bream. However, the underlying molecular mechanism of ammonia stress on the growth is still unclear. Therefore, this study lays the foundation for further research.

The liver of fish is the main detoxification and metabolic organ. It is also the gathering place of various pollutants and toxins. A high concentration of ammonia nitrogen in a water environment is transported to the liver and participates in metabolism through the hepatic

portal vein. Therefore, the liver is the main damaged organ under ammonia nitrogen stress. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which transfer amino groups to form oxaloacetate, are the most important aminotransferases and are found in the liver cytosol [34]. A previous study also found that ALT activity in the liver is thousands of times higher than in serum [35]. Therefore, increased AST and ALT in the serum suggests that the fish liver is injured. In this present study, the AST and ALT contents in the liver of fish in stress groups were significantly lower than that of fish in the control group. This result indicated that the function of hepatic cell metabolism of the fish was weakened by the ammonia nitrogen stress. Furthermore, ammonia nitrogen stress had no effect on the AST and ALT contents in the plasma of fish compared to the control group in this study. This suggested that no injury occurred in the liver of the fish exposed to ammonia nitrogen. This was because serum AST and ALT activities increased only when the liver was injured [36].

The increase in environmental stress factors can induce the production of reactive oxygen species (ROS). The generation of oxygen free radicals, derivatives of a large number of metabolites, causes serious damage to the tissue function of the body. In fact, the major key to coping with changes in environmental factors in a water environment is to remove oxygen free radicals in the organism via the antioxidant enzyme system [37]. If the ROS level is uncontrolled by the antioxidant defense system, the increased ROS causes oxidative stress [18]. Generally, the antioxidant enzyme system in fish consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and others. SOD is responsible for catalyzing the superoxide radicals into O_2 and H_2O_2, and then CAT decomposes H_2O_2 into O_2 and H_2O [38]. In the present study, the T-AOC, SOD, GPX, and CAT contents of fish in the stress groups were significantly lower than those of fish in the control group. This suggests that ammonia nitrogen decreased the antioxidant capacity of the fish [39]. Therefore, weak free radical scavenging activity appeared in fish exposed to ammonia nitrogen stress. A similar result was reported in hybrid Tilapia (*Oreochromis niloticus* × *Oreochromis areus*) [40]. Interestingly, the malondialdehyde (MDA) levels of fish in the stress groups were significantly higher than that of fish in the control group in this present study. MDA is mainly regarded as an indicator of lipid peroxidation, as it can injure the structure and normal function of cells [41]. This result indicated that the hepatocyte function was impaired when the fish were under ammonia nitrogen exposure. This explanation is supported by the results of antioxidant genes and metabolic enzyme activities in this study. In addition, we also analyzed the effect of ammonia nitrogen stress on antioxidant capacity in the fish at the genetic level. Our results revealed that subacute ammonia nitrogen stress significantly decreased the relative expression levels of *SOD* and *CAT* but upregulated the *NOX2* and *Bach1* mRNA levels in the livers of the blunt snout bream. *NOX2* and *Bach1* contribute to oxidative stress [42], and their upregulated expression levels are indicative of increased ROS production. This result demonstrated that ammonia nitrogen stress weakened the antioxidant capacity of the fish again.

The fish immune system plays an important role in preventing pathogens from entering the body and consists of adaptive immunity and innate immunity [43]. The innate immune system is responsible for the removal of environmental pollutants, and it contains lysozyme, acid phosphatase, albumin, and others. In fish, lysozyme is an essential component of the innate immune system because it can decompose the cell walls of bacteria [44]. ACP is one of the vital marker enzymes of lysozyme's ability to break down invading organisms and participates in the first line of nonspecific immunity [45]. ACP has been shown to participate in the response of red claw crayfish to cold stress, and its immune enzyme activity is inhibited at low temperatures [46]. Globulin, as one of the major components of plasma proteins, plays an important role in the immune response of freshwater fish, and thus its content could reflect the immune status of fish [28]. In the present study, ammonia nitrogen stress significantly decreased the lysozyme, ACP, globulin, and albumin activities in the stress groups compared with the control group. This result indicated that ammonia nitrogen stress decreased the innate immunity of the fish. This explanation was probably

