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Impacts of circadian rhythm and melatonin on the specific activities of immune and antioxidant enzymes of the Chinese mitten crab (*Eriocheir sinensis*)

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ABSTRACT

Many physiological functions of crustaceans show a rhythmic change to adapt to daily environmental cycles. However, daily variation in the immune and antioxidant status and its possible correlation with circulatory melatonin levels during the daily cycle have not been reported in the Chinese mitten crab, *Eriocheir sinensis*. In this study, the specific activities of immune and antioxidant enzymes of *E. sinensis* during the 24 h cycle and its relationship with injected doses of melatonin were evaluated. The results showed that the immune parameters in the hemolymph, such as total hemolymph count, alkaline phosphatase, lysozyme, acid phosphatase, and phenol oxidase, exhibited bimodal patterns during the 24 h cycle, these parameters were synchronized with the activity of antioxidant enzymes such as malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase, and catalase. However, there was only one peak in the muscle (during 1200–1600 h) and gills (during 0400–0800 h). The survival rate reached approximately 80% in 5 days when melatonin concentrations were lower than 0.05 g/L, significantly decreasing as melatonin concentrations increased. Four hours after melatonin injection, MDA levels in the muscle and hemolymph were significantly lower than those in the control group. Eight hours after melatonin injection, SOD levels in the hemolymph were significantly higher than those in the control group. These findings highlight the importance of considering circadian regulation of innate immunity when comparing immune responses at fixed times.

1. Introduction

Most organisms display physiological and behavioral rhythms during the 24 h light/dark cycle. In crustaceans, circadian rhythms have been reported to affect various physiological functions such as sensitivity of sensory organs [1,2], antioxidant defense systems [2,3], blood sugar regulation [4], oxygen consumption [5], and heart rate [6]. However, there is a lack of reports on circadian rhythms in the immune and antioxidant systems of crustaceans, which are believed to be closely involved in host defense against potential pathogens [7,8]. Therefore, in the present study, we aimed to investigate the antioxidant and immune functions in the diurnal variations in crustaceans.

It is well known that the circadian rhythm information is regulated in organisms, at least in part, by a biogenic amine, melatonin, which is also involved in the regulation of many physiological functions in crustaceans [1]. Geihs, Vargas, Maciel, Caldas, Cruz, Primel, Monserrat and Nery [9] and Maciel, Ramos, Geihs, Vargas, Cruz, Meyer-Rochow, Vakkuri, Allodi, Monserrat and Nery [10] first demonstrated the relationship between melatonin and activity of antioxidant enzymes in crustaceans, but only muscle and gills were tested. Although those enzymes are present in the hemolymph [11] and optic lobe [12] to assist in the protection of the host tissues against reactive oxygen species (ROS), little is known about the regulatory mechanisms of melatonin in these tissues. Moreover, the influence of melatonin concentration and actuation duration on ROS remains unknown. Furthermore, there are no reports available on the melatonin regulation of the immune system in crustaceans.

The Chinese mitten crab, *Eriocheir sinensis*, is an economically important decapod crustacean species in China and has spread to Europe and America as an invasive species [13]. In our previous study, we showed that, similar to other crustaceans, *E. sinensis* is active and molt at night [14]. However, the circadian rhythm of specific enzymes

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Abbreviations

AKP	alkaline phosphatase
ACP	acid phosphatase
PO	phenol oxidase
LZM	lysozyme
ROS	reactive oxygen species
SOD	superoxide dismutase
CAT	catalase
GPx	glutathione peroxidase
GSH	reduced glutathione
MDA	malondialdehyde
THC	total hemolymph count

activities remains unknown. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA), represent the first line of defense of antioxidative stress; furthermore, they are potentially involved in the regulation of redox and innate immune responses in *E. sinensis* [15,16]. Immune enzymes, such as lysozyme (LZM), phenol oxidase (PO), acid phosphatase (ACP), and alkaline phosphatase (AKP), represent the first line of defense in nonspecific immune responses. Therefore, further studies are required to elucidate the influence of circadian rhythm on status of specific enzymes activities. It is well known that melatonin participates in circadian rhythms, immunity and antioxidant defense in crustacean. In *E. sinensis*, both haemolymph and eyestalks are exhibited significant peaks at 12:00 and 24:00 [17,18]. However, to the best of our knowledge, the mechanism of melatonin regulation in the immune system of *E. sinensis* has not been studied.

2. Materials and methods

2.1. Crabs

A total of 420 *Eriocheir sinensis* individuals (45.63 ± 6.59 g) were sampled from rice fields in Panjin City, Liaoning Province, China, in August 2017, and transported to the aquaculture laboratory at Shenyang Agricultural University. They were acclimated in 300 L square fiberglass recirculation tanks (12 tanks in total, each containing 30 crabs) at 18 °C ± 0.5 °C and a 12:12 h light:dark cycle. Six of these tanks were used for circadian rhythm experiments, and the remaining six were used for melatonin injection experiments. These tanks were linked to four recirculation systems, each with a mechanical filter, UV sterilizer, and central temperature system. Light was controlled by automatic timing switch (turn on at 8:00, turn off at 18:00), and light intensity increase gradually. The crabs were allotted 2 weeks of acclimation, after which they were starved for 24 h prior to the experiment.

