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PrePub-Article

Effects of changing temperature on the physiological and biochemical properties of *Harmonia axyridis* larvae

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With 5 figures and 3 tables

Abstract: *Harmonia axyridis* (Pallas) is an important natural enemy insect. Using fourth-instar larvae, we investigated whether changing temperature (5, 15 and 25°C) can enhance the cold tolerance of *H. axyridis* and determined optimal storage temperature conditions. Larvae were exposed to five altering temperature regimes:15/5 (15°C/12 h:5°C/24 h), 5/15/5 (5°C/12 h:15°C/12 h:5°C/24 h), 15/25/5 (15°C/12 h:25°C/12 h:5°C/24 h), 15/5/25/5 (15°C/12 h:5°C/12 h:25°C/12 h:5°C/24 h), and 5/25/15/5 (5°C/12 h:25°C/12 h:15°C/12 h:5°C/24 h). Compared with the larvae of control treatment (5°C/24 h), the larvae had lower supercooling points (SCPs) in the 15/25/5 and 5/25/15/5 groups, higher glycerol content in all treated groups (except for group 5/15/5), lower fat content in the 15/5/25/5 group, lower water content in the 15/25/5 and 15/5/25/5 groups, higher trehalose content in the 15/5, 15/25/5, and 15/5/25/5 groups, higher glucose content in the 15/25/5 group, and higher glycogen content in the 15/25/5, 15/5/25/5, and 5/25/15/5 groups. Compared with the control larvae, TRE1 activity of all treated larvae was significantly enhanced except for those in the 5/15/5 group. Also, the expression levels of three soluble trehalase genes (*TRE1-1, TRE1-2* and *TRE1-3*) and one membrane-bound trehalase gene (*TRE2*) increased after the changing temperature treatment, whereas *TRE1-4, TRE1-5*, and one membrane-bound like trehalase gene (*TRE2-like*) mRNA expression levels were very low. These results suggest that fourth-instar *H. axyridis* larvae exposed to a succession of different temperatures, including low temperatures, survived by reducing the SCP, accumulating carbohydrates and glycerol, and consuming fat. The temperature combination 15/25/5°C provided optimal storage conditions. These findings provide valuable insights for further elucidation of the cold resistance mechanism of ladybeetles and for obtaining an extended shelf life under low-temperature storage.

Keywords: Coccinellidae, trehalose metabolism, glycerol, RT-qPCR

1 Introduction

Aphids are major agricultural pests worldwide (van Emden et al. 2017; Hullé et al. 2020). They could induce notably extensive pesticide applications in crops (Johnson et al. 2009; Ragsdale et al. 2011; Heimpel et al. 2013), with multiple potential associated side effects on non-target arthropods (Lu et al. 2012; Jam & Saber 2018; Mohammed et al. 2018, and see Desneux et al. 2007 for a thorough review) as well as on targeted pests (e.g. hormesis effect or the selection of resistant populations, Guedes et al. 2016; Qu et al. 2015; Ullah et al. 2019a; 2019b), and this despite that natural enemies could be useful in the framework of biocontrol and Integrated Pest Management programs (Desneux et al. 2006; 2019; Ali et al. 2018; Jaworski et al. 2019). The ladybeetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an important natural enemy that can be used to effectively control whiteflies, mites, aphids, and other pests (Koch 2003; Wang et al. 2017a; Chen et al. 2019), with effective limitation of aphid population growth in various crops (Koch 2003; Costamagna et al. 2008; Koch & Costamagna 2017). However, factors such as cannibalism, need of artificial diet, and low-temperature storage pose challenges for scaling up the commercial production of *H. axyridis* (Wu et al. 2016, Ovchinnikov et al. 2019).

Among insects that are resistant to freezing, the content of cryoprotective penetrants such as sorbitol, glycerol, trehalose, and proline has been found to be significantly increased

