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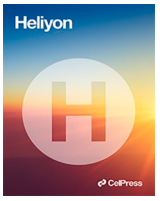

Research article

Response of antioxidation and immunity to combined influences of pH and ammonia nitrogen in the spotted babylon (*Babylonia areolata*)

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ABSTRACT

Spotted babylon were exposed to three different pH levels (7.0, 8.0 and 9.0) and four different concentrations of ammonia nitrogen (0.02, 1.02, 5.10 and 10.20 mg/L) in seawater to determine their acute toxicity and physiological responses to environmental fluctuation. The study evaluated four antioxidant enzymes: catalase (CAT), alkaline, superoxide dismutase (SOD), peroxidase (POD) and glutathione peroxidase (GSH-PX), and two immunoenzymes: acid phosphatase (ACP) and phosphatase (AKP). Over time, the immunoenzyme activity was significantly affected by pH and ammonia nitrogen concentration. After being exposed to pH and ammonia nitrogen, the spotted babylon showed signs of unresponsiveness to external stimuli, reduced vitality, slow movement, and an inability to maintain an upright position. Over time, the spotted babylon exhibited a trend of increasing and then decreasing GSH-PX, CAT, and SOD activities to adapt to the changing environment and enhance its immunity. On the contrary, the POD and ACP activities exhibited a decreasing trend initially, followed by an increasing trend over time and the AKP activity showed a gradual increase with time. The combined effect of pH and ammonia was found to be stronger than the effect of either factor alone. The interaction between pH and ammonia increased the activity of the spotted babylon antioxidant enzymes, induced oxidative stress, and reduced the ability of the spotted babylon's non-specific immune system to reverse it. Thus, the reverse-back of the spotted babylon was higher when pH and ammonia stress were dual than when pH or ammonia were single-factor stresses. The study results will establish a theoretical basis for analyzing the risk of multiple factors to the spotted babylon, and also enrich the basic information about the shellfish immune system.

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1. Introduction

The spotted babylon, also known as ivory shell, belongs to the family Babyloniidae. The marine gastropod is typically found in tropical and subtropical waters off the coasts of Japan and Southeast Asia and China. On the coast of southeast China, it is an important farmed species and an economically important shellfish. This seafood is rich in nutrients and has delicious flavour, making it a popular choice. It also has a large market demand [1–4]. Over recent years, due to breakthroughs and improvements in breeding technology, there has been a rapid development of industrial breeding of spotted babylon in the southeast coastal areas of China.

Ammonia nitrogen exists in the water column in NH_4^+ and NH_3 forms, with NH_4^+ being less toxic to crustaceans and NH_3 having a toxic solid effect on crustaceans [5]. NH_3 is lipid-soluble and can cross cell membranes, causing damage to the non-specific immune system of crustaceans and leading to greater susceptibility of crustaceans to disease [6]. Studies have shown that most aquatic organisms are susceptible to ammonia toxicity, and there have been many studies on the effects of ammonia on the disease resistance of marine organisms (e.g., fish and shrimp) [7,8]. Several mechanisms of ammonia toxicity have been summarized, including gill and liver damage, acid-base disturbances in the body, and disturbances in the electrochemical gradient [9]. The spotted babylon is usually raised in an indoor environment and fed with miscellaneous fish. This high-density culture produces a large amount of residual feed, and the increased amount of manure and excreta can lead to elevated ammonia nitrogen levels [10,11]. It usually causes toxicity to the spotted babylon, including tissue damage, bacterial disease, and reduced reproductive capacity, which reduces its survival [12]. Excess ammonia nitrogen adversely affects aquatic organisms and is a major limiting factor for aquaculture operations.

pH plays a vital in acid-base homeostasis in aquatic animals, and its rise or decrease interferes with the organism's acid-base balance, ion regulation, and ammonia excretion [13]. Osmoregulation assays are a useful tool for monitoring the physiological status of shellfish and the effects of stress. Therefore, it is essential to maintain normal osmotic pressure in shellfish during culture [14]. In industrial shellfish farming, the pH of the water body can be lowered due to a range of factors, including shellfish mortality, excessive algae feeding, and harmful algal blooms in seawater. In addition, many aquatic plants are planted during the breeding process to purify the water. Photosynthesis consumes large amounts of carbon dioxide (CO2) in the water, raising the pH of the water column [15]. Alterations in pH can cause stress to aquatic organisms, either chronic or acute, which can affect their and immunological function and activity [16].