Prior to the melatonin experiment, 70 crabs (53.25 ± 8.42 g) were randomly divided into seven groups for the preliminary experiment. To determine a safe dose of melatonin injection, each group consisted of 10 crabs were injected with 2 mL melatonin solution with concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2, and 5 g/L at 0900 h. Dead crabs were recorded during the 5-day experiment. According to the results of the above preliminary experiment, the melatonin experiment consisted of three levels, at melatonin concentrations of 0.0001 g/L, 0.001 g/L, and 0.01 g/L, as well as a control without melatonin. For melatonin injection experiments, six individuals were randomly taken from each of the four groups for sample collection, where muscles and hemolymph were collected at 1 h, 2 h, 4 h, 8 h, and 12 h after the injection time at 8:00. Standard melatonin was purchased from Sigma-Aldrich Chemical Co. (USA) and dissolved in crustacean saline (0.21 M NaCl, 13.6 mM KCl, 8.6 mM H_3BO_3, 4.75 mM NaOH, 20 mM $MgSO_4{\cdot}7H_2O$; pH 7.2). Each crab was injected in the fifth pair of epipodites with 2 mL of solution.

2.2. Sample preparation

For circadian rhythm experiments, the hemolymph, muscle, and gills of 10 *E. sinensis* individuals from each tank were obtained every 4 h over 24 h. All samples were collected under natural light or under dim red light at night. For melatonin injection experiments, six individuals were randomly taken from each of the four groups for sample collection, where muscles and hemolymph were collected at 1 h, 2 h, 4 h, 8 h, and 12 h after the injection time.

Hemolymph was drawn using a sterile 1 mL syringe from the unsclerotized membrane of the right third pleopod and was immediately diluted 1:1 with sterile anticoagulant (30 mM trisodium citrate, 338 mM NaCl, 115 mM glucose, and 10 mM EDTA). The serum was collected and stored at −20 °C until the antioxidant capacity could be evaluated. The muscles and gills were collected and stored at −20 °C for analysis.

2.3. Total hemolymph count measurement

The total hemolymph count (THC) was obtained using a drop of anticoagulated hemolymph placed on a hemocytometer and an inverted phase-contrast microscope (Olympus IX-71, Tokyo, Japan). Treatments

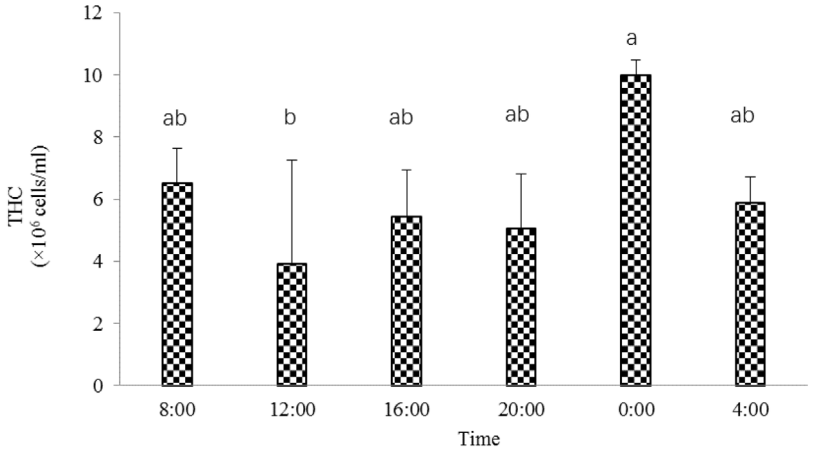

Fig. 1. Daily variation in the total hemolymph count (THC) in *Eriocheir sinensis*. The values are expressed as the means ± SD (n = 10). Different letters placed above the column represent significant differences between times ($P < 0.05$).