reasonable because non-ionic ammonia in water can easily penetrate cell members and produce toxic effects [2]. A previous study also found that excessive ammonia nitrogen in the environment could affect the immune system [47]. In this study, we further found that the relative expression levels of *Leap 1* and *Leap 2* in the control group were significantly higher than that of fish in the stress groups. Antimicrobial peptide 1 (*Leap*-1) and antimicrobial peptide 2 (*Leap*-2) have been identified from the livers of different animals [48]. Previous research showed that antimicrobial peptides have broad-spectrum antibacterial properties [49]. This result indicated that ammonia nitrogen stress decreased the innate immunity of fish again. However, research regarding the mechanism of ammonia nitrogen stress affecting immunity is still lacking.

5. Conclusions

The present research reveals that subacute ammonia nitrogen stress not only inhibited the growth but also decreased the antioxidant capacity and immunity of the blunt snout bream under study. However, it is still unknown how ammonia nitrogen stress affects the immune response of fish. Therefore, we will further study the signal pathway of the immune mechanism regulated by ammonia nitrogen stress.

Author Contributions: Methodology, X.Y. and C.W.; writing—original draft, X.Y.; data curation, Q.W. and M.D.; project administration, supervision, C.W.; validation, X.X. and H.W.; formal analysis, X.X. and H.W.; All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Natural Science Foundation of China (Grant No. 32302980), the General Project of Basic Science (natural science) of Colleges and Universities in Jiangsu Province (Grant No. 21KJB240002), and the College Students' innovation and entrepreneurship training program of the Huaiyin Institute of Technology (Grant No. 202411049032Z).

Institutional Review Board Statement: The use of the fish in this study was approved by the Animal Care and Use Committee of the Huaiyin Institute of Technology (Huai'an, China) [No.202305] in 2023. All experimental operations involving experimental fish were conducted based on the Introductions for the Care and Use of Laboratory Animals in China.

Informed Consent Statement: Not applicable. This study did not involve humans.

Data Availability Statement: All data generated during this study are included in this article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Armstrong, D.A.; Chippendale, D.; Knight, A.W.; Colt, J.E. Interaction of ionized and un-ionized ammonia on short-term survival and growth of prawn larvae, *Macrobrachium rosenbergii*. *Biol. Bull.* **1978**, *154*, 15–31. [CrossRef] [PubMed]
2. Benli, A.C.K.; Koksal, G.; Ozkul, A. Sublethal ammonia exposure of Nile tilapia (*Oreochromis niloticus* L.): Effects on gill, liver and kidney histology. *Chemosphere* **2008**, *72*, 1355–1358. [CrossRef] [PubMed]
3. Cong, M.; Wu, H.F.; Cao, T.F.; Ji, C.L.; Lv, J.S. Effects of ammonia nitrogen on gill mitochondria in clam *Ruditapes philippinarum*. *Environ. Toxicol. Pharmacol.* **2019**, *65*, 46–52. [CrossRef] [PubMed]
4. Sitja-Bobadilla, A.; Alvarez-Pellitero, P. Experimental transmission of *Sparicotyle chrysophrii* (Monogenea: Polyopisthocotylea) to gilthead seabream (*Sparus aurata*) and histopathology of the infection. *Folia Parasitol.* **2009**, *56*, 143–151. [CrossRef] [PubMed]
5. Cheng, C.H.; Ma, H.L.; Su, Y.L.; Deng, Y.Q.; Feng, J.; Xie, J.W.; Chen, X.L.; Guo, Z.X. Ammonia toxicity in the mud crab (*Scylla paramamosain*): The mechanistic insight from physiology to transcriptome analysis. *Ecotoxicol. Environ. Saf.* **2019**, *179*, 9–16. [CrossRef]
6. Lang, T.; Peters, G.; Hoffmann, R.; Meyer, E. Experimental investigations on the toxicity of ammonia: Effects on ventilation frequency, growth, epidermal mucous cells, and gill structure of rainbow trout *Salmo gairdneri*. *Dis. Aquat. Org.* **1987**, *3*, 159–165. [CrossRef]
7. Hang, Y.L.; Shang, Z.H.; Wang, G.Y.; You, K.; Mi, D. High concentrations of environmental ammonia induced changes in large-scale loach (*Paramisgurnus dabryanus*) immunity. *Ecol. Evol.* **2021**, *11*, 8614–8622. [CrossRef]
8. Jiang, M.; Li, L.; Shen, X.Q.; Wu, Q.Y.; Niu, J.X. Effect of ammonia stress on immunity indicators of juvenile *Mugil cephalus*. *Oceanol. Limnol. Sin.* **2014**, *45*, 529–535.
9. Hu, Y.; Huang, Y.; Zhong, L.; Xiao, T.Y.; Wen, H.; Huan, Z.L.; Mao, X.W.; Li, J.L. Effects of ammonia stress on the gill Na^+/K^+-ATPase, microstructure and some serum physiological-biochemical indices of juvenile black carp (*Mylopharyngodon piceus*). *J. Fish. China* **2012**, *36*, 538–545. [CrossRef]