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after cold treatments (Yoder et al. 2006, Michaud & Denlinger 2007, Overgaard et al. 2007, Koštál et al. 2011a, Teets et al. 2012, Vesala et al. 2012, Koštál et al. 2016). To minimize injury from low temperature exposure, coccinellids, similar to other insect species, have evolved a series of behavioral and physiological strategies, such as seeking shelter, dormancy, altering cell membrane fluidity, and accumulating sugars and polyols (Hamedi et al. 2013). Watanabe et al. (2002) revealed that a short-term low-temperature storage can alter the content of polyols in *H. axyridis* to enhance its cold resistance. In cold winters, the survival strategy of *H. axyridis* adults involves seeking sheltered locations, such as in the crevices of rocks, in concrete buildings and caves, and in the folds of fallen leaves, to obtain a protective microclimate (Obata 1986, Sakurai et al. 1993, Berkvens et al. 2010, Durieux et al. 2015). In nature, there are several other species of ladybeetles that exhibit aggregation behavior, including *Coccinella septempunctata* (Coleoptera: Coccinellidae), *Ceratomegilla undecimnotata* (Schneider) (Coleoptera: Coccinellidae), *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae), *Hippodamia convergens* (Guérin-Méneville) (Coleoptera: Coccinellidae), *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), and *Aiolocaria mirabilis* (Motschulsky) (Coleoptera: Coccinellidae) (Hodek 1973, Copp 1983, Hemptinne 1985, Hodek 1996, Honek et al. 2007). In terms of physiological changes, the adults of *H. axyridis* enter a diapause state during winter and are characterized by an increased in fat body, a decreased respiration rate, and an atrophied corpus allatum. Additionally, females have reduced ovaria (Sakurai et al. 1992, Hodek & Honek 1996, Iperti & Bertand 2001). As diapause continues, the fat body is slowly depleted to provide subsistence energy, the body weight is subsequently reduced, and the digestive tract is emptied (Iperti & Bertand 2001).

Trehalose, which comprises between 80% and 90% of carbohydrates in insect hemolymph (Hottiger et al. 1987, Thompson 2003), plays an important role in the regulation of insect cold resistance. It can both promote the conversion of some carbohydrates into lipids and amino acids through energy metabolism (Tang et al. 2018), and form a unique sugar protective film on the cell surface that can effectively protect against protein invariant inactivation (Wyatt 1967, Thompson 2003). Trehalose can be hydrolyzed to glucose by trehalase (TRE), and is synthesized in fat bodies mainly by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) via the TPS/TPP pathway (Shukla et al. 2015, Shi et al. 2017, Yang et al. 2017). In insects, TRE occurs in the form of soluble trehalase (TRE1) and membrane-bound trehalase (TRE2) (Nardelli et al. 2019). In silkworm larvae (Lepidoptera: Bombycidae), the midgut contains mainly TRE2 (Mitsumasu et al. 2005), whereas a class of membrane-bound like TRE (TRE2-like) with no transmembranal structure and high homology with membrane-bound proteins has been found in *Locusta migratoria manilensis* (Orthoptera: Acrididae). This TRE2-like protein is very likely to be an evolutionary intermediate type between TRE1 and TRE2 (Liu et al. 2016). This is consistent with the findings of Wegener et al. (2003), who reported that the activity of TRE2 in vitro can be subdivided into an overt fraction and a latent fraction.

Theoretically, if the ambient temperature drops to the critical thermal minimum (CT_{min}), insects enter a state in which there is an initial loss of neuromuscular function (chill coma) and motor cessation (Hazell & Bale 2011, Jakobs et al. 2015, Andersen et al. 2017). However, these insects gradually recover after returned to normal temperatures, with the recovery time generally being dependent upon the duration and intensity of cold exposure (Overgaard & MacMillan 2017). In contrast, the exposure of ladybeetles to high temperatures results in a shortened development time, decreased survival, and reduced fat accumulation (Krengel et al. 2012, Knapp & Nedvěd 2013). Conversely, the developmental period of *Chilocorus bipustulatus* (Coleoptera: Coccinellidae) was found to be prolonged with decreasing temperature, and the survival during total preimaginal development at 15°C was higher than that at 35°C (Eliopoulos et al. 2010). Additionally, larvae of the mite *Dermacentor variabilis* (Acari: Ixodidae) exposed to a temperature of -5°C showed high survival (70%) (Rosendale et al. 2016). The findings of these studies therefore indicate that maintenance of a low temperature is an important strategy for prolonging the survival time of insect larvae.

The present study aimed to determine the optimal pre-storage temperature treatments (acclimatization) for a long-term storage of *H. axyridis* larvae, at 5°C. We measured the effects of a series of different temperature regimes during the early stage of storage on the supercooling point (SCP), freezing point, fat content, glycerol content, and trehalose metabolism of *H. axyridis* larvae. These parameters are closely related to insect cold tolerance. The findings of this study may help enhance cold storage of *H. axyridis* larvae and also provide insights into physiological mechanisms of cold tolerance in this species.