In an intensive rearing environment, photosynthesis and respiration cause pH fluctuations in the water column while influencing NH_3 concentrations to vary over 24 h. The increase in pH causes the rapid conversion of NH_4^+ to more stable NH_3 [17], and thus, pH determines the ratio between NH_3 and NH_4^+ and the NH_3 concentration. Studies have shown that the toxicity of ammonia nitrogen increases nearly 10-fold when the pH increases by 1.0 [18]. The undissociated and uncharged NH_3 molecules are lipid-soluble and, therefore, can easily pass through their membranes into cells and disrupt cellular metabolism. Biofilms are relatively impervious to ionized and hydrated forms of NH_4^+, which are usually considered less harmful to biological growth [19]. The toxicity of ammonia nitrogen to crustaceans is similar to the toxicity range of fish. Not only if threshold values are exceeded, but especially if they interact with chemical or abiotic stressors, changes in pH and ammonium levels can pose a risk to aquatic organisms. The spotted babylon is mainly cultured in the flow-through system on the southern coast of China. Changes in pH and ammonia nitrogen in the system can adversely affect the spotted babylon's physiology and growth processes, thus reducing its survival rate.

The aim of this study was to investigate the effects of dual stressors, pH and ammonia-nitrogen, on the behaviour of the spotted babylon and its stress-related enzymes. We determined the changes in the spotted babylon's immune activity through pH and ammonia stress tests to understand the spotted babylon's immune response better, provide some theoretical basis for its disease control, and provide a reference for the in-depth study of the spotted babylon.

2. Materials and methodology

2.1. Animals

The experiment was performed with the spotted babylon of weight (2.10 ± 0.27) g and length (2.40 ± 0.12) cm, which was acclimated for 2 d. This trial had been conducted at the Tropical Aquatic Research and Development Center, the South Sea Fisheries Research Institute, and the Chinese Academy of Fisheries Sciences (Lingshui, Hainan). During the experimental period, the water quality parameters were as follows: temperature (26.00 ± 1.00) °C, dissolved oxygen (DO) > 6.50 mg/L, ammonia nitrogen <0.01 mg/L, salinity (33.00 ± 0.80) ‰, nitrite nitrogen <0.04 mg/L. The test water used was seawater that had undergone sedimentation and sand filtration. All other physicochemical indices met the standard for fishery aquaculture water.

2.2. Experimental design and sampling

The experiment involved placing 1080 healthy and vigorously spotted babylon, with 30 individuals per tank, into 36 tanks with a capacity of 4 L each. This test was a 3 × 4 test, and each treatment was set in triplicate. The pH values selected for the experiment were 7.0, 8.0, and 9.0, while the ammonia nitrogen concentrations were 0.02, 1.02, 5.10, and 10.20 mg/L. Controls were defined as pH 8 and ammonia 0.02 mg/L. These values were chosen based on the tolerance limits of the spotted babylon [18,20]. Ammonia nitrogen concentration was prepared by adding NH_4Cl to seawater. The pH is adjusted by adding NaOH and $NaHCO_3$. Frozen mixed fish were fed daily at 9:00 a.m., and water changed 1 h after feeding, with each change exceeding 50% of the volume of the culture bucket. Remove leftover feed, faeces and dead spotted babylon from the breeding bucket in good time. Interval observations were conducted to record the behaviour and reverse-back rate of spotted babylon in each group. Every 3 h, the pH and ammonia nitrogen of the seawater

were measured and adjusted. The pH and total ammonia nitrogen concentration were measured using a pH meter (SMART SENSOR, Dongguan, China) and a multi-parameter analyzer (Octadem, Wuxi, China), respectively. Soft tissues from three individuals in each tank were sampled at 0, 24, 48, 72 and 96 h and mixed to detect changes in biochemical indices.

2.3. Behavioral observation

At 0, 24, 48, 72 and 96 h, behavioural changes were observed in the spotted Babylon. Reverse-backed means that the spotted babylon sank plunge to the bottom and was unable to remain upright. The rate of reverse-backed has been calculated in the following way:

Reverse back rate (%) = (Nt/N1) × 100%

In the equation, N_1 and N_t are the number of reverse-backed snails at the beginning of the experiment and at the observation time, respectively.

2.4. Biochemical index analysis

For each sample, 0.5 g was added to 1 mL of saline at a concentration of 0.5% by mass for grinding, and the grinding solution was centrifuged at 4000 r/min for 10 min at 4 °C. The supernatant was divided into two portions, placed in clean EP (Eppendorf) tubes to avoid repeated freezing and thawing during data measurement, and stored in a refrigerator at −80 °C for spare parts. Coomassie blue staining was used to determine the protein content of the homogenate [21]. Peroxidase (POD; A084-1-1; colorimetric method), acid phosphatase (ACP; A060-2-2; microenzymatic assay), total superoxide dismutase (SOD; A001-3-1; WST-1 method), alkaline phosphatase (AKP; A059-2-2; microenzymatic assay), catalase (CAT; A007-1-1; Ammonium molybdate method) and glutathione peroxidase (GSH-PX; A005-1-2; colorimetric method) were measured following the instructions of the manufacturer according to kits available on the market (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). Analyses of all parameters were carried out in triplicate.

