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Temperature regulates circadian rhythms of immune responses in red swamp crayfish *Procambarus clarkii*

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ABSTRACT

As an ectothermic animal, crayfish immunity and their resistance to pathogen can be significantly affected by environmental factors such as light and temperature. It has been found for a long time that multiple immune parameters of animals and human are circadian-regulated by light-entrained circadian rhythm. Whether temperature also affects the immune rhythm of animals still remains unclear. In the present study, we investigated the effect of temperature cycles on the rhythm of crayfish immunity and their resistance. Survival experiments demonstrated that temperature cycles of 24 °C and 18 °C effectively entrained the circadian rhythm of crayfish resistance to *Aeromonas hydrophila* in constant dark. After being exposed to temperature cycles, the crayfish injected at different time points exhibited significant difference in resistance to *A. hydrophila*. Bacterial growth and total hemocyte count (THC) also showed circadian variation in crayfish subjected to temperature cycles, but phenoloxidase (PO) activity didn't show rhythmic change under the same conditions. Quantitative real-time PCR revealed that basal expression of *crustin1* and *astacidin* in crayfish subjected to temperature cycles was circadian-rhythmic, but induced expression by *A. hydrophila* didn't show the same rhythm. In contrast, crayfish maintained at constant temperature showed completely arrhythmic in bacterial resistance, immune parameters mentioned above and the expression of antimicrobial peptides. The results present here collectively indicated that temperature cycles entrained circadian rhythm of some immune parameters and shaped crayfish resistance to bacteria.

1. Introduction

Up to date, the immune research of crustacean focus on identification of immune components, immune responses to major pathogens and the interaction mechanism between host and pathogens [1,2]. Actually, since crustaceans are ectothermic animals, environmental factors affect crustacean immunity and shape their resistance to pathogen [3,4]. Especially, environmental temperature varies greatly between days and nights or between seasons, which makes it important to investigate the effect of environmental temperature cycles on crustacean immunity. However, nothing is known about the regulation mechanism of temperature cycles to crustacean immunity.

Circadian clock is known to regulate many physiological processes and hence synchronizes physiology and behavior of animals [5–7]. Temperature and light are the main zeitgibers to entrain circadian rhythm by influencing the expression of some transcriptional factors [8–12]. These transcriptional factors are involved in two interlocked transcriptional feedback loops which result in molecular oscillations to control physiological and behavioral rhythmicity [7]. The molecular oscillations are further adjusted and fine-tuned to synchronize with environmental factors by post-transcriptional and post-translational mechanisms. It remains to be known whether circadian clock mediates the effect of environmental factors on crustacean immunity and resistance to pathogen infection.

It has been found that many immune-related diseases of human exhibit circadian rhythmicity such as cancer [13], asthma [14], metabolic disorders [15], and cardio-cerebrovascular diseases [16]. Many immune parameters such as lymphocyte proliferation [17], numbers of circulating leukocytes [18], the activity of natural killer cell [19], cytokine levels [20] have also been observed to show circadian variation. More and more evidences indicate that disorder

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of circadian rhythm influences immune responses in both vertebrate and invertebrate.

Although lots of evidences indicate that many animal diseases and immune components exhibit rhythmic changes, all these data are based on the light-entrained circadian clock. Temperature has been verified to be an effective zeitgiber of biological circadian clock both in invertebrate and vertebrate [8–11,21]. It remains to be known whether temperature can also affect circadian rhythm of animal diseases and immune components.

Here, we showed that temperature cycles altered survival of crayfish from bacterial infection, and some of immune parameters and expression of antimicrobial peptides (AMP) exhibited synchronization to temperature cycles. The data presented here indicated that temperature could entrain circadian rhythm in crayfish immunity.

2. Materials and methods

2.1. Experimental animals and bacteria

The intermolt crayfish (*Procambarus clarkii*) used in the present study were purchased from local market in Qingdao, China. The animals were maintained in aerated water under natural light/dark cycles at 21 °C to acclimatize for two weeks before being used in the experiments. Crayfish were fed once every day with clam meat which was heated to inactivate enzymes and microorganisms prior to being fed to crayfish.

Aeromonas hydrophila used in the present study were originally isolated from water of artificial lake within Qingdao Agricultural University and cultured in Luria–Bertani's rich nutrient medium supplemented with 100 μg L^{-1} ampicillin overnight at 28 °C. Subsequently, bacteria were washed three times with CFS (Crayfish Saline Buffer, 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2 mM $NaHCO_3$, pH 6.8) [22] to remove the culture medium and dissolved in CFS. Bacterial concentration was measured with viable plate counts and recorded by colony forming units (CFUs) per milliliter.