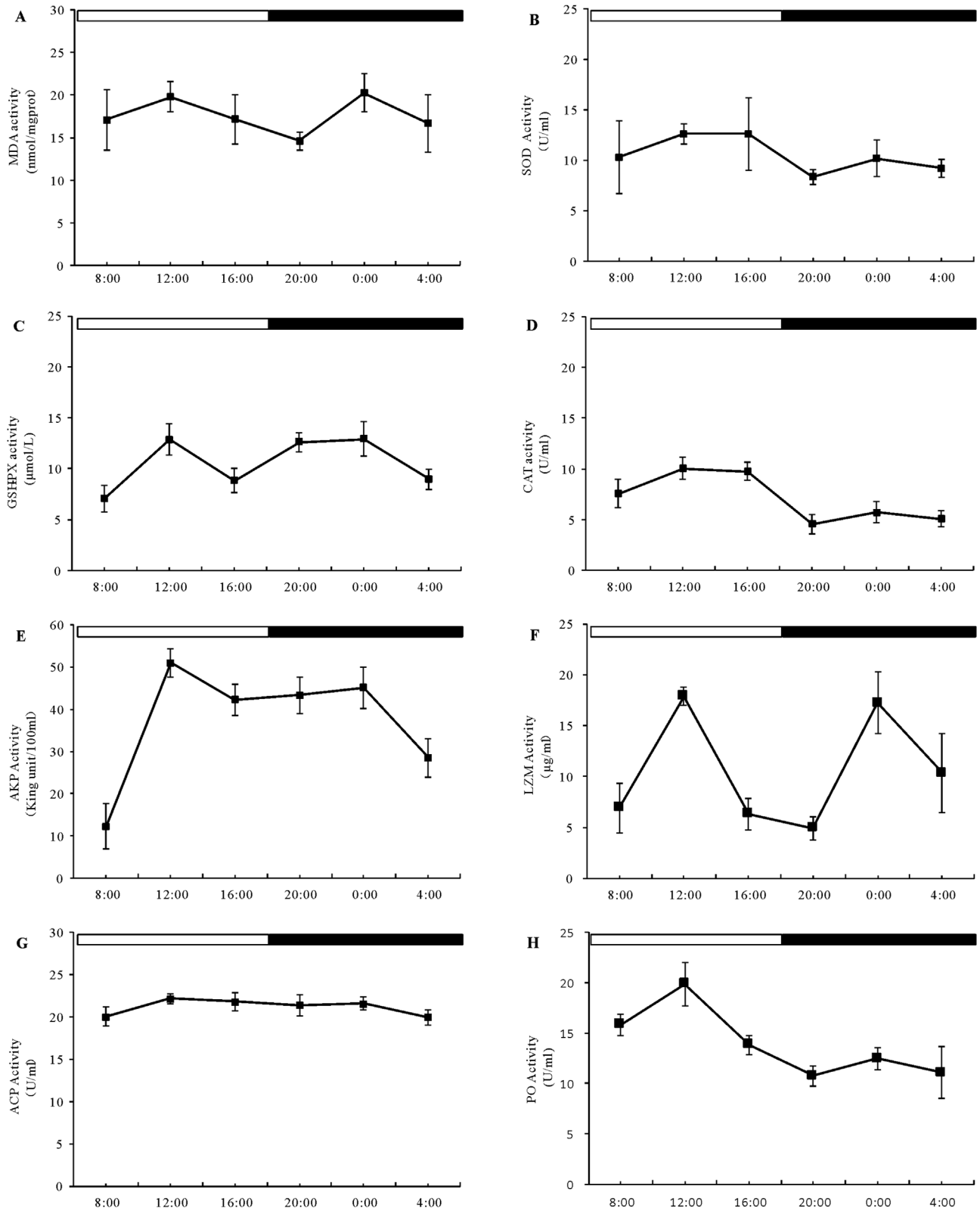

Fig. 2. Daily variation in the MDA, SOD, GPx, CAT, AKP, LZM, ACP, and PO activity levels in the hemolymph of *E. sinensis*. The values are expressed as the means ± SD (n = 10). (A) Malondialdehyde (MDA) activity; (B) superoxide dismutase (SOD) activity; (C) glutathione peroxidase (GPx) activity; (D) catalase (CAT) activity; (E) alkaline phosphatase (AKP) activity; (F) lysozyme (LZM) activity; (G) acid phosphatase (ACP) activity; (H) phenol oxidase (PO) activity.