2.5. Statistical analysis

Samples followed a normal distribution and were randomly and independently selected. Data were presented as mean ± standard deviation (mean ± SD). SPSS 21.0 was used for statistical analysis. Data were initially analyzed using a multivariate analysis of variance (MANOVA). Where there was a significant difference between treatments, the Duncan method was used to compare means at the $P < 0.05$ level of significance.

3. Results

3.1. Influence of pH and ammoniacal nitrogen stress on spotted babylon behaviour

In the group subjected to the pH and ammonia nitrogen stress test, Spotted Babylon exhibited varying degrees of stress response to alterations in pH and ammonia nitrogen. Spotted Babblers exhibited sluggish responses to external stimuli, slowed locomotion, reduced wall climbing, sinking to the base of the tank and an inability to stand upright, which were the main characteristics of spotted babblers following pH and ammonia nitrogen stress. As time progressed, both pH = 7 and pH = 9 showed increased reverse-back rates compared to control (pH = 8), and both pH = 9 and pH = 7 showed higher reverse-back rates. With the increase in ammonia nitrogen concentration, the spotted babylon's reverse-back rate also increased. The test group started to act slowly at 48 h and reverse back at 72 h, while in thereference group (pH = 8.0, ammonia nitrogen concentration 0.02 mg/L), reverse back only appeared at 96 h with the

Table 1
Effects of pH (7.0, 8.0 and 9.0) and ammonia nitrogen (0.02, 1.02, 5.10 and 10.20 mg/L) Spotted Babylon behaviour and survival.

		Reverse back rate/%					Behaviour				
		0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
pH = 7.0	0.02 mg/L	0	0	0	1 ± 0.6	3 ± 0.2	Normal	Normal	Slow	Slow	Reverse back
	1.02 mg/L	0	0	0	2 ± 0.3	4 ± 0.1	Normal	Normal	Slow	Reverse back	Reverse back
	5.10 mg/L	0	0	0	3 ± 0.4	6 ± 0.7	Normal	Normal	Slow	Reverse back	Reverse back
	10.20 mg/L	0	0	0	5 ± 0.1	8 ± 0.6	Normal	Normal	Slow	Reverse back	Reverse back
pH = 8.0	0.02 mg/L	0	0	0	0	1 ± 0.1	Normal	Normal	Normal	Normal	Slow
	1.02 mg/L	0	0	0	0	2 ± 0.5	Normal	Normal	Normal	Slow	Reverse back
	5.10 mg/L	0	0	0	0	4 ± 0.1	Normal	Normal	Normal	Slow	Reverse back
	10.20 mg/L	0	0	0	4 ± 0.1	6 ± 0.4	Normal	Normal	Slow	Reverse back	Reverse back
pH = 9.0	0.02 mg/L	0	0	0	2 ± 0.4	5 ± 0.1	Normal	Normal	Slow	Slow	Reverse back
	1.02 mg/L	0	0	0	3 ± 0.4	4 ± 0.7	Normal	Normal	Slow	Slow	Reverse back
	5.10 mg/L	0	0	0	5 ± 0.2	7 ± 0.2	Normal	Normal	Slow	Reverse back	Reverse back
	10.20 mg/L	0	0	0	6 ± 0.4	9 ± 0.8	Normal	Normal	Slow	Reverse back	Reverse back

Note: Reverse back means that the spotted Babylon is in an unhealthy condition with the belly side up and the shell side down.

extension of time. Control group compared, which had a pH of 8.0 and 0.02 mg/L ammonia, the spotted babylon exhibited a higher rate of back flopping over time at pH = 9.0 (0.02–10.20 mg/L ammonia) than at pH = 7.0 (0.02–10.20 mg/L ammonia). The toxic effect of ammonia nitrogen increases with higher pH and concentration, resulting in a higher reverse-back rate for the spotted babylon (Table 1).

3.2. Influence of pH and ammonia nitrogen stress on ACP activity of the spotted babylon

The ACP activity of spotted babylon was significantly affected by different pH, ammonia nitrogen and treatment time ($P < 0.05$). There is an interaction between pH and ammonia nitrogen concentration (Table 2). ACP activity peaked after 72 h of treatment at pH = 7.0 and 9.0 and ammonia concentrations between 0.02 and 10.20 mg/L and then declined. At the late stage of stress, the experimental group with ACP activity showed higher levels than all other groups when exposed to an ammonia concentration of 5.10 mg/L. ACP activity changed most at pH = 9.0 and ammonia concentrations between 0.02 and 10.20 mg/L. Overall, there has been a general decrease and then an increase in ACP activity over time (Fig. 1).

3.3. Influence of pH and ammonia nitrogen stress on AKP activity of the spotted babylon

The influence of different pH, ammonia nitrogen and treatment time on the viability of Spotted Babylon AKP was significant ($P < 0.05$) (Table 3). AKP activity gradually increased after 48 h at pH = 7.0 and ammonia concentrations ranging from 1.02 to 10.20 mg/L. The AKP activity also increased gradually at 48h when pH = 8.0 and 9.0, and the ammonia concentration was 0.02–10.20 mg/L. AKP activity increased as pH increased at ammonia concentrations of 5.10–10.20 mg/L. There was a general trend of a gradual increase with increasing time of AKP activity, but different patterns of change were observed in the different groups of the experiment (Fig. 2).