2.2. Immune challenge with A. hydrophila

For immune challenge, crayfish were exposed to temperature cycles of 24°C/18 °C 12 h/12 h for two weeks and then were kept at constant temperature of 21 °C. Each crayfish was injected with *A. hydrophila* at an amount of 2.0×10^9 CFUs via base of the fourth walking leg. Four groups were injected at CT05, CT07, CT19 and CT21, respectively (CT means circadian time and refers to the stage being removed zeitgibers. Here, it is the stage of constant temperature of 21 °C after temperature cycles. CT05, CT07, CT19 and CT21 refer to 5, 7, 19 and 21 h respectively from beginning of constant temperature of 21 °C). To rule out that the survival differences were only driven by the temperature changes, animals were maintained at constant temperature of 21 °C during and after injection with bacteria. Crayfish were monitored after injection and survival rates were recorded every two hours. Three control groups with the same injection procedure were maintained at constant temperature of 24 °C, 21 °C and 18 °C, respectively. The experiment was repeated three times and total 100 crayfish were used in each time point. All crayfish were kept in dark throughout the experiment. Further, to confirm temperature cycles as a zeitgeber, reverse temperature cycles (the temperature cycles were shifted by 12 h) were also used to treated crayfish and the survival rates were also calculated after injection with bacteria using the same procedure mentioned above. Survival rates of crayfish injected at different time points were first assessed by one-way ANOVA to determine the significance of differences. All pairs of groups were compared by Tukey–Kramer HSD analysis. Statistical analysis was carried out with SPSS11.0 software. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the mean ± SE.

2.3. Bacterial titer

Hemolymph was collected using a 1 ml syringe containing anticoagulant at 3, 6 and 12 h post infection respectively and put on ice immediately to limit further bacterial growth. Before hemolymph collection, crayfish were wiped and washed using pre-cold 75% ethanol to remove bacteria on the surface. Hemolymph was collected from 5 crayfish at each time point for determination of bacterial titer. Serially dilution of the mixed hemolymph were dotted onto the LB plate containing ampicillin sodium of 100 μg ml^{-1} and then incubated at 28 °C overnight. Visible colonies were counted to calculate the bacterial titer. To obtain more reliable data, three independent experiments were performed for each time point. Pairwise comparison between CT05 and CT19 or between temperature cycles and constant temperature were performed using two tailed Student's t-test. Differences were considered significant at $P < 0.05$.

2.4. Total hemocyte count

To measure total hemocyte count (THC) in hemolymph of crayfish, hemolymph was sampled at different time points after treatment with 24°C/18 °C 12 h/12 h temperature cycles, and fixed with 10% formalin in CFS according to Watthanasurorot et al. [22]. Hemolymph was dropped onto a clean hemocytometer and THC was examined under phase-contrast microscope. For each time points, five crayfish were checked. The experiments were repeated in three consecutive subjective days (72 h). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey–Kramer HSD test. Statistical analysis was performed with SPSS11.0 software. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the mean ± SE.

2.5. Assay of phenoloxidase activity

Hemolymph was collected from crayfish subjected to temperature cycles 24°C/18 °C 12 h/12 h treatment at 3 different time points after injected with bacteria or CFS and mixed with equal volume of anticoagulant. To measure phenoloxidase (PO; EC1.14.18.1) activity, 100 μl of the mixture was incubated with 3 mg mL^{-1} L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma) for 10 min at room temperature. Then phenoloxidase activity was determined by measured the absorbance of reaction solution at 490 nm and recorded as $\Delta A490 \cdot mg^{-1}$ protein$\cdot min^{-1}$. Total protein of hemolymph was determined using Bradford method with bovine serum albumin as standard. The data of PO activity were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey–Kramer HSD test. Statistical analysis was performed with SPSS11.0 software. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the mean ± SE.

2.6. Quantitative RT-PCR

To evaluate the expression of antimicrobial peptides, crayfish were infected with heat-killed *A. hydrophila* at CT05, CT07, CT19 and CT21. At 6 h after injection, hemolymph was collected from ventral sinus of crayfish with 1 ml syringe containing 200 μl anticoagulant and centrifuged to obtain hemocytes. Hemocytes from three crayfish were pooled together as a sample for RNA preparation. Total RNA was extracted using RNAiso plus (TaKaRa, Japan) following the manufacturer's instruction and RNA integrity was checked by electrophoresis on 1% agarose gel. Two μg RNA was used

Table 1
Primers used in the present study.