2 Materials and methods

2.1 Insects

A laboratory population of *H. axyridis* was established mainly from the collections at the campus of Hangzhou Normal University, Hangzhou, China (30°9′12″N, 120°23′26″E), from June to September in 2016. The population was maintained in a climate chamber under 25 ± 1°C, a relative humidity of 70% ± 5%, and 14:10 h L:D photoperiod. Approximately 200 ladybeetles were retained in each insect breeding cage (45 cm × 45 cm × 45 cm) and supplied with a sufficient number of aphids (*Aphis glycines*) each morning. Fourth instar nymphs of *H. axyridis* were used for all bioassays. Previously, it has been established that a temperature of 5°C might represent a signal for *H. axyridis*, and that prior

to experiencing this temperature, individuals must undergo sufficient physical changes to enable them to withstand cold stress during the winter (Shi et al. 2016). Therefore, in the present study, 5°C was used as the final storage temperature. Today, in order to improve the cold tolerance of insects, cold acclimation or rapid low temperature stress is often used. We suspect that if 25°C is added and the storage device is filled with caramel popcorn, whether this will have a positive effect on the energy metabolism of the larvae after resuscitation. At the same time, we also added another temperature of 15°C, which is an intermediate temperature of 5 to 25°C. In consequence, the combination of the three temperatures has increased, which helps to screen out suitable pretreatment conditions. The fourth instar larvae were exposed to a temperature of 5°C for 24 h as a control treatment and five experimental treatments were established (Table 1).

Test larvae were first placed in a cylindrical plastic tube (10 cm × 2 cm), with a gauzed top. The tube was wrapped with newspapers to protect it from light and filled with caramel popcorn to both provide energy and prevent self-cannibalism by the larvae. The larvae were then exposed subsequently to 5°C, 15°C, and/or 25°C in a refrigerator (Siemens, Germany), a MIR-554-PC cryogenic incubator (Panasonic, Japan), and a MLR-352H-PC plant incubator (Panasonic, Japan), respectively. Given that it was not possible to adjust the lighting of the refrigerator, in order to establish consistent illumination conditions among treatments, all the tubes were wrapped with newspaper, and thus the larvae were stored in a dark environment. Thirty *H. axyridis* larvae were placed in each tube, and there were three replicates for each treatment.

2.2 RNA isolation and synthesis of first-strand cDNA

Total RNA was extracted from larvae (three individuals per sample) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was examined for purity and concentration using agarose gel electrophoresis and a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Good quality RNA samples were stored in a –80°C freezer. First-strand cDNA was reversed transcribed using a Prime Script® reverse transcription (RT) reagent kit and an Eraser RT kit (Narishige, Japan). The cDNA was temporarily stored at –20°C and subsequently stored at –80°C for long-term preservation.

2.3 Real-time quantitative PCR (RT-qPCR)

For RT-qPCR analyses, 10 µL reaction mixtures containing 0.4 µL each of the reverse (R) and forward (F) primers (10 pmol each), 1 µL of template DNA, 3.2 µL of sterile water, and 5 µL of SYBR Green Premix Ex Taq (Takara, Japan) were used. The specific primers (Table 2) were synthesized by Shanghai Invitrogen Corporation. The reaction procedure was denaturation at 95°C for 5 s and annealing at 58°C for 30 s (39 cycles), and finally, a melting curve was constructed at 65°C. *H. axyridis rp49* (*ribosomal protein 49 gene*, AB552923) was used as a reference gene (Osanai-Futahashi et al. 2012), and the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

2.4 Determination of carbohydrate content and TRE activity

2.4.1 Material handling

Three *H. axyridis* larvae were placed in a 1.5 mL Eppendorf tube, to which 100 µL of phosphate-buffered saline (PBS, 20 mM, pH 6.0) was added. The larvae were then grounded on ice using an electric grinder, followed immediately by the addition of 200 µL of PBS, and subsequent disruption using a sonicator for 30–60 s. Following the addition of 700 µL of PBS, the preparation was centrifuged for 20 min at 1000 × *g* and 4°C, and 500 µL of the resulting supernatant was used for trehalose, glycogen, and protein content analysis. A 350 µL aliquot of the supernatant was further centrifuged for 60 min

Table 1. Description of the experimental treatments used to examine the effects of changing temperature on the physiological and biochemical properties of *Harmonia axyridis* larvae.