3.4. Influence of pH and ammonia nitrogen stress on POD activity of the spotted babylon

The POD activity of the spotted babylon was significantly affected by different pH, ammonia nitrogen, and treatment time ($P < 0.05$) (Table 4). As time progressed, the activity of the POD first decreased, then rose, and then reduced to the lowest level at 24 h. The POD activity showed the highest change at a pH of 9.0 and an ammonia concentration of 0.02–10.20 mg/L. At pH levels of 8.0 and 9.0, the POD activity was more significant in the experimental group than in the control group (pH = 8.0, ammonia concentration 0.02 mg/L; ammonia concentration 1.02–10.20 mg/L). Overall, there was a trend of decrease and then increase in POD activity over time (Fig. 3).

3.5. Influence of pH and ammonia nitrogen stress on GSH-PX activity of the spotted babylon

The GSH-PX activity of the spotted babylon was significantly affected by different pH, ammonia nitrogen, and treatment time ($P < 0.05$) (Table 5). With increasing time, the activity of GSH-PX increased and then decreased, reaching its extreme maximum value at 72 h. GSH-PX activity increased with increasing ammonia nitrogen concentration at the same pH. The activity of GSH-PX was significantly higher in the other groups compared to the control group (pH = 8.0, ammonia concentration 0.02 mg/L). There was an overall increase in GSH-PX activity with increasing pH during the same time. GSH-PX activity tended to improve and then decrease over time in each treatment group (Fig. 4).

3.6. Influence of pH and ammonia nitrogen stress on SOD activity of the spotted babylon

The SOD activity of the spotted babylon was significantly affected by different pH, ammonia nitrogen, and treatment time ($P < 0.05$) (Table 6). SOD activity changed significantly with time, showing a tendency to increase and then decrease. When pH = 7.0, there was no significant change in SOD activity among the groups. When the pH was constant, the SOD activity increased as the concentration of ammonia nitrogen increased. At a pH of 8.0 and a concentration of ammonia nitrogen of 10.20 mg/L, the most significant change in the activity of SOD was observed in the experimental group. At pH 8.0–9.0 and 10.20 mg/L ammonia, the highest shift in

Table 2
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of ACP in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	368.477	102.320	0.001
Intercept	1	28834.842	8006.983	0.001
Time	4	1044.870	290.144	0.001
pH	2	221.790	61.588	0.001
ammonia nitrogen	3	207.083	57.504	0.001
Time*pH	8	484.151	134.441	0.001
Time*ammonia nitrogen	12	179.123	49.740	0.001
pH*ammonia nitrogen	6	354.020	98.306	0.001
Time*pH*ammonia nitrogen	23	387.457	107.591	0.001
Error	121	3.601		

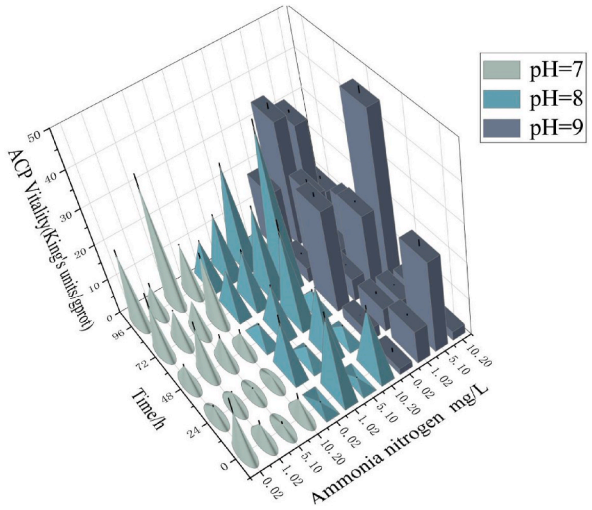

Fig. 1. Influence on ACP activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of AKP in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	18326.188	22.381	0.001
Intercept	1	556829.733	680.023	0.001
Time	4	174419.668	213.008	0.001
pH	2	10196.819	12.453	0.001
ammonia nitrogen	3	3975.982	4.856	0.001
Time*pH	8	20554.003	25.101	0.001
Time*ammonia nitrogen	12	5166.939	6.310	0.001
pH*ammonia nitrogen	6	2419.637	2.955	0.001
Time*pH*ammonia nitrogen	23	3134.312	3.828	0.001
Error	121	818.840		