10. Jia, R.; Liu, B.L.; Han, C.; Huang, B.; Lei, J.L. Effects of ammonia exposure on stress and immune response in juvenile turbot (*Scophthalmus maximus*). *Aquac. Res.* **2017**, *48*, 3149–3162. [CrossRef]
11. Mckenzie, D.; Shingles, A.; Taylor, E. Sub-lethal plasma ammonia accumulation and the exercise performance of salmonids. *Comp. Biochem. Physiol. A* **2003**, *135*, 515–526. [CrossRef] [PubMed]
12. Roumieh, R.; Barakat, A.; Abdelmeguid, N.E.; Ghanawi, J.; Patrick, S.I. Acute and chronic effects of aqueous ammonia on marbled spinefoot rabbitfish, *Siganus rivulatus* (Forsskål 1775). *Aquac. Res.* **2013**, *44*, 1777–1790. [CrossRef]
13. Reinecke, M.; Björnsson, B.T.; Dickhoff, W.W.; McCormick, S.D.; Navarro, I.; Power, D.M.; Gutiérrez, J. Growth hormone and insulin-like growth factors in fish: Where we are and where to go. *Gen. Comp. Endocrinol.* **2005**, *142*, 20–24. [CrossRef] [PubMed]
14. Hou, J.; Su, Y.J.; Lin, W.; Guo, H.H.; Xie, P.; Chen, J.; Gu, Z.M.; Li, L. Microcystin-LR retards gonadal maturation through disrupting the growth hormone/insulin-like growth factors system in zebrafish. *Ecotoxicol. Environ. Saf.* **2017**, *139*, 27–35. [CrossRef] [PubMed]
15. Zhou, X.; Dong, Y.W.; Wang, F.; Dong, S.F. The effect of high ammonia concentration on gill structure alternation and expression of SOD and HSP90 genes in grass carp, *Ctenopharyngodon idella*. *Acta Hydrobiol. Sin.* **2013**, *37*, 321–330.
16. Zhang, J.; Shen, H.; Wang, X.; Wu, J.; Xue, Y. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. *Chemosphere* **2004**, *55*, 167–174. [CrossRef]
17. Lu, K.L.; Wang, L.N.; Zhang, D.D.; Liu, W.B.; Xu, W.N. Berberine attenuates oxidative stress and hepatocytes apoptosis via protecting mitochondria in blunt snout bream (*Megalobrama amblycephala*) fed high-fat diets. *Fish Physiol. Biochem.* **2017**, *43*, 65–76. [CrossRef]
18. Sheikhzadeh, N.; Tayefi-Nasrabadi, H.; Oushani, A.K.; Enferadi, M.H. Effects of *Haematococcus pluvialis* supplementation on antioxidant system and metabolism in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **2012**, *38*, 413–419. [CrossRef]
19. Yuan, X.Y.; Wang, C.L.; Liu, W.B.; Jiang, G.Z.; Dai, Y.J. Evaluation of yeast hydrolysate as a substitute to dietary fish meal of juvenile Jian carp (*Cyprinus carpio* var. Jian): Protein synthesis via TOR pathway. *Aquac. Nutr.* **2021**, *27*, 1853–1860. [CrossRef]
20. El-Shafai, S.A.; El-Gohary, F.A.; Nasr, F.A.; Steen, N.P.V.D.; Gijzen, H.J. Chronic ammonia toxicity to duckweed-fed tilapia (*Oreochromis niloticus*). *Aquaculture* **2004**, *232*, 117–127. [CrossRef]
21. Rietman, S.; Frankel, S.A. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.* **1957**, *28*, 56–63. [CrossRef] [PubMed]
22. Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **1974**, *47*, 469–474. [CrossRef] [PubMed]
23. Sinha, A.K. Colorimetric assay of catalase. *Anal. Biochem.* **1972**, *47*, 389–394. [CrossRef] [PubMed]