Name	Treatment description
5	Development took place at 25°C, and subsequently the 4th-instar larvae were exposed to 5°C for 24 h (control treatment)
15/5	Development took place at 25°C, and subsequently the 4th-instar larvae were exposed to 15°C for 12 h, and then exposed to 5°C for 24 h
5/15/5	Development took place at 25°C, and subsequently the 4th-instar larvae were exposed to 5°C for 12 h, then exposed to 15°C for 12 h, and finally stored at 5°C for 24 h
15/25/5	Development took place at 25°C, and subsequently the 4th-instar larvae were exposed to 15°C for 12 h, then exposed to 25°C for 12 h, and finally stored at 5°C for 24 h
15/5/25/5	Development took place at 25°C, and subsequently the 4th-instar larvae were sequentially exposed to 15, 5, and 25°C for 12 h each, and finally stored at 5°C for 24 h
5/25/15/5	Development took place at 25°C, and subsequently the 4th-instar larvae were sequentially exposed to 5, 25, and 15°C for 12 h each, and finally stored at 5°C for 24 h

Table 2. Primer sequences used for real-time Quantitative PCR analysis of *Harmonia axyridis* larvae (Shi et al. 2017).

Primer Name	Forward Primer (5′-3′)	Reverse Primer(5′-3′)
RT*TRE1-1*	CTTCGCCAGTCAAATCGTCA	CCGTTTGGGACATTCCAGAT
RT*TRE1-2*	TGACAACTTCCAACCTGGTAATG	TTCCTTCGAGACATCTGGCTTA
RT*TRE1-3*	ACAGTCCCTCAGAATCTATCGTC	GGAGCCAAGTCTCAAGCTCATC
RT*TRE1-4*	TTACTGCCAGTTTGATGACCAT	CATTTCGCTAATCAGAAGACCCT
RT*TRE1-5*	TGATGATGAGGTACGACGAGA	GTAGCAAGGACCTAACAAACTG
RT*TRE2-like*	TTCCAGGTGGGAGATTCAGG	GGGATCAATGTAGGAGGCTGTG
RT*TRE2*	CAATCAGGGTGCTGTAATGTCG	CGTAGTTGGCTCATTCGTTTCC
RT*TPS*	GACCCTGACGAAGCCATACC	AAAGTTCCATTACACGCAC
RT*rp49*	GCGATCGCTATGGAAAACTC	TACGATTTTGCATCAACAGT

at 20,800 × *g* and 4°C, and the resulting supernatant was used for the analysis of glucose content, TRE1 activity, and protein 1 content. The pellet was first washed with 200 μL of PBS to obtain a suspension, which was then used for the analyses of TRE2 activity and protein 2 content.

2.4.2 TRE1 and TRE2 activity

Analyses of TRE1 and TRE2 activity were performed using a Glucose (GO) Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Initially, 300 μL of a reaction solution containing 60 μL TRE1 or TRE2 extract, 75 μL 40 mM standard trehalose solution (Sigma-Aldrich Co., St. Louis, MO), and 165 μL PBS (20 mM) was prepared. The mixture was then placed in a water bath at 37°C for 60 min and then 100°C for 5 min. Thereafter, 50 μL of the mixture was added to 100 μL of the glucose analysis reagent, and placed in a water bath at 37°C for 30 min. Finally, the reaction was terminated by the addition of 100 μL of 12N H_2SO_4, and the absorbance was measured at 540 nm. In addition, standard glucose solutions of different concentrations were prepared according to the kit instructions for construction of a standard curve.

2.4.3 Protein content

Protein concentrations were determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A standard curve was prepared by initially diluting 2 mg/mL BCA to 0.5 mg/mL with PBS, followed by the preparation of standard solutions of different concentrations according to the kit instructions. A 200 μL aliquot of working solution was then added to 20 μL each of sample to be analyzed and the standard solutions. After incubation in a water bath at 37°C for 30 min, the absorbance was measured at 562 nm. TRE activity was expressed as the amount of glucose contained per milligram of protein.