Primers	Sequence (5′-3′)
EF2-F	TATCGTGCCGAGATGCTTTA
EF2-R	TCTGACCACTGCCAACCTTT
RPS14-F	AGTTCAGAAGGAGGAGGTGC
RPS14-R	AGTGATGCGGACAATGGTTT
β-actin-F	TGTGCGACTCTGGTGATGGT
β-actin-R	AGCGGTGGTGGTGAAGGAAT
Crustin1-F	CCATGCCTAAGTTTGAACCC
Crustin1-R	TGTCACATAGCACCTCCCTC
Astacidin-F	GACGGCTTCCCTTCCCTCTT
Astacidin-R	CCATACGCCTGACCACCTTG

for cDNA synthesis with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to manufacturer's manual. For remove DNA contamination of RNA, RNA was treated with gDNA Eraser provided in PrimeScript™RT reagent Kit before cDNA synthsis. The resultant cDNA was diluted by 10-fold and 1 μl diluted cDNA was used in a total of 20 μl reaction mixture for quantitative real-time PCR. PCR reactions were performed using SYBR® Premix Ex Taq™ (TaKaRa, Japan) on LightCycler 480 (Roche, USA) with the following conditions: a cycle of 5 min at 95 °C followed by 40 cycles of 10 s at 94 °C, 10 s at 55 °C, and 10 s at 72 °C. Primers used for quantitative real time PCR were shown in Table 1. Each PCR reaction was performed in triplicate and melting curve was analyzed using a procedure with the conditions: 95 °C for 5 s, 65 °C for 1min, and 97 °C continuous to determine the specificity of PCR amplification. For the qPCR analysis, seven candidate internal reference genes including *ribosomal protein S14* (RPS14), elongation factor2 (EF2), Histone3, cyclophilinA (CycA), 18sRNA, ribosomal protein L9 (RPL9), and β-actin was evaluated on their expression stability using the NormFinder program and three of them *RSP14* (accession number D14609), *β-actin* (accession number D14612) and *EF2* (accession number EU921011) were used in subsequent qRT-PCR analysis for normalize the antimicrobial peptide (AMP) expression values. The