24. Dabas, A.; Nagpure, N.; Kumar, R.; Kushwaha, B.; Kumar, P.; Lakra, W. Assessment of tissue-specific effect of cadmium on antioxidant defense system and lipid peroxidation in freshwater murrel, *Channa punctatus*. *Fish Physiol. Biochem.* **2012**, *38*, 469–482. [CrossRef]
25. Satho, K. Serum lipid peroxidation in cerebrovascular disorders determined by a new colorimetric method. *Clin. Chim. Acta* **1978**, *190*, 37–43. [CrossRef]
26. Chen, Q.Q.; Liu, W.B.; Zhou, M.; Dai, Y.J.; Xu, C.; Tian, H.Y.; Xu, W.N. Effects of berberine on the growth and immune performance in response to ammonia stress and high-fat dietary in blunt snout bream *Megalobrama amblycephala*. *Fish Shellfish Immunol.* **2016**, *55*, 165–172. [CrossRef]
27. Obach, A.; Quentel, C.; Laurencin, F.B. Effects of alpha-tocopherol and dietary oxidized fish oil on the immune response of sea bass *Dicentrarchus labrax*. *Dis. Aquat. Org.* **1993**, *15*, 175–185. [CrossRef]
28. Yuan, X.Y.; Jiang, G.Z.; Cheng, H.H.; Cao, X.F.; Shi, H.J.; Liu, W.B. An evaluation of replacing fish meal with cottonseed meal protein hydrolysate in diet for juvenile blunt snout bream (*Megalobrama amblycephala*): Growth, antioxidant, innate immunity and disease resistance. *Aquac. Nutr.* **2019**, *25*, 1334–1344. [CrossRef]
29. Sun, S.; Ge, X.; Zhu, J.; Xuan, F.; Jiang, X. Identification and mRNA expression of antioxidant enzyme genes associated with the oxidative stress response in the Wuchang bream (*Megalobrama amblycephala* Yih) in response to acute nitrite exposure. *Comp. Biochem. Physiol. C* **2014**, *159*, 69–77. [CrossRef]
30. Zhao, Z.; Xie, J.; Liu, B.; Ge, X.P.; Song, C.Y.; Ren, M.C.; Zhou, Q.L.; Miao, L.H.; Zhang, H.M.; Shan, F.; et al. The effects of emodin on cell viability, respiratory burst and gene expression of Nrf2-Keap1 signaling molecules in the peripheral blood leukocytes of blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immunol.* **2017**, *62*, 75–85. [CrossRef]
31. Paust, L.O.; Foss, A.; Imsland, A.K. Effects of chronic and periodic exposure to ammonia on growth, food conversion efficiency and blood physiology in juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **2011**, *315*, 400–406. [CrossRef]
32. Peng, R.B.; Wang, P.S.; Le, K.X.; Wang, Y.; Jiang, X.M. Acute and chronic effects of ammonia on juvenile cuttlefish, Sepia pharaonis. *J. World Aquac. Soc.* **2017**, *48*, 602–610. [CrossRef]
33. Figueiredo, M.D.A.; Lanes, C.F.C.; Almeida, D.V.; Proietti, M.C.; Marins, L.F. The effect of GH overexpression on GHR and IGF-I gene regulation in different genotypes of GH-transgenic zebrafish. *Comp. Biochem. Physiol. D* **2007**, *2*, 228–233. [CrossRef]
34. Cai, L.S.; Wang, L.; Song, K.; Lu, K.L.; Zhang, C.X.; Rahimnejad, S. Evaluation of protein requirement of spotted seabass (*Lateolabrax maculatus*) under two temperatures, and the liver transcriptome response to thermal stress. *Aquaculture* **2020**, *516*, 734615. [CrossRef]
35. Kim, W.R.; Flamm, S.L.; Di Bisceglie, A.M.; Bodenheimer, H.C. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology* **2008**, *47*, 1363–1370. [CrossRef]