2.4.4 Trehalose content

The trehalose content was determined using the anthrone method. Initially, a 40 mM trehalose solution (Sigma-Aldrich Co., St. Louis, MO) was diluted to 1.6, 0.8, 0.6, 0.4, 0.2, 0.1, and 0.05 mM in PBS to prepare a standard curve, and then 10 μL of each dilution was analyzed. The supernatant was added to a 1.5 mL centrifuge tube, followed by 10 μL 1% H_2SO_4, and then the mixture was placed in a water bath at 90°C for 10 min, and subsequently in an ice bath for 3 min, followed by the addition with 10 μL 30% potassium hydroxide (KOH) and incubation in a water bath at 90°C for10 min. The mixture was then placed in an ice bath for 3 min and, finally, 200 μL of the developer (0.02 g anthrone and 10 mL 80% H_2SO_4) was added followed by incubation in a water bath at 90°C for 10 min. After cooling, the absorbance of the reaction mixture was measured at 630 nm.

2.4.5 Glucose and glycogen content

Glucose content was measured using a Glucose (GO) Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). A standard curve was initially prepared, and 0, 1, 2, 3, 4, and 5 μL of glucose standard solution was added to 1.5 mL Eppendorf tubes, and then the volume was supplemented with 50 mL with PBS. Fifty microliters of the test solution was added to a separate 1.5 mL Eppendorf tube. Thereafter, glucose analysis reagents were separately added to each of the aforementioned tubes, which were then placed in an incubator at 37°C for 30 min. Finally, 100 μL of 12N H_2SO_4 was added to terminate the reaction, and the absorbance was measured at 540 nm. The glycogen content was determined using the starch transglucosidase method. Initially, a standard 0.1 U/μL starch transglucosidase solution was prepared from a solution containing 1 mg 70 U/mg in 700 μL distilled water. Thereafter, 100 μL of the sample was added to 20 μL of the enzyme solution and incubated in a water bath at 40°C for 4 h, and 50 μL of the glucose solution produced by the reaction was measured using the glucose content measurement method described above.

2.5 Supercooling point

The thermocouple method was used to determine the supercooling point (SCP) of larvae (Ju & Du 2002, Liu et al.

2005). When the temperature of the insect falls below the SCP, the body fluid begins to freeze spontaneously, and the external release of latent heat can be measured to determine the SCP and freezing points of the insect. A supercooling curve was constructed following the instructions of the SUN-V intelligent insect supercooling point tester (Beijing Pengcheng Electronic Technology Center), the SCP and the freezing points can be readily determined.

2.6 Water, fat, and glycerol content

The water and fat content of larvae was determined using a modified version of the method described by Jiang et al. (2015). The method used for determinations of glycerol content was that described by Zhang et al. (2013) and Jiang et al. (2015). Initially, we determined the fresh mass (FM) of each replicate, after which the sample was dried in a 60°C oven for 48 h. Having determined the dry mass (DM) of samples, the water content (%) was calculated as (FM-DM)/FM × 100%. The dried larvae were then grounded to powder in liquid nitrogen and samples of the powder were placed in 10 mL centrifuge tubes, followed by the addition of 2 mL chloroform and 1 mL methanol. The mixture was then centrifuged for 10 min at 5000 r/min and 25°C. The resulting supernatant was then collected, and the remaining precipitate resuspended in 2 mL chloroform and 1 mL methanol, followed by centrifugation. The supernatant was again removed, and the remaining precipitate was dried in an oven at 60°C for 24 h to a constant weight (Lean Dry Mass, LDM). The fat content (%) was calculated as (DM - LDM)/DM × 100%.

2.7 Statistical analysis

Each treatment of cDNA was performed using three parallel samples, and for each cDNA sample, three RT-qPCR analyses were performed to determine CT values. If the differences among the three values were less than 1, the three values were considered valid. Conversely, if the differences were large, the experiment was repeated. All experiments in this study were performed with three biological replicates, and the data presented in figures are expressed as the mean + standard deviation (SD) of the three replicates. The original data were analyzed using Microsoft Office Excel 2010 software, and having evaluated the data for normality and homogeneity of variance, they were analyzed statistically using the Tukey and Student-Newman-Keuls methods of one-way ANOVA (using the measured physiological index as the dependent variable and the treatment as a factor) of IBM SPSS statistics 20 software ($P < 0.05$, indicated by *; $P < 0.01$, indicated by **). Different lowercase letters in Table 3 indicate a significant difference at the 0.05 level after the test. Finally, histograms were drawn using SigmaPlot 10.0 software.