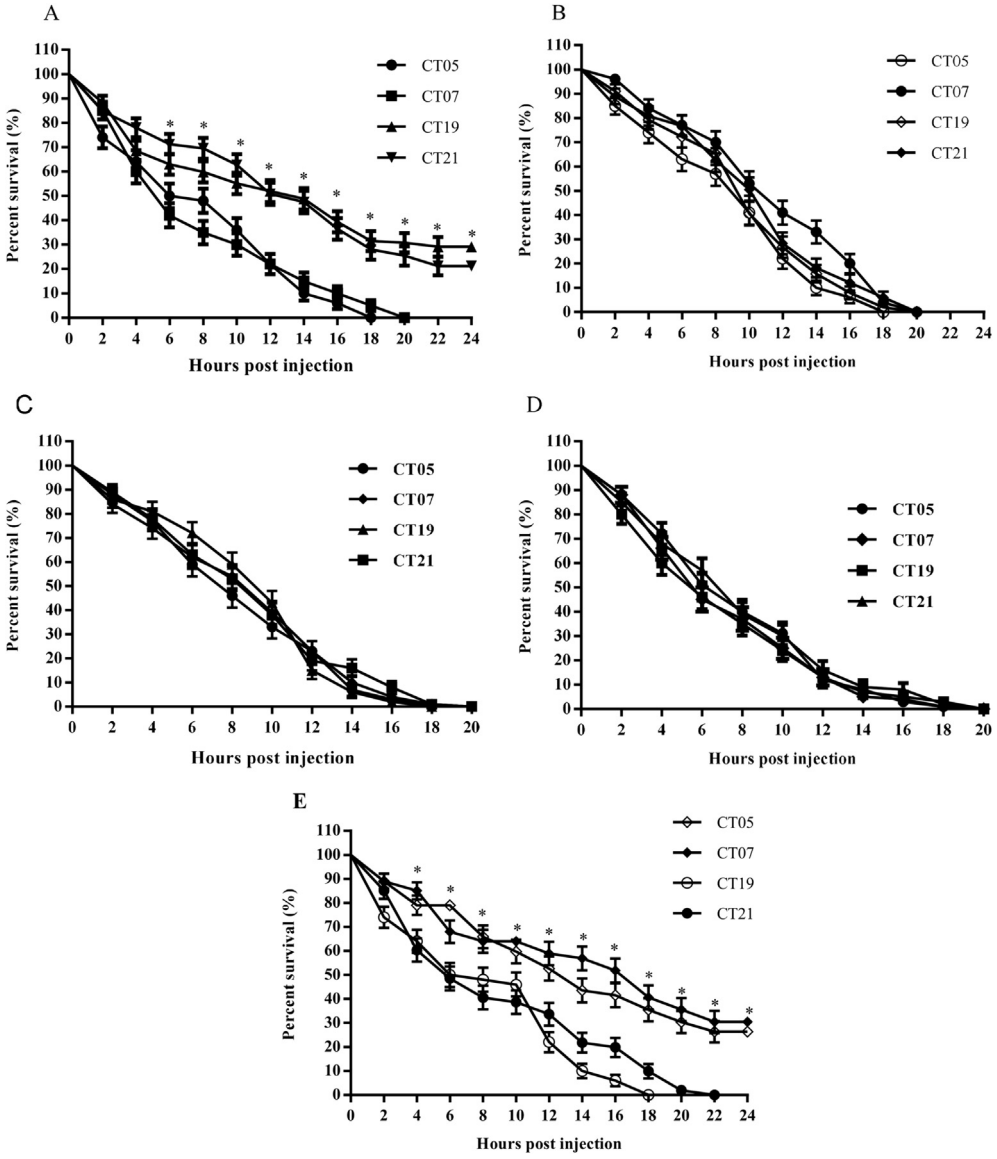

Fig. 1. Time course in the proportion of crayfish surviving after infection with *A. hydrophila* at CT05, CT07, CT19 and CT21. Crayfish groups were subjected to A, temperature cycles of 24°C/18 °C 12 h/12 h; B, C, D, constant temperature 18 °C, 21 °C, 24 °C respectively or E, reverse temperature cycles under 18 °C/24 °C 12 h/12 h. The data were collected from three independent experiments and the experiments were performed in constant dark. One hundred crayfish in total were used at each time points in three independent experiments, and error bars indicate SE.

PCR efficiency for each gene was evaluated using relative standard curve with serially diluted cDNA as template. Fluorescence crossing points were calculated with the second derivative maximum method using the Lightcycler 1.5 software included with the equipment, and relative quantifications were carried out with corrected PCR efficiencies. For basal expression, animals of CT05 group under temperature cycles were used as calibrators. For induced expression, the animals used as calibrators were injected CFS at each time point corresponding the animals injected with bacteria and sampled at the same time post injection. The data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey–Kramer HSD test. Statistical analysis was performed with SPSS11.0 software. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the mean ± SE.

3. Results

3.1. Temperature cycles resulted in resistance differences between different time points

After being subjected to temperature cycles of 24°C/18 °C 12 h/12 h, crayfish injected with bacteria at different time points exhibited significantly different survival rate. CT19 and CT21 groups showed significantly higher survival rates than CT05 and CT07 groups (Fig. 1A). In contrast, the animals maintained at constant temperature all the time exhibited no significant difference in survival rates after being challenged at the different time points as mentioned above (Fig. 1B–D). To further confirm the differences of survival rate were due to the effect of temperature cycles, crayfish were exposed to reverse temperature cycles (24 °C and 18 °C phases were shifted by 12 h) for 1 week to adjust to a new temperature rhythm and followed by injection with bacteria at the four time points mentioned above. The change of temperature cycles markedly altered survival rates of crayfish. As expected, significantly higher survival rate was observed in crayfish injected at CT05 and CT07 (Fig. 1E).

3.2. Temperature cycles affected growth of A. hydrophila in crayfish

Crayfish exposed temperature cycles exhibited significantly different bacterial titers between CT05 and CT19 groups (Fig. 2A). The difference of bacterial titers was observed at 12 h after injection at which the bacterial titer of CT05 group was significantly higher than that of CT19 group ($P < 0.05$). No significant difference was observed at 3 and 6 h after injection. By contrast, animals maintained at constant temperature of 21 °C all along didn't show significantly different bacterial titers between crayfish groups infected at different time points (Fig. 2B). Moreover, bacterial titer in crayfish subjected to temperature cycles was significantly lower ($P < 0.01$) than that in crayfish kept at constant temperature of 21 °C (Fig. 2C).

3.3. THC was circadian-fluctuated by treatment with temperature cycles

To determine if total hemocyte count (THC) is related with difference of survival rates under different temperature conditions, total number of circulating hemocytes in crayfish were examined every three hours at constant temperature stage of 21 °C after exposure to temperature cycles of 24°C/18 °C 12 h/12 h. THC in hemolymph exhibited circadian fluctuation after exposure to temperature cycles (Fig. 3). Two peak values were observed at CT18 and CT0, respectively. The lowest value of THC appeared at CT12 when the crayfish were transferred from 24 °C to 18 °C. At 24 °C