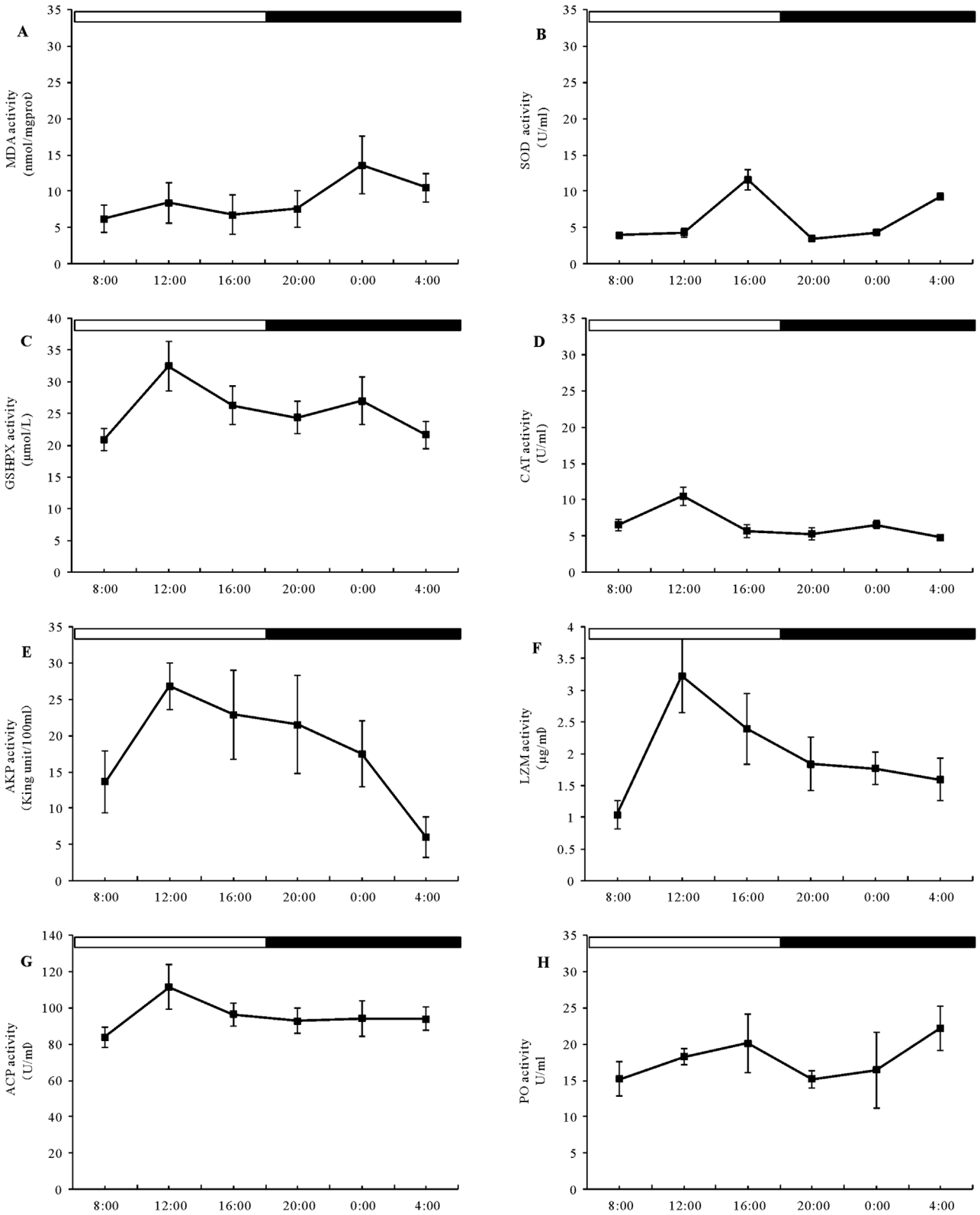

Fig. 3. Daily variation in the MDA, SOD, GPx, CAT, AKP, LZM, ACP, and PO activity levels in the muscle of *E. sinensis*. The values are expressed as the means ± SD (n = 10). (A) Malondialdehyde (MDA) activity; (B) superoxide dismutase (SOD) activity; (C) glutathione peroxidase (GPx) activity; (D) catalase (CAT) activity; (E) alkaline phosphatase (AKP) activity; (F) lysozyme (LZM) activity; (G) acid phosphatase (ACP) activity; (H) phenol oxidase (PO) activity.

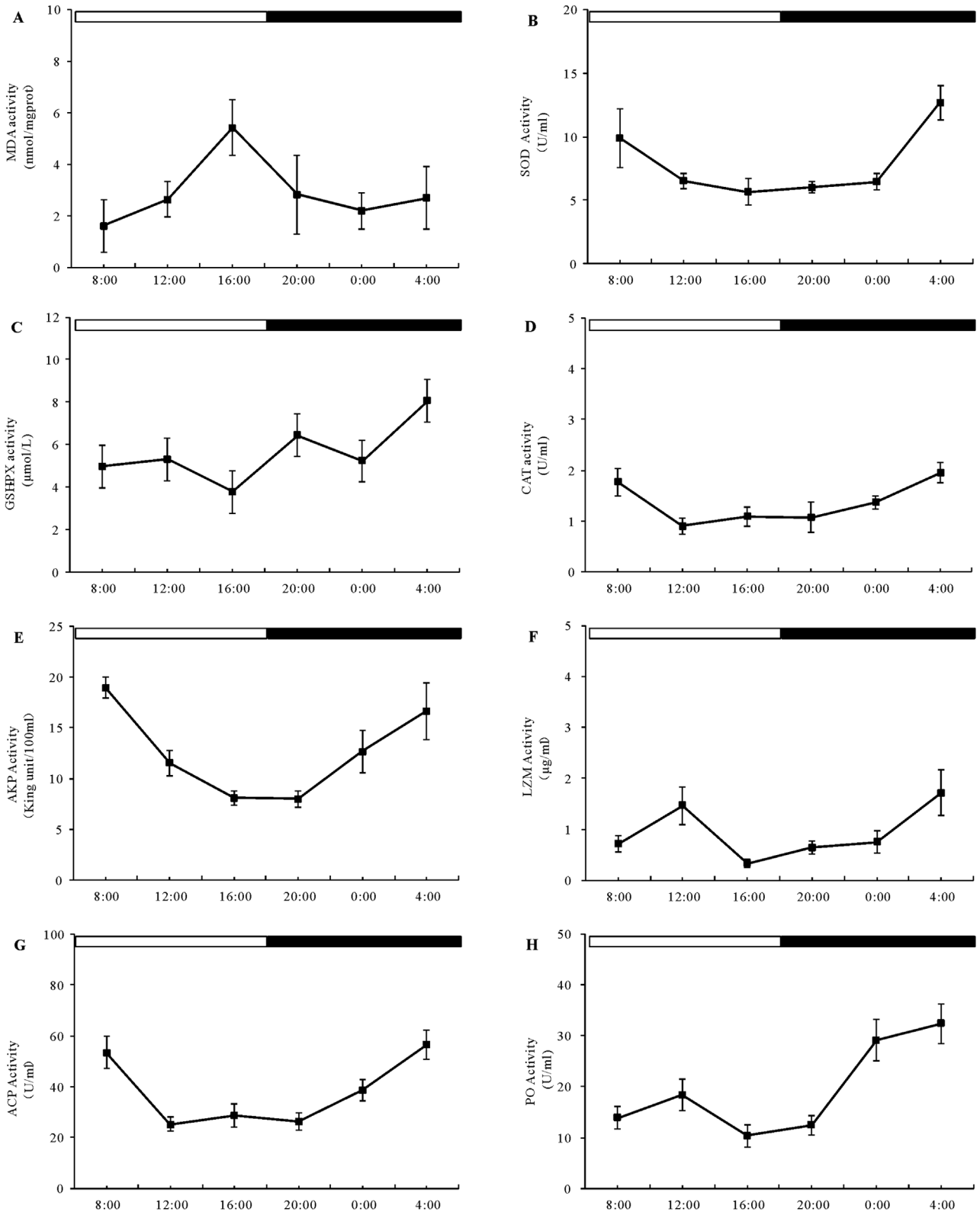

Fig. 4. Daily variation in the MDA, SOD, GPx, CAT, AKP, LZM, ACP, and PO activity levels in the gills of *E. sinensis*. The values are expressed as the means ± SD (n = 10). (A) Malondialdehyde (MDA) activity; (B) superoxide dismutase (SOD) activity; (C) glutathione peroxidase (GPx) activity; (D) catalase (CAT) activity; (E) alkaline phosphatase (AKP) activity; (F) lysozyme (LZM) activity; (G) acid phosphatase (ACP) activity; (H) phenol oxidase (PO) activity.

were measured in triplicate for each sample.