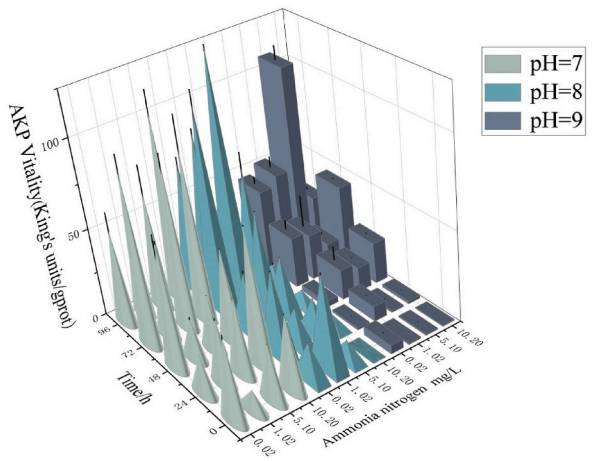

Fig. 2. Influence on AKP activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of POD in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	13.109	58.591	0.001
Intercept	1	626.111	2798.389	0.001
Time	4	96.778	432.545	0.001
pH	2	14.479	64.713	0.001
ammonia nitrogen	3	2.168	9.689	0.001
Time*pH	8	12.421	55.515	0.001
Time*ammonia nitrogen	12	6.358	28.416	0.001
pH*ammonia nitrogen	6	4.659	20.824	0.001
Time*pH*ammonia nitrogen	23	5.409	24.175	0.001
Error	121	0.224		

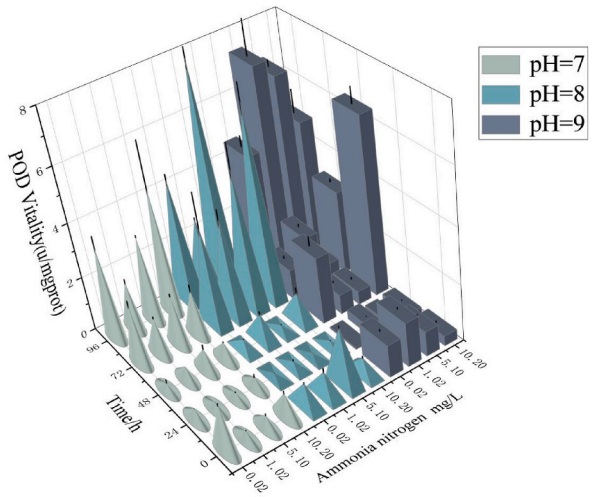

Fig. 3. Influence on POD activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of GSH-PX in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	4277670.677	198.566	0.001
Intercept	1	154438517.800	7168.894	0.001
Time	4	19117978.160	887.439	0.001
pH	2	108646.739	5.043	0.001
ammonia nitrogen	3	2691875.271	124.954	0.001
Time*pH	8	3910160.446	181.506	0.001
Time*ammonia nitrogen	12	1947083.335	90.382	0.001
pH*ammonia nitrogen	6	3432858.719	159.350	0.001
Time*pH*ammonia nitrogen	23	3296261.147	153.009	0.001
Error	121	21542.865		

POD activity was observed. Overall, the SOD activity of each treatment group tended to increase and then decrease with time (Fig. 5).

3.7. Influence of pH and ammonia nitrogen stress on CAT activity of the spotted babylon

The CAT activity of the spotted babylon was significantly affected by different pH, ammonia nitrogen, and treatment time ($P < 0.05$) (Table 7). The activity of CAT varied more significantly with ammonia nitrogen at pH 9.0 than at pH 7.0 and 8.0. At pH (7.0–9.0) and ammonia nitrogen concentration (0.02–10.20 mg/L) there was no significant change in CAT activity over 24 h. At pH values of 7.0 and 8.0, after 48 h and with an ammonia concentration ranging from 0.02 to 10.20 mg/L, CAT activity increased gradually over time, reaching its peak at 72 h before gradually decreasing. CAT activity increased with increasing ammonia nitrogen concentration when pH was held constant. With time, the CAT activity of each of the treatment groups showed a general tendency increase and then to

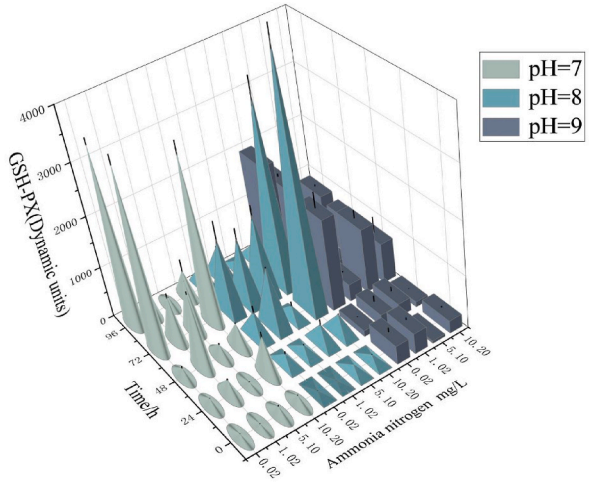

Fig. 4. Influence on GSH-PX activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of SOD in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	41.630	171.469	0.001
Intercept	1	3383.155	13934.980	0.001
Time	4	53.131	218.845	0.001
pH	2	203.272	837.263	0.001
ammonia nitrogen	3	43.060	177.361	0.001
Time*pH	8	57.245	235.787	0.001
Time*ammonia nitrogen	12	35.618	146.708	0.001
pH*ammonia nitrogen	6	37.734	155.424	0.001
Time*pH*ammonia nitrogen	23	21.938	90.362	0.001
Error	121	0.243		