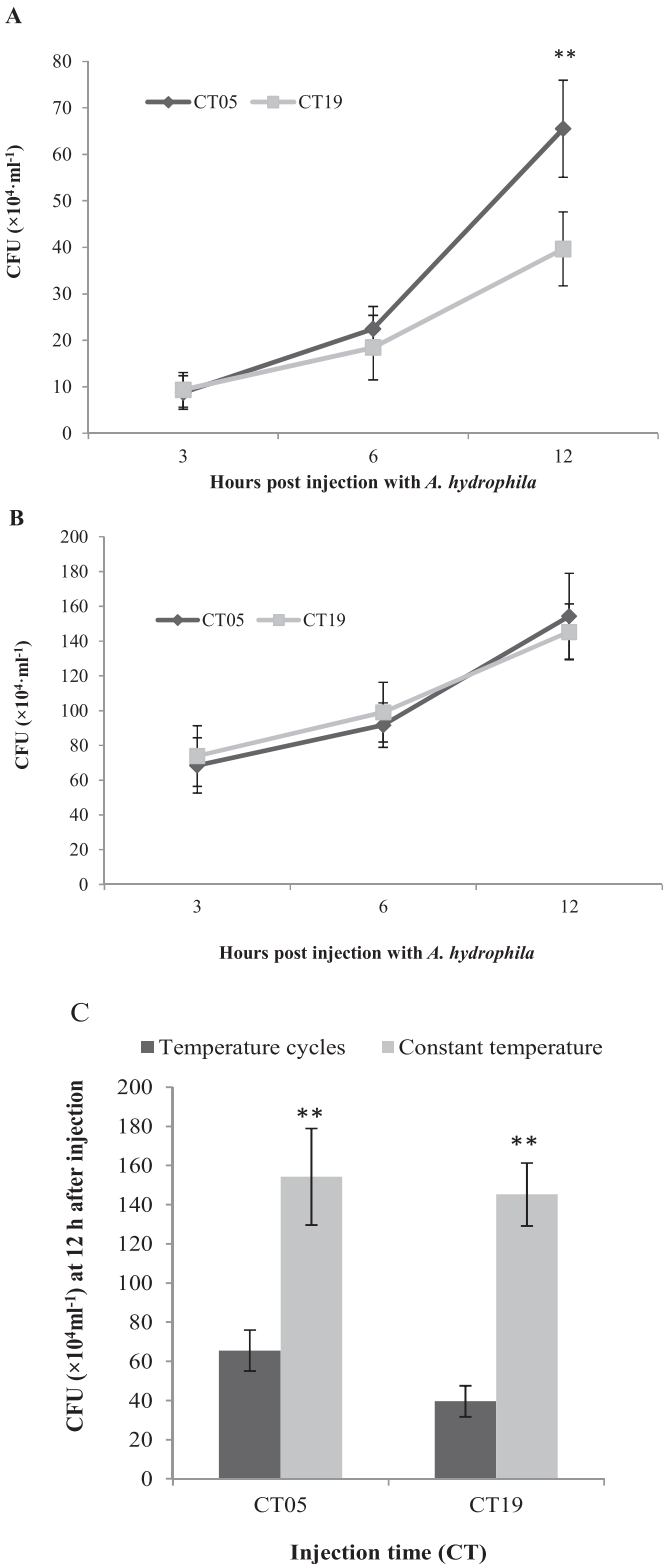

Fig. 2. Temporal change of bacterial titers in crayfish after infection with *A. hydrophila*. A. Crayfish was exposed to temperature cycles of 24°C/18 °C 12 h/12 h for one week and followed by constant temperature of 21 °C. B. Crayfish was kept at constant temperature all along. C. Comparison of temperature cycles and constant temperature for their effect on bacterial titer in crayfish. Crayfish were infected at either CT05 or CT19 and bacterial titer was measured at the indicated times. Asterisks (*) and different letters indicate significantly difference (n = 15, $P < 0.05$), double asterisks indicate significantly different at $P < 0.01$ level. Experiments were repeated three times. Error bars indicate SE.

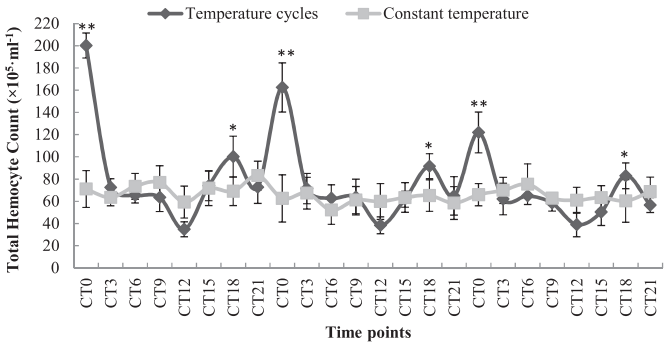

Fig. 3. Temporal change of THC in crayfish examined at different time points. Crayfish was exposed to temperature cycles of 24°C/18 °C 12 h/12 h for one week and followed by constant temperature of 21 °C and the crayfish used as control were maintained at 21 °C all along. Experiments were performed in dark and finished within 72 h corresponding three temperature cycles. Asterisks (*) indicate significant differences of THC in crayfish subjected to temperature cycles compared to trough value at CT12, Error bars indicate SE (n = 5 $P < 0.05$).

stage, especially from CT03 to CT12, THC maintained relative low level. After that, continuously increased THC was observed up to CT18 at which one of THC peak was observed. The highest value of THC appeared at CT0 when the crayfish were transferred from 18 °C to 24 °C. In contrast, THC changes showed arrhythmic in crayfish kept at constant temperature of 21 °C all the time.