2.4. Antioxidant defense systems parameters

Commercial kits obtained for CAT, MDA, SOD, and GPx from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used to measure their respective activity levels. For enzyme activity, muscle and gills were weighed and homogenized (1:10 w/v) in a cold (4 °C) buffer solution. The hemolymph of the last centrifugation were used for analysis.

2.5. Nonspecific immune system parameters

The LZM, PO, ACP, and AKP activities were assayed using the corresponding detection kits (Nanjing Jiancheng Biological Product, Nanjing, China) according to the manufacturer's guidelines. PO activity was measured as described by Ashida [19]. Briefly, 10 μL of serum, 300 μL phosphate buffer (0.1 M, pH 6.0), and 10 μL of dihydroxyphenylalanine solution (0.01 M) were added to 96-well microtiter plates, and the absorbance (optical density at 490 nm [OD_{490}]) was determined every 2 min. An increase of 0.001/min in the OD_{490} was regarded as 1 unit of activity.

2.6. Statistical analyses

Statistical analyses were performed by analysis of variance (ANOVA). Significant differences between means were determined by Duncan's test, and the significance level was set at $P < 0.05$. All statistical analyses were performed using SPSS 20.0 software (Version 22.0; Chicago, IL, USA).

3. Results

3.1. Rhythmic variations of THC

The THC in *E. sinensis* exhibited circadian rhythms (Fig. 1). Although during most part of the day, the THC values were not significantly different from each other, the value at 1200 h ($3.91 \pm 3.34 \times 10^6$ cells/mL) was significantly ($P < 0.05$) lower than that at 0000 h ($9.99 \pm 0.49 \times 10^6$ cells/mL).

3.2. Rhythmic variations of specific enzyme activities

Specific enzyme activities exhibited circadian rhythms in the hemolymph, muscle, and gills of *E. sinensis*. In the hemolymph, GPX, SOD, and LZM had two significant ($P < 0.05$) peaks separated by 12 h, and MDA, AKP, and PO showed a similar trend (Fig. 2). While at 1200 h, all the enzymes reached maximum values, the activity levels of MDA, SOD, GPX, and LZM had a second peak at 0000 h. In the muscle, GPX, CAT, AKP, LZM, and ACP activities were found to be significantly higher at 1200 h than at other times ($P < 0.05$) (Fig. 3). However, MDA and SOD activities were found to have a significant peak at 0000 and 1600 h, respectively. Except MDA, all specific enzymes in the gills showed a higher activity from 0000 to 0800 h (early in the morning) (Fig. 4). The activity of MDA, GPX, and CAT in the hemolymph and muscles were significantly ($P < 0.05$) higher than that in the gills. Moreover, the activity of AKP and LZM in the hemolymph were significantly ($P < 0.05$) higher than that in the gills and muscles. Furthermore, the activity of AKP, LZM, ACP, and PO in the hemolymph all showed the same circadian rhythm trend as those in the muscle.

3.3. Survival rate at different melatonin concentrations

The effect of different concentrations of melatonin injections on the survival rate in crabs was measured (Fig. 5). When the melatonin injection concentration was lower than 0.05 g/L, the survival rate reached approximately 80% in 5 days. However, almost none of the crabs could survive past 5 days when the melatonin injection concentration was higher than 1 g/L. In the 5 g/L group, 90% of the crabs died within 24 h. Moreover, crabs injected with 0.0001 g/L, 0.001 g/L, and 0.01 g/L showed no behavioral abnormality compared with the control group. Therefore, melatonin injections at concentrations of 0.0001 g/L, 0.001 g/L, and 0.01 g/L were considered safe for *E. sinensis*.

3.4. Effects of melatonin on antioxidant enzyme activity

After melatonin injection, antioxidant enzyme activity in the hemolymph of none of the test groups differed significantly from the control group, except for the 4 h post-injection group of MDA, which showed significantly lower activity than that of the control group ($P < 0.05$), and the 8 h post-injection group of SOD, which showed significantly higher activity than that of the control group ($P < 0.05$) (Fig. 6).

Similar to the hemolymph results, after melatonin injection, antioxidant enzyme activity in the muscle showed no differences between samples from 1 h post-injection, 2 h post-injection, and 12 h post-injection groups (Fig. 7). However, 4 h post injection, the MDA activity of the 0.01 g/L melatonin injection group was significantly lower than that of the control group ($P < 0.05$), and the SOD activity of the 0.00 1 g/L and 0.0001 g/L groups were significantly higher than that of the control group ($P < 0.05$). Four hours post injection, CAT activity in the 0.0001 g/L melatonin injection group was significantly higher than that in the control group, but no differences were observed between samples of the 0.001 g/L and 0.01 g/L groups.