36. Li, J.Y.; Zhang, D.D.; Xu, W.N.; Jiang, G.Z.; Zhang, C.N.; Li, X.F.; Liu, W.B. Effects of dietary choline supplementation on growth performance and hepatic lipid transport in blunt snout bream (*Megalobrama amblycephala*) fed high-fat diets. *Aquaculture* **2014**, *434*, 340–347. [CrossRef]
37. Rahimnejad, S.; Yuan, X.Y.; Liu, W.B.; Jiang, G.Z.; Cao, X.F.; Dai, Y.J.; Wang, C.C.; Hesham, E.D. Evaluation of antioxidant capacity and immunomodulatory effects of yeast hydrolysates for hepatocytes of blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immun.* **2020**, *106*, 142–148. [CrossRef]
38. Kiron, V. Fish immune system and its nutritional modulation for preventive health care. *Anim. Feed. Sci. Technol.* **2012**, *173*, 111–133. [CrossRef]
39. Zhang, W.X.; Xia, S.L.; Zhu, J.; Miao, L.H.; Ren, M.C.; Lin, Y.; Ge, X.P.; Sun, S.M. Growth performance, physiological response and histology changes of juvenile blunt snout bream, *Megalobrama amblycephala* exposed to chronic ammonia. *Aquaculture* **2019**, *506*, 424–436. [CrossRef]
40. Han, C.Y.; Zheng, Q.M.; Chen, G.D.; Liu, L.X. Effect of ammonia-nitrogen stress on non-specific immunity of hybrid Tilapia (*Oreochromis niloticus* × *Oreochromis areus*). *Anim. Husb. Feed. Sci.* **2015**, *7*, 226–229.
41. Parvez, S.; Raisuddin, S. Protein carbonyls: Novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). *Environ. Toxicol. Pharmacol.* **2005**, *20*, 112–117. [CrossRef] [PubMed]
42. Wei, Y.H.; Gong, J.S.; Xu, Z.H.; Duh, E.J. Nrf2 promotes reparative angiogenesis through regulation of NADPH oxidase-2 in oxygen-induced retinopathy. *Free Radic. Biol. Med.* **2016**, *99*, 234–243. [CrossRef] [PubMed]
43. Ellis, A.E. Innate host defense mechanisms of fish against viruses and bacteria. *Dev. Comp. Immunol.* **2001**, *25*, 827–839. [CrossRef]
44. Gómez, G.D.; Balcázar, J.L. A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunol. Med. Microbiol.* **2008**, *52*, 145–154. [CrossRef]
45. Yang, W.L.; Jiang, S.; Yang, Q.B.; Huang, J.H.; Shi, J.Z.; Li, Y.D.; Yang, Y.K.; Zhou, F.L. Effects of partial substitution of fish meal with soybean products and chicken meal on growth, antioxidant capacity and intestinal microbiota of *Penaeus monodon*. *Fishes* **2024**, *9*, 42. [CrossRef]
46. Wu, D.L.; Huang, Y.H.; Chen, Q.; Jiang, Q.C.; Li, Y.M.; Zhao, Y.L. Effects and transcriptional responses in the hepatopancreas of red claw crayfish *Cherax quadricarinatus* under cold stress. *J. Therm. Biol.* **2019**, *85*, 102404. [CrossRef] [PubMed]
47. Yue, F.; Pan, L.Q.; Xie, P.; Zheng, D.B.; Li, J. Immune responses and expression of immune-related genes in swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N stress. *Comp. Biochem. Physiol. A* **2010**, *157*, 246–251. [CrossRef]
48. Liang, T.; Ji, W.; Zhang, G.R.; Wei, K.J.; Feng, K.; Wang, W.M.; Zou, G.W. Molecular cloning and expression analysis of liver-expressed antimicrobial peptide 1 (LEAP-1) and LEAP-2 genes in the blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immunol.* **2013**, *35*, 553–563. [CrossRef]
49. Liu, T.; Gao, Y.; Wang, R.; Xu, T. Characterization, evolution and functional analysis of the liver-expressed antimicrobial peptide 2 (LEAP-2) gene in miiuy croaker. *Fish Shellfish Immunol.* **2014**, *41*, 191–199. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.