3.4. Prophenoloxidase-activating system was not circadian-regulated by temperature cycles

To test if prophenoloxidase-activating system is circadian-regulated by temperature cycles in crayfish injected with bacteria, the phenoloxidase (PO) activity was investigated 3 h, 6 h and 9 h after injection with heat-killed *Aeromonas hydrophila*. Significantly enhanced PO activities were observed in crayfish subjected to either temperature cycles or constant temperature compared to that of control crayfish injected with CFS. However, no significant difference of PO activity was found between groups injected at different time points irrespective of temperature conditions or what was injected (bacteria or CFS) (Fig. 4A and B). This data suggested that prophenoloxidase-activating system was not circadian-regulated by temperature cycles in crayfish.

Although no circadian-regulated PO activity was observed, different PO activity was observed between crayfish groups subjected to different temperature conditions. In the groups subjected to temperature cycles, PO activity maintained at a relatively stable level from 6 h to 9 h after injection. Unlike the groups subjected to temperature cycles, groups kept at constant temperature showed a continuously increasing PO activity from 3 h to 9 h after injection (Fig. 4C).

3.5. Basal expression of two AMP genes but not induced-expression by A. hydrophila is temperature-regulated

To test if AMP expression contributes to resistance difference of crayfish to bacterial infection, we examined the basal (uninjected) and bacteria-induced expression of two AMP genes, *crustin1* (accession number: GQ301201) and *astacidin* (accession number: GQ301199). The basal expression was examined at four time points CT05, CT07, CT19, and CT21 using quantitative real time RT-PCR. For crayfish subjected to temperature cycles, CT19 and CT21 groups exhibited significantly higher basal expression of *crustin1* and *astacidin* than other two groups (Fig. 5A and B). In contrast, different expression of *crustin1* and *astacidin* between different