3 Results

3.1 Effect of changing temperature treatment on the SCP and freezing point of larvae

In general, both the SCP ($df = 5$; $F = 6.029$; $P = 0.005$) and freezing points ($df = 5$; $F = 1.476$; $P = 0.268$) of the *H. axyridis* larvae exposed to different altering temperature (5, 15 and/or 25°C) were reduced when compare to the control treatment at constant 5°C (Fig. 1). The SCPs of the15/25/5 and 5/25/15/5 groups were significantly lower than the control treatment (Fig. 1A).

3.2 Effects of changing temperature treatment on the water, glycerol, and fat content of larvae

Exposure to changing temperatures affects the water ($df = 5$; $F = 16.702$; $P < 0.001$), glycerol ($df = 5$; $F = 9.866$; $P = 0.001$) and fat ($df = 5$; $F = 4.028$; $P = 0.025$) content of *H. axyridis* larvae. Compared to the control, the glycerol content increased in all treatments except the 5/15/5 group (Fig. 2A); fat content was reduced only in the 15/5/25/5 group (Fig. 2B) and the water content was reduced in the 15/25/5 and 15/5/25/5 groups (Table 3).

3.3 Effects of changing temperature treatment on the trehalose, glucose, and glycogen content of larvae

We also examined changes in trehalose ($df = 5$; $F = 10.1822$; $P = 0.001$), glucose ($df = 5$; $F = 4.109$; $P = 0.032$) and glycogen ($df = 5$; $F = 32.407$; $P < 0.001$) content in larvae. Compared with the larvae in the control treatment at 5°C, there was a significant increase in the contents of trehalose, glucose, and glycogen in the 15/25/5 group (Fig. 3), whereas a significant increase was found in the trehalose and glycogen content of the 15/5/25/5 group (Fig. 3). In contrast, only the trehalose content showed a significant increase in the 15/5 group, and only the glycogen content showed a significant increase in the 5/25/15/5 group (Fig. 3).

Table 3. Changes in the water content of larvae under different temperature treatments.

Groups	Control	15/5	5/15/5	15/25/5	15/5/25/5	5/25/15/5
Water Content (%)	74.18 ± 0.0320[a]	72.54 ± 0.0165[a]	76.19 ± 0.0069[a]	64.98 ± 0.0163[b]	64.90 ± 0.0183[b]	72.95 ± 0.0240[a]

Results are reported as the mean ± SD. Means followed by the same letter for different groups are not significantly different according to one-way ANOVA analysis ($P < 0.05$).

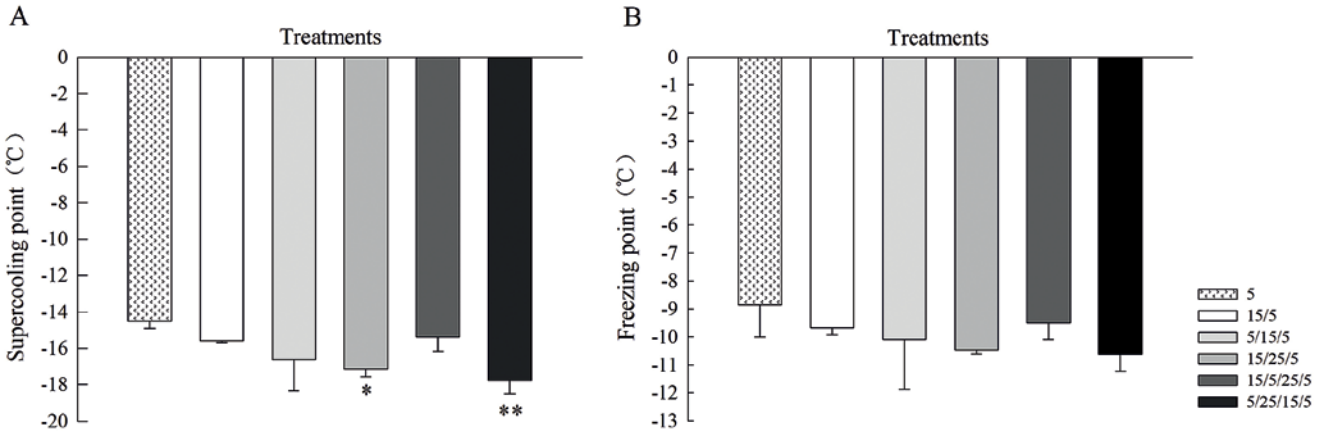

Fig. 1. Changes in the supercooling point and freezing point of fourth-instar *Harmonia axyridis* larvae under different temperature treatments. **(A)** supercooling point, and **(B)** freezing point. Each bar represents the mean (+ SD) of three samples. Asterisks show significant differences between control and experimental treatments (**P < 0.01; *P < 0.05).