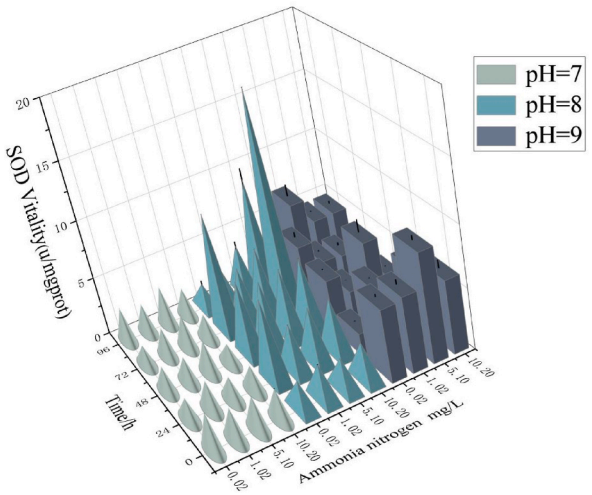

Fig. 5. Influence on SOD activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 7
Multivariate analysis of variance (ANOVA) was used to analyse the influence of pH and ammonia stress on the activity of CAT in spotted babylon.

Source	df	Mean Square	F	Sig.
Corrected Model	58	381.971	1139.881	0.001
Intercept	1	33834.378	100968.801	0.001
Time	4	2620.759	7820.888	0.001
pH	2	244.609	729.963	0.001
ammonia nitrogen	3	238.595	712.016	0.001
Time*pH	8	306.580	914.898	0.001
Time*ammonia nitrogen	12	248.383	741.226	0.001
pH*ammonia nitrogen	6	255.612	762.800	0.001
Time*pH*ammonia nitrogen	23	153.403	457.785	0.001
Error	121	0.335		

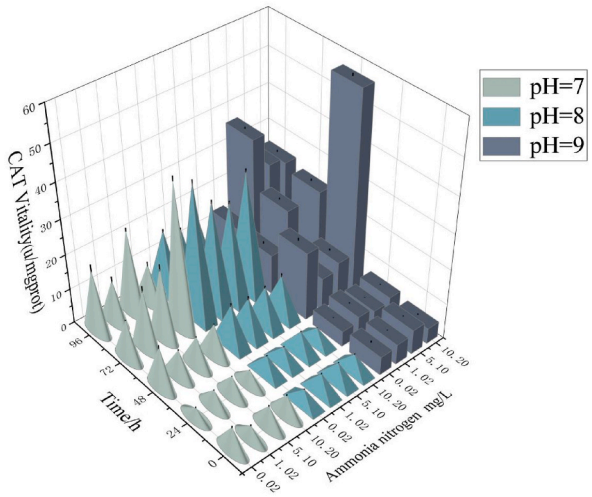

Fig. 6. Influence on CAT activity of spotted babylon by pH and ammonia nitrogen stress. An orange colour indicates a pH of 7.0, a grey colour indicates a pH of 8.0 and a dark green colour indicates a pH of 9.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decrease (Fig. 6).

4. Discussion

4.1. Behavioral changes in spotted babylons

The aquatic environment determines how farmed animals grow and develop. Inappropriate water conditions can cause metabolic stress and harm aquatic organism growth [22]. Fluctuations in pH and ammonia concentrations can significantly affect marine organisms, potentially resulting in compromised immunity or even mortality. During the experiment, the spotted babylon showed reversed and retarded movement as the time was prolonged. This may be related to the fact that changes in pH and ammonia nitrogen in the water disrupt the spotted babylon fluid balance and interfere with osmotic pressure homeostasis, leading to physiological dysfunction. There is an interaction between two environmental factors, pH and ammonia, when exposure to alkaline pH decreases the rate of conversion of NH_3 to NH_4^+, and H^+ reacts with NH_3 and converts to its ionized form, thus inhibiting NH_3 efflux and increases tissue NH_3 accumulation [23]. The study found that spotted Babylon's reverse back rate was higher at pH 7.0 and 9.0 compared to pH 8.0 for 96 h and at a specific ammonia nitrogen concentration. For instance, the reverse back rate for pH 9.0 at 10.20 mg/L ammonia concentration and pH 7.0 at 0.02 mg/L ammonia nitrogen concentration is 9 and 3 times higher, respectively, than that for pH 8.0 at 0.02 mg/L ammonia nitrogen concentration. Evidence suggests that the interaction between pH and ammoniacal nitrogen affects Spotted Babylon. In particular, the combined impact of pH and ammonia nitrogen is more significant than the impact of either factor individually. High concentrations of ammonia nitrogen can stimulate the neuroendocrine system of shellfish, which responds by regulating the antioxidant system, osmolarity-regulating enzymes, and energy metabolism [24]. Aquatic organisms can regulate their osmotic pressure using an internal buffering system to maintain their ionic and acid-base balance. This enables them to adapt to changes in their external surroundings to a degree [25]. Exceeding the threshold of the buffer can, however, disrupt the physiological acid-base and ionic levels of the body, upsetting the equilibrium and affecting the behaviour of cultured organisms, causing them to swim more slowly, feed less frequently or even die. [26,27]. The spotted babylon showed some tolerance to environmental change. The