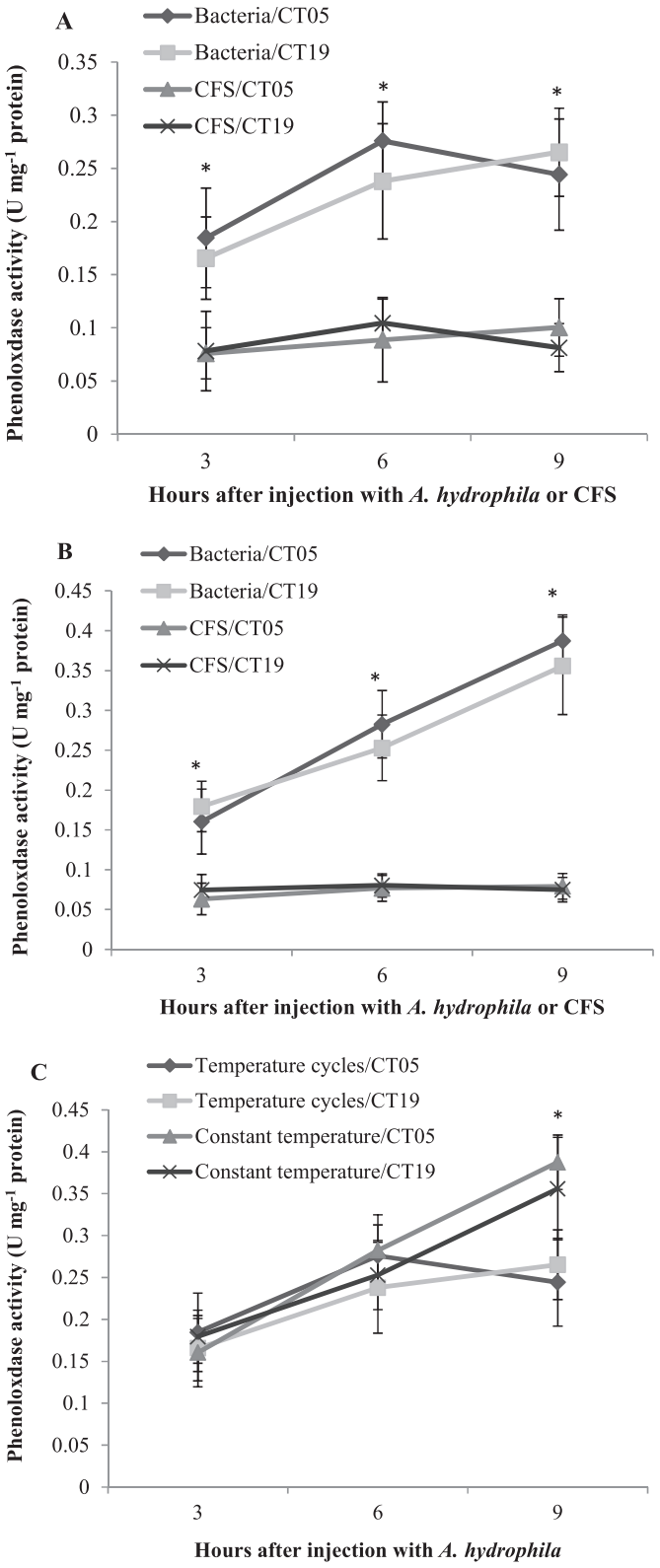

Fig. 4. Temporal change of PO activity in hemolymph of crayfish after injection with *A. hydrophila* at different time points. A. Temperature cycles of 24°C/18 °C 12 h/12 h for one week and followed by constant temperature of 21 °C. B. Crayfish was kept at constant temperature all along. C. Comparison of PO activity between temperature cycles and constant temperature. Crayfish were infected at either CT05 or CT19 and PO activity was measured at the indicated times. Experiments were repeated three times. Asterisks (*) indicate significant differences between groups injected with bacteria and CFS in A and B or between groups subjected to temperature cycles and constant temperature in C (n = 15, $P < 0.05$). Error bars indicate SE.

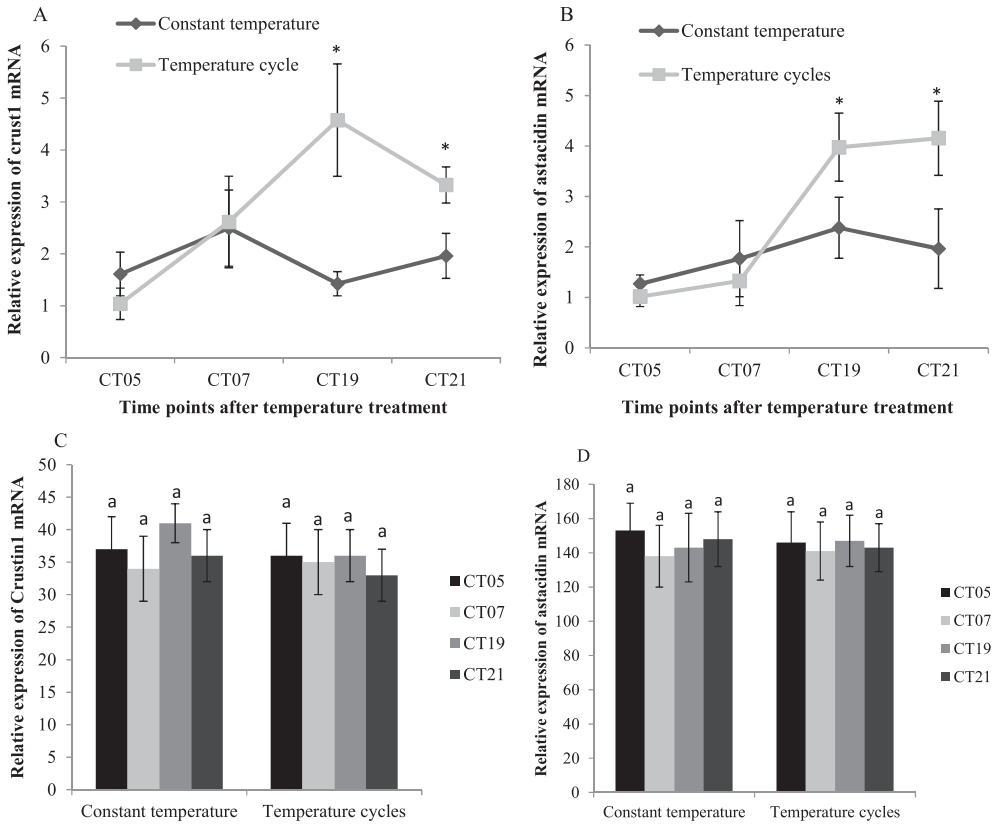

Fig. 5. Basal and induced expression of *crustin1* and *astacidin* in crayfish subjected to different temperature conditions. A, B. Basal expression of *crustin1* and *astacidin*. Expression analysis was performed at CT05, CT07, CT19 and CT21, respectively. Asterisks (*) indicate significantly difference compared to CT05 group of temperature cycles (n = 15, $P < 0.05$); C, D. Induced expression of *crustin1* and *astacidin* by heat-killed *A. hydrophila*. Crayfish were challenged at CT05, CT07, CT19 and CT21, respectively, and gene expression was analyzed at 6 h after injection. The relative expression values were calculated with animals injected with CFS at each time point corresponding treated animals as calibrator. Same letters indicate no significant difference (n = 15, $P > 0.05$). Experiments were repeated three times within consecutive 72 h. Error bars indicate SE.

groups wasn't observed in the case of constant temperature (Fig. 5C and D). For bacteria-induced expression, crayfish were injected with heat-killed *A. hydrophila* at four time points CT05, CT07, CT19, and CT21, and sampled at 6 h after injection for measuring gene expression using quantitative real-time PCR. Under either temperature cycles or constant temperature, the expression of *crustin1* and *astacidin* were significantly induced by *A. hydrophila*, but the expression of both *crustin1* and *astacidin* didn't show significantly difference between different time points.