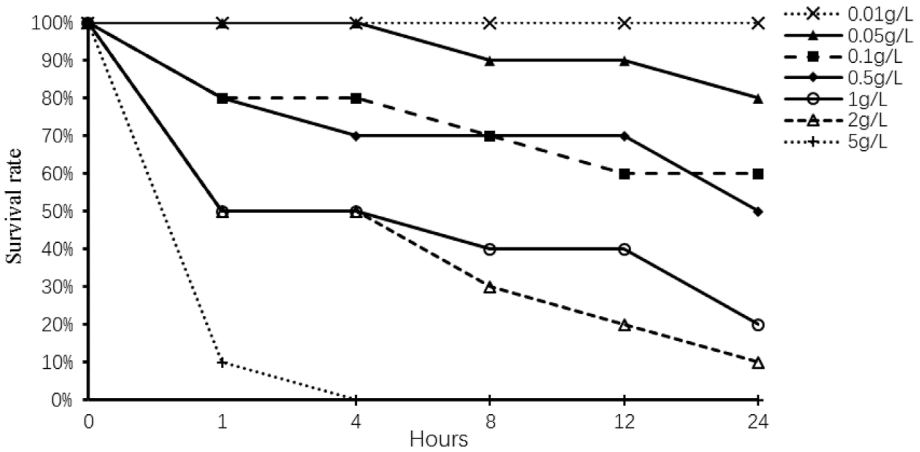

Fig. 5. Survival rate of *E. sinensis* under melatonin injections of different concentrations (mean ± SD; n = 6).

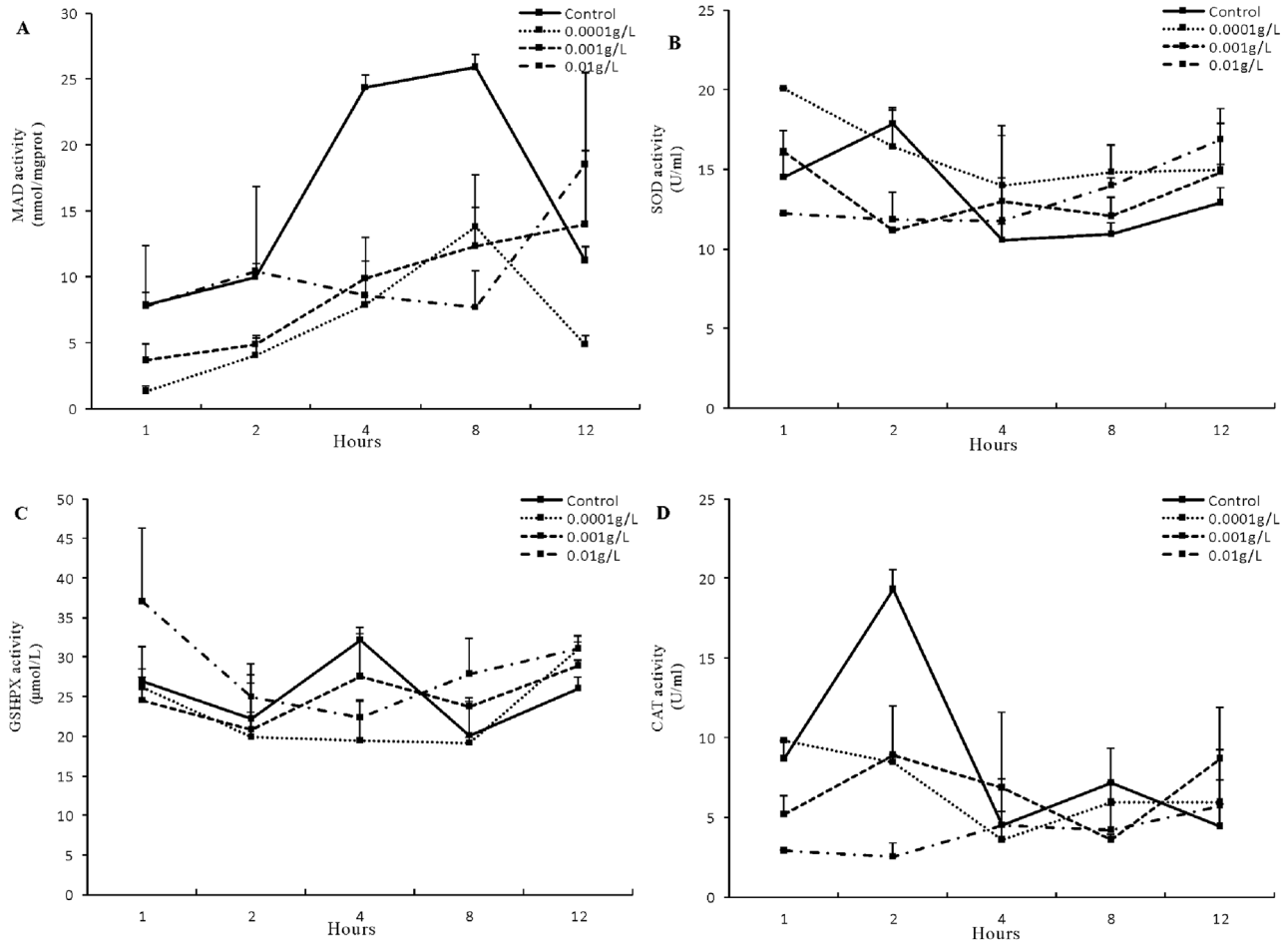

Fig. 6. Activity of MDA, SOD, GPx, and CAT in the hemolymph of *E. sinensis* under different melatonin doses. The values are expressed as the means ± SD (n = 6). (A) Malondialdehyde (MDA) activity; (B) superoxide dismutase (SOD) activity; (C) glutathione peroxidase (GPx) activity; (D) catalase (CAT) activity.