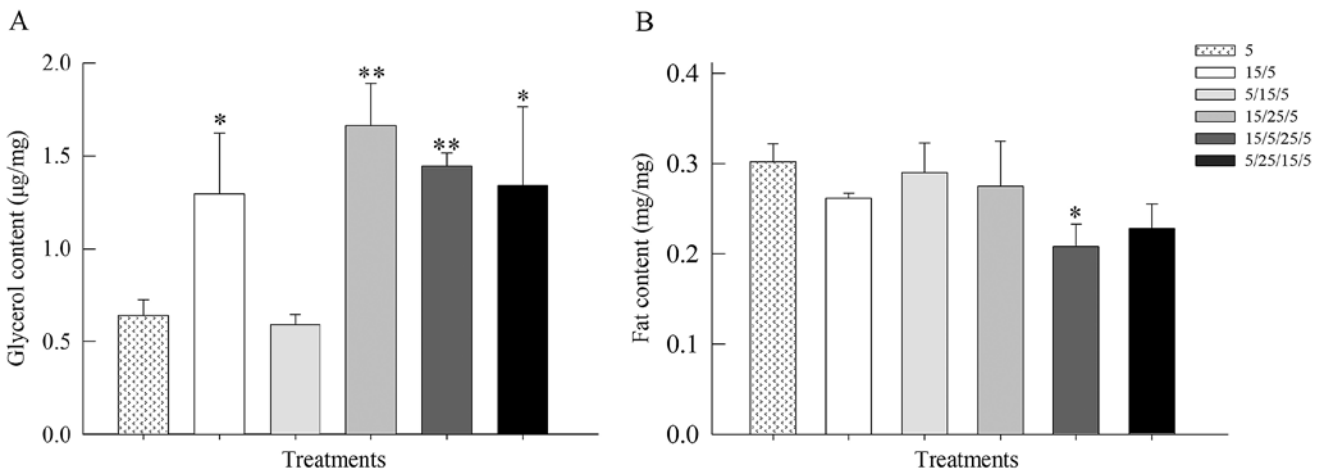

Fig. 2. Changes in the glycerol and fat content of fourth-instar *Harmonia axyridis* larvae under different temperature treatments. **(A)** glycerol content, and **(B)** fat content. Each bar represents the mean (+ SD) of three samples. Asterisks show significant differences between control and experimental treatments (**P < 0.01; *P < 0.05).

3.4 Effects of changing temperature treatment on the TRE1 and TRE2 activities in larvae

Whereas the TRE1 activity (df = 5; F = 16.702; P < 0.001) of the 5/15/5 group larvae was broadly comparable with that of the control larvae treatment, significantly increased levels were observed in the remaining four groups (Fig. 4A). In contrast, no significant difference was detected in the TRE2 activity (df = 5; F = 1.691; P = 0.232) among the treatment groups when compared with the control treatment (Fig. 4B).

3.5 Effects of changing temperature treatment on the relative expression of trehalose metabolism-related genes

The expression level of each of the selected trehalose metabolism related genes in the control treatment was set to 1 and then the relative expression levels of these genes in the treatment groups were examined. It was accordingly found that the relative expression level of *TRE1-1* showed a significant increase (P < 0.001) in the 15/25/5 and 5/25/15/5