4. Discussion

In this study, we explored the effect of temperature cycles on circadian rhythm of crayfish resistance to bacterial infection. We found that temperature cycles resulted in different resistance to bacterial infection in crayfish injection different time points. Moreover, these resistance differences were related to THC and basal AMPs expression, but not proPO activating system. To our knowledge, this is the first report about the regulation of crayfish resistance to bacterial infection by temperature cycles.

This temperature-entrained rhythm is comparable to light-entrained rhythm in a previous report [22] in which crayfish resistance to bacterial infection was significantly different between crayfish groups injected at different times of day and the high survival was observed in crayfish injected at dark stage which is corresponding low temperature stage in the present study. The resistance differences observed at different time points couldn't be the results of only temperature change, because the injections were performed under constant temperature so that all animals were kept at the same temperature during and after injection, which also indicated that the rhythm was endogenously driven, for this rhythm of crayfish resistance persisted from temperature cycles to constant temperature.

Among the immune parameters of red swamp crayfish, bacterial titers, THC and basal expression of AMPs exhibited circadian rhythm which suggested that these parameters might be related with resistance rhythm of crayfish entrained by temperature cycles. After being subjected to temperature cycles, significantly differences of bacterial titers were observed between crayfish groups infected at different time points. The same phenomenon was repeatedly observed within three temperature cycles and was not found in crayfish groups kept at constant temperature. This indicated that temperature cycles resulted in rhythmic difference of bacterial titers. Moreover, crayfish subjected to temperature cycles showed significantly higher bacterial titers than that kept in constant temperature regardless of injection time. This indicated that exposure to temperature cycles contributed to bacterial resistance of crayfish.

Hemocytes play a central role in innate immunity of crayfish [23]. Total hemocyte count is a key indicator for crayfish to defense against bacterial infection. In a previous report, THC of crayfish showed circadian rhythm during day cycles [22]. Since mechanism of light-entrained circadian rhythm is different from that of temperature-entrained circadian rhythm [8,24], we wondered whether THC also showed circadian rhythm after being exposed to temperature cycles. The data presented here indicated that THC was completely arrhythmic when crayfish was placed into constant dark and constant temperature for a week and THC rhythm could

be entrained by temperature cycles. The temperature-entrained rhythm of THC appeared two peaks during a temperature cycle, which is different from that reported in previous study [22] in which only one THC peak was observed during a day cycle. This may due to the different entrainment mechanism between LD and temperature cycles. However, these results suggested that temperature cycles could entrain the THC rhythm of crayfish and might thereby affect the immune responses to bacterial infection.

Whether AMP expression is circadian-regulated varies as observed in *drosophila* and mosquito. Some are circadian-regulated by LD cycles or fluctuating temperature [25,26], but some others aren't. We showed that basal expression of two AMP of crayfish was circadian rhythmic , but induced expression was arrhythmic. This suggested that basal expression of the two AMP genes was circadian-regulated by temperature cycles, but induced expression not. The similar phenomenon was also found in previous study on drosophila *Drs* under LD cycles [26]. We still don't know why the induced expression of AMP was not circadian-regulated, but the results presented here suggested that the two AMPs contributed to the basal defense of crayfish and thereby to the resistance to bacterial infection.

PO activity didn't showed circadian rhythm after being subjected to temperature cycle, which suggested that PO activity was not the main factor contributing to resistance rhythm entrained by temperature cycles. This was similar to drosophila melanization response under LD cycles, an innate immune response driven by prophenoloxidase activating system, which is not circadian-regulated either [26]. However, an interesting phenomenon is that PO activity of infected crayfish exposed temperature cycles exhibited significantly different pattern from that kept at constant temperature. The PO activity of the latter exhibited an increasing trend all along. In contrast, PO activity of infected crayfish exposed temperature cycles showed a decrease of PO activity 6 h after infection. Excessive activation of PO activity produces more ROS which is thought to be detrimental to animals [27,28]. The decrease of PO activity 6 h after infection under temperature cycles might be a representation of PO control. This suggested that temperature cycles might be more profitable to crayfish resistance than constant temperature. This point was supported by the data of bacterial titers which were shown significantly lower in infected crayfish subjected to temperature cycles than that in infected crayfish kept at constant temperature.

In summary, we concluded that temperature cycles could effective entrained the circadian rhythm of crayfish immunity. Crayfish are more resistant at low temperature stage (18 °C) during a temperature cycle than at high temperature stage (24 °C). Further research will be needed for understand the molecular mechanism of temperature regulation on immune rhythm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.05.025.

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