4. Discussion

The immune and antioxidant systems serve to identify and protect against pathogens and oxidative stress to ensure appropriate response in organisms. Thus, any rhythmic variation in these systems is associated with the circadian rhythm. Although circadian modulation may influence the innate immune response in *Drosophila* [20], the relationship between circadian regulation and immune response in crustaceans remains unclear. Crustaceans possess an innate immune system intimately related to their hemolymph, which contains circulating hemocytes involved in cellular immune reactions in addition to a variety of specific enzymes, such as immune enzymes and antioxidant enzymes [21–23]. Several studies on crustaceans have demonstrated that such hematological parameters are important for assessing the immune response [24–26].

In a previous report, THC, the most commonly used performance parameter for evaluating cellular immunity in crayfish, showed a circadian rhythm during day–night cycles [12,27]. The present study also demonstrated that in *E. sinensis*, THC followed a circadian rhythm and exhibited two peaks (1200 and 0000 h), which dovetails with the immune-related enzyme activity in the hemolymph, including AKP and LZM. However, SOD, CAT, and PO in the hemolymph of *E. sinensis* are only active during the light phase, probably as a measure to be prepared for the increase in the metabolic rate that occurs later, similar to that in *Lithodes santolla* [3].

The gills of a crab provide the first line of antioxidant defense, because this organ is the first to come into contact with any changes that occur in the water [5,28]. In general, the antioxidant defense system of their gills is more active during the dark phase, showing peaks of enzymatic activity and non-enzymatic antioxidants, similar to *L. santolla* [3]. In the present study, the relative activities of SOD, GPX, and CAT in the gills of *E. sinensis* also showed a similar profile, indicating higher metabolic activity during the night, probably due to an increase in oxygen uptake, similar to most crabs [2,5]. According to the activities of AKP, LZM, ACP, and PO in the present study, daily variation in immune stress occurs coinciding with the antioxidant enzymes. The locomotor muscle is one of the most energetically expensive tissues in animals. Hence, it is expected that this tissue has an effective antioxidant defense system to avoid oxidative damage during the day cycle. In the present study, although enzymatic activity in the muscle of *E. sinensis* was followed by periodic rhythms, similar to most crabs (except MDA, SOD and PO), all enzyme activities peaked at 1200 h during the light phase in the muscle, which was opposite to the response of the gills.

In present study, the relative activity of the enzymes showed significant differences in the different tissues of *E. sinensis*. The activities of MDA, GPX, and CAT in the hemolymph and muscle were significantly higher than those in the gills in *E. sinensis*, which is contrary to the conclusion of studies on the lithodid crab, *L. santolla* [3,29]. This could be attributed to the fact that *L. santolla* inhabits the intertidal zone and has air exposure for long periods of time, thereby requiring a higher antioxidant activity. Moreover, *L. santolla* is more sensitive to PO than other crustacean species [30]. However, the relative activities of AKP and ACP in the muscle were significantly higher than those in the hemolymph and gills. In general, the activities of AKP and ACP were sensitive to environmental salinity and dopamine in the muscle of crabs