antioxidant enzyme activities in the spotted babylon were significantly increased in the first 72 h, which indicates that pH and ammonia nitrogen stimulated the spotted babylon to produce a large amount of highly reactive oxygen species. At an early stage, the spotted babylon organism can still respond to outside cues effectively to protect cells by producing antioxidant enzymes for the timely removal of superoxide anions [26]. Therefore, in the early stages, the spotted babylon has no significant symptoms. When ecological factors change, living things undergo several different stress responses, in which the production of oxygen free radicals during these responses can cause damage to the organism [27]. Over time, changes in pH, ammonia-nitrogen concentrations, and pH-ammonia-nitrogen interactions caused oxidative and tissue damage to the spotted babylons, disrupting cellular function. Therefore, in actual factory farming, controlling the pH and ammonia nitrogen of the culture water, as well as purifying the polluted water within 72 h, will effectively reduce the negative effects of the refined water on the spotted babylon.

4.2. Changes in antioxidant enzyme activity

Shellfish only have innate immunity, so antioxidant enzymes (SOD, CAT, etc.) play an essential role in protecting cells from free radical damage [28]. It is important to avoid the accumulation of ammonia nitrogen in the cells, as high concentrations of ammonia nitrogen can be toxic to shellfish, inhibiting their metabolism and causing an influx of ammonia nitrogen into the cells [29,30]. In comparison with a low pH, a high pH inhibits the resistance to external ammonia nitrogen and the ammonia nitrogen metabolism, resulting in an easy entry and a difficult exit of ammonia nitrogen from the environment. The elevated levels of ammonia nitrogen result in oxidative stress due to the production of reactive oxygen species (ROS) [31]. The immune system of shellfish is usually sensitive to different environmental stresses, so when exposed to environmental stresses, the organism produces excess ROS, leading to oxidative damage [32]. When excess ROS are produced, the organism activates its antioxidant system to eliminate the excess ROS and maintain proper functioning. SOD and POD, as necessary endogenous antioxidant enzymes, convert O_2 to H_2O_2 and O_2, and GSH-PX and CAT convert H_2O_2 to harmless H_2O and O_2 [33]. Moreover, the strength of the defence of shellfish against pathogens has been assessed by the activity of SOD, CAT, POD, and GSH-PX [34]. During the experiment, it was observed that the SOD and POD activities of the spotted babylon increased gradually as the ammonia concentration ranged from 0.02 to 10.20 mg/L in the pH = 8.0 and 9.0 groups. The activities of SOD and POD were also higher in the pH = 9.0 group as compared to the pH = 8.0 group. When shellfish are exposed to environmental stress, the activities of SOD and POD in the tissues increase significantly to adapt to the environment [35]. The GSH-PX and CAT activities, on the other hand, also showed an increasing trend followed by a decreasing trend in the experiment. GSH-PX and CAT activity also tended to increase and decrease during desiccation and submersion in *Marsupenaeus japonicus* [36]. The data are an indication that the ROS system is crucial in the innate defence against environmental stress.

The study demonstrated that the spotted babylon's POD activity increased significantly following exposure to pH and ammonia stress, reaching its maximum value at 96 h. Similarly, POD activity was significantly increased in black tiger shrimp in response to the oxidation of Vibrio parahaemolyticus [37]. The pattern of changes in POD activity was similar, although the timing of the peak was different. The increase in POD activity can potentially reduce the damage caused by free radicals in normal cells, thereby enhancing the immune function and detoxification of the shellfish against disease infections [38]. At lower combinations of ammonia nitrogen and pH, the increased POD activity could remove the produced O^{2-}. The increased activity of POD may be able to compensate for the decreased activity of SOD. Therefore, in this experiment, the SOD activity increased gradually with time and gradually reduced at 72 h. The trend of POD activity was the opposite. The increase in GSH-PX and CAT activity can remove H_2O_2 increased by the elevated POD and SOD activity. At pH 9.0 and ammonia concentrations between 1.02 and 10.20 mg/L, the activity of GSH-PX and CAT is greater than that of the controls (pH = 8.0, ammonia concentration = 0.02 mg/L). These trends were in line with the changes observed in the activities of the POD and the SOD. This suggests that GSH-PX and CAT are more closely related to POD and SOD. The increase in antioxidant enzyme activity indicates enhanced antioxidant capacity [39]. The interaction between ammonia nitrogen and pH induces a state of oxidative stress. This results in excess ROS that cannot be efficiently scavenged, leading to a reduction in antioxidant capacity and an imbalance between ROS and antioxidant systems [40]. Zhou et al. also found that high salt disrupts the balance between ROS production and utilization in clams. Excess ROS can attack their surrounding biomolecules, leading to oxidative damage [41].