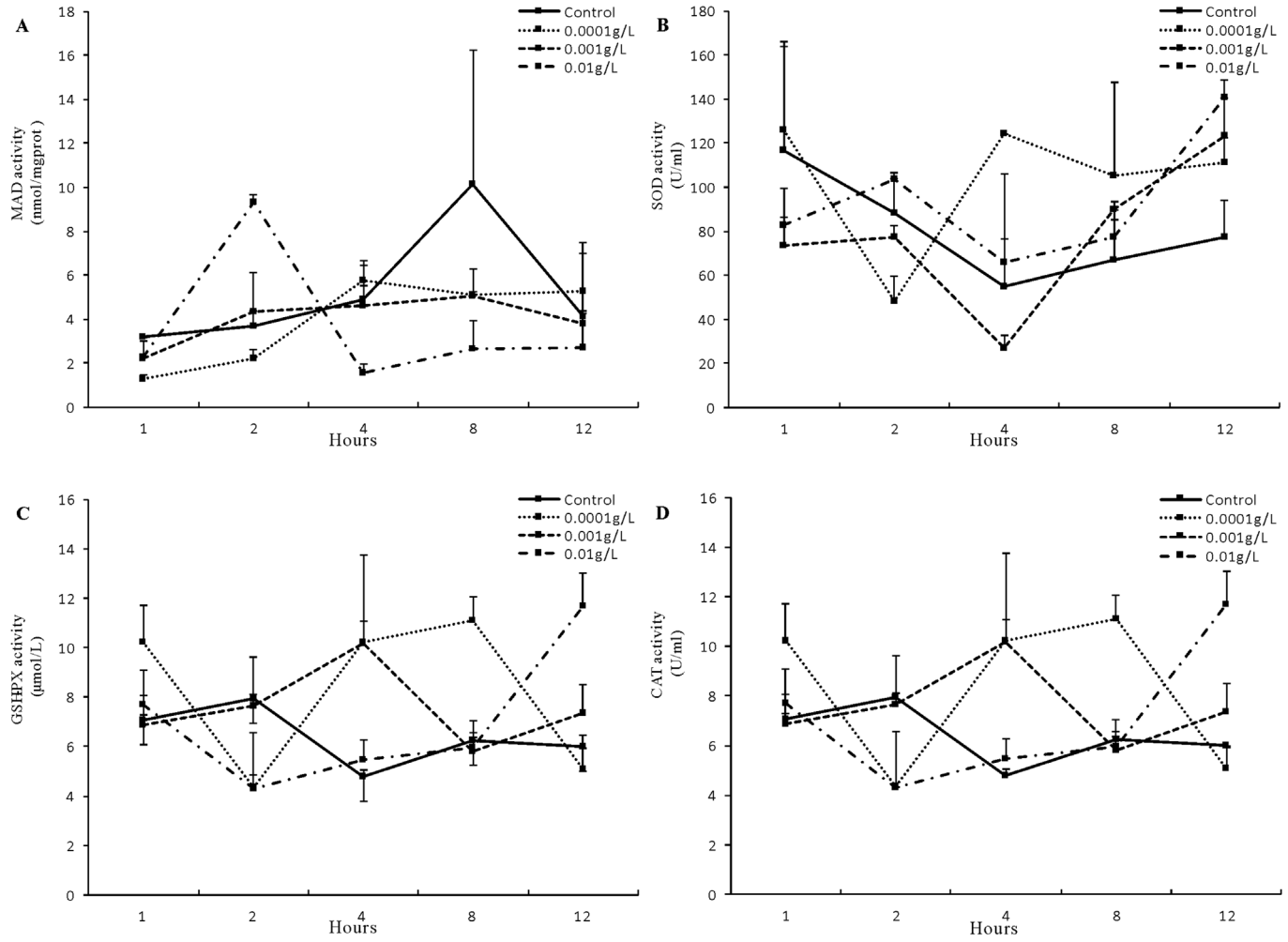

Fig. 7. Activity of MDA, SOD, GPx, and CAT in the muscle of *E. sinensis* in response to different melatonin doses. The values are expressed as the means ± SD (n = 6). (A) Malondialdehyde (MDA) activity; (B) superoxide dismutase (SOD) activity; (C) glutathione peroxidase (GPx) activity; (D) catalase (CAT) activity.

[31,32].

Exogenous melatonin regulates certain antioxidant enzymes, such as SOD and GPx, and neutralize both reactive oxygen and reactive nitrogen species thereby improves antioxidant defense system in crustacean [1]. Although the oxidant defense effects of melatonin may be related to its ability to scavenge free radicals, high concentration melatonin may become a toxic affecting the lifespan of animals [33]. Melatonin concentration above 100 mg/L reduces the lifespan of *Caenorhabditis elegans* [34]; in addition, the mortality is elevated with melatonin concentration in *Drosophila melanogaster* [35]. In present study, crabs injected with 0.0001 g/L, 0.001 g/L, and 0.01 g/L showed no behavioral abnormality compared with the control group and no mortality were observed during 5-days toxic experiment. Therefore, melatonin injections at concentrations of 0.0001 g/L, 0.001 g/L, and 0.01 g/L were considered safe for *E. sinensis*.

When the melatonin injection concentration was lower than 0.05 g/L, the survival rate reached approximately 80% in 5 days. However, almost none of the crabs could survive past 5 days when the melatonin injection concentration was higher than 1 g/L. In the 5 g/L group, 90% of the crabs died within 24 h. In addition, crabs injected with 0.0001 g/L, 0.001 g/L, and 0.01 g/L showed no behavioral abnormality compared with the control group. Therefore, melatonin injections at concentrations of 0.0001 g/L, 0.001 g/L, and 0.01 g/L were considered safe for *E. sinensis*.

Although melatonin is known to induce hyperglycemia [36], promote cheliped regeneration, digestive ability, and immunity during short-term injection [37] in *E. sinensis*, its antioxidant function still unclear. In present study, melatonin in the hemolymph of *E. sinensis* followed a circadian rhythm, which is consistent with our previous studies [17,18]. Therefore, the influence of this most important circadian rhythm on physiological parameters should be considered for the duration after melatonin treatment. In the present study, although the melatonin concentrations (0.0001 g/L, 0.001 g/L, and 0.01 g/L) injected were far higher than the naturally secreted level (0.00001–0.0001 g/L), the antioxidant enzyme activity in the hemolymph and muscles of all concentration groups showed no significant differences compared with the control group after 1 and 2 h post injection. These results indicate that melatonin may not significantly impact the antioxidant capacity of *E. sinensis* in a short time (1–2 h) after treatment. However, the 4 h post-injection group of MDA, showed significantly lower activity than that of the control group ($P < 0.05$), and the 8 h post-injection group of SOD, showed significantly higher activity than that of the control group ($P < 0.05$). In addition, antioxidant enzyme activity in the muscles also showed differences between samples from the 4 and 8 h post-injection groups. According our previously study, melatonin showd peaks at 12:00 and 16:00 under natural condition, which was also our sample collection time at 4 h and 8 h after the injection time at 8:00 [17,18]. Melatonin injection did not increase locomotor activity in *Uca pugilator*, until approximately 6 h later, indicating that the pharmacological dosage persisted in the crabs' systems and had showed effects later during the incline and peak of activity but not the trough [38]. The findings of the present study

completely elucidate the antioxidant and immune functions in the diurnal variations of *E. sinensis* and therefore warrants further research.

In summary, the specific activities of immune and antioxidant enzymes in the hemolymph, muscle, and gills of the Chinese mitten crab, *E. sinensis*, followed a circadian rhythm. Our findings indicate that the antioxidant and immune status may also exhibit two peaks (1200 and 0000 h) during daily cycle, which was synchronized with melatonin rhythm. The survival rate decreased as the melatonin dose increased. Moreover, antioxidant enzymes such as MDA and SOD were significantly lower in the muscle and hemolymph 4–8 h after injection compared to the control group. These findings highlight the importance of comparing the immune and antioxidant responses of crustaceans at fixed times and to also consider the circadian regulation of innate immunity.

Declaration of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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