4.3. Changes in immunoenzymes activity

ACP and AKP are phosphatases in the lysosomal and endosomal systems of shellfish and other aquatic animals and are essential immune marker enzymes and detoxification enzymes in marine animals. In animals, it is extensively involved in cell phagocytosis, immune response, regulation of energy metabolism, cell damage and repair, and other life processes [44]. ACP and AKP are essential components of the detoxification system in animals, as well as essential reference indicators of the immune function and health status of animals [42]. Xu et al. found that *Chinese horseshoe crabs* showed significantly higher ACP and AKP activities under copper supplementation conditions [43]. In this study, the ACP and AKP enzyme activities of spotted babylon tended to change in the same way as in the control group (pH = 8.0, ammonia nitrogen 0.02 mg/L) in the other experimental groups under different conditions, all of which showed different degrees of improvement. Furthermore, the ACP activity was significantly higher at a pH of 9.0 and an ammonia nitrogen concentration ranging from 1.02 to 10.20 mg/L, compared to pH 7.0 and 8.0 with the same ammonia nitrogen concentration. It is suggested that chemical environmental pollutants activate the non-specific immune system of the spotted babylon, leading to enhanced non-specific immunity. It has been shown that ACP is released when xenobiotics and other environmental factors cause physiological or structural changes, including lysosomal fragility [44]. At 72 h after exposure to ammonia stress, AKP activity significantly increased. This suggests that pH and ammonia stress-induced physiological or structural changes in the spotted babylon. In addition, the spotted babylon needs to consume more energy in response to external adverse stimuli to meet the energy

requirements of its stress metabolism. Therefore, the elevated AKP activity is an energy-compensatory effect of the spotted babylon in response to external adverse factors and an adaptive mechanism for the spotted babylon to tolerate pH and ammonia stress. To adjust to the external environment, *Eriocheir sinensis* ACP and AKP activities also increased significantly during saline stress [45]. It was noted that prolonged stress can cause enhanced or even severely higher than normal levels of immune function in the animal organism, and in *Ctenopharyngodon idellus*, AKP and ACP activity increased dramatically after 56 days of feeding 1% honeysuckle (Lonicera caerulea L.) compared to the control [46]. At the end of the study, the activities of ACP and AKP increased significantly in the group with a pH of 9.0 and an ammonia nitrogen concentration of 10.20 mg/L. The reverse-backrate of spotted babylon also reached its highest value. The spotted babylon's non-specific immune regulation has reached its limit, which has reduced various physiological functions. As a result, the body is unable to maintain a normal physiological state.

5. Conclusion

This study investigates at both biochemical and protein levels the combined effects of pH and ammoniacal nitrogen on the antioxidant and immune response of the spotted babylon. This study demonstrated the impact of the interaction between pH and ammonia nitrogen on various antioxidant and immune indices. The combined effect of pH and ammonia nitrogen increases oxidative stress and generates excess ROS. To scavenge excess ROS, antioxidant enzyme systems are activated. Simultaneously,the immune system is activated to maintain overall health. These findings provide a new understanding of the effects of the interactions generated under various culture conditions on the immune mechanisms and antioxidants of the spotted babylon. It also provides fundamental information for evaluating environmental stresses in cultured shellfish. There are few reports on the effects of the interaction between pH and ammonia on the growth and physiological metabolism of the spotted babylon. This suggests that spotted babylon aquaculture should not only focus on changes in pH as a single water quality indicator, but also pay attention to the correlation between water quality indicators. This will help in selecting spotted babylon as a suitable aquaculture water body. To improve the efficiency of Spotted Babylon culture, We will investigate methods to reduce pH and ammonia nitrogen-induced oxidative stress on Spotted Babylon are being investigated.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Institutional review board statement

The animal study was reviewed and approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The ethical code is CAFS (2020TD55).

Informed consent statement

Not applicable.

CRediT authorship contribution statement

Ruixia Ding: Writing – original draft, Methodology, Data curation. **Rui Yang:** Visualization, Software, Methodology. **Zhengyi Fu:** Writing – review & editing, Formal analysis. **Wang Zhao:** Supervision, Project administration, Investigation. **Minghao Li:** Validation, Methodology, Investigation. **Gang Yu:** Supervision, Resources, Project administration. **Zhenhua Ma:** Writing – review & editing, Supervision. **Zemin Bai:** Writing – review & editing, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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