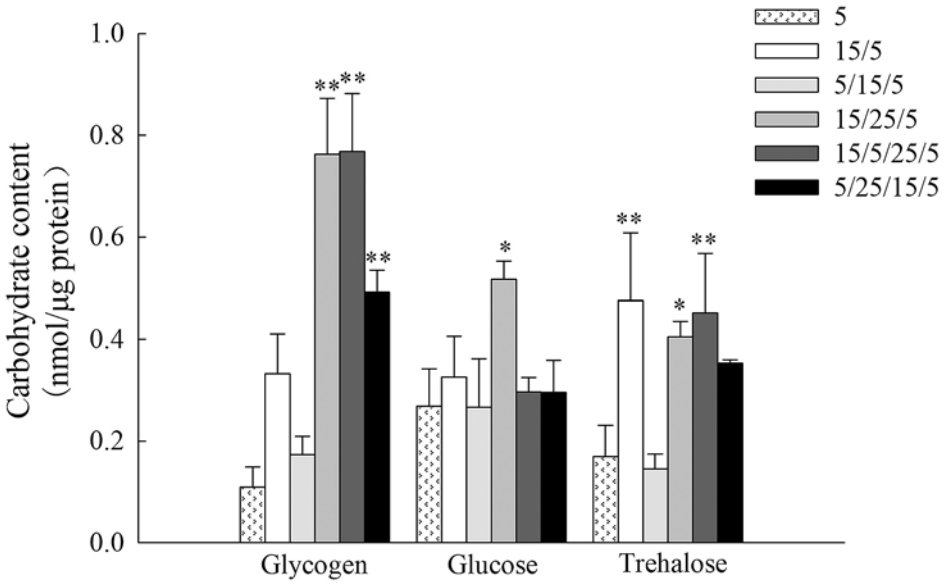

Fig. 3. Changes in the content of carbohydrates in fourth-instar *Harmonia axyridis* larvae under different temperature treatments. Each bar represents the mean (+ SD) of three samples. Asterisks show significant differences between control and experimental treatments (**P < 0.01; *P < 0.05).

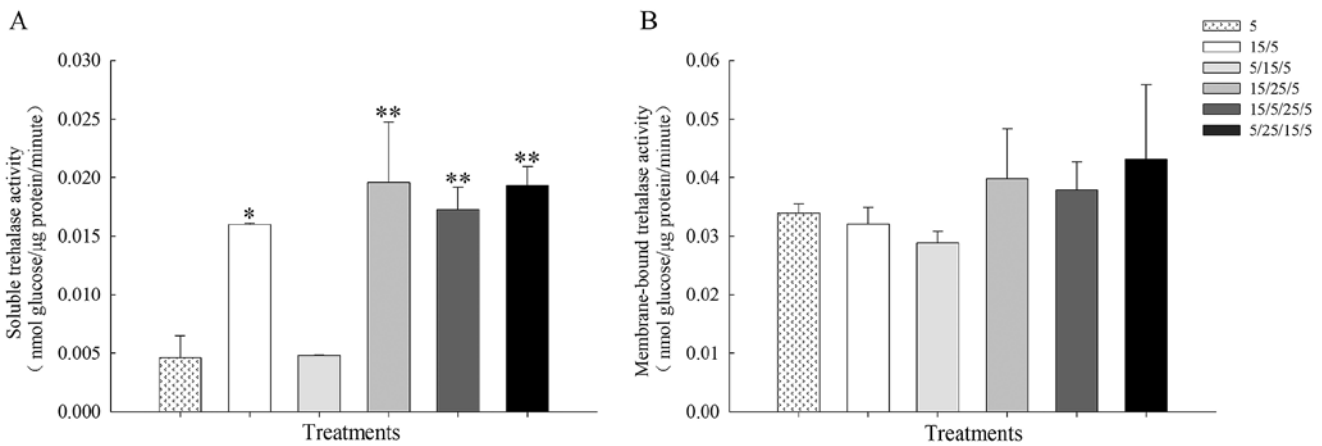

Fig. 4. Changes in the trehalase activity of fourth-instar *Harmonia axyridis* larvae induced by different temperatures. **(A)** soluble trehalase activity, and **(B)** membrane-bound trehalase activity. Each bar represents the mean (+ SD) of three samples. Asterisks show significant differences between control and experimental treatments (**P < 0.01; *P < 0.05).

groups (Fig. 5A), whereas that of *TRE1-2* was significantly increased in the 15/5 (P = 0.022) and 15/25/5 (P < 0.001) groups (Fig. 5A). Additionally, the relative expression level of *TRE1-3* was found to have increased significantly (P < 0.001) in the 15/5 group (Fig. 5A). With the exceptions of *TRE1-4* in group 15/5 (P = 0.095) and *TRE1-5* in group 15/5/25/5 (P = 0.070), the expression levels of these two genes in other treatment groups were observed to be significantly higher (P < 0.001) than those in the control treatment (Fig. 5A). Moreover, in all treatment groups, the relative expression of *TRE2-like* mRNA was significantly lower (P < 0.001) than that of the control treatment and remained at an extremely low level (Fig. 5B). With respect to *TRE2*, it was found that the expression level was significantly higher in the 15/5 group (P < 0.001) than that in the control treatment (Fig. 5B). Finally, the relative expression level of *TPS* in the 15/5 group showed a significant increase (P < 0.001), whereas in groups 15/25/5 (P = 0.001), 15/5/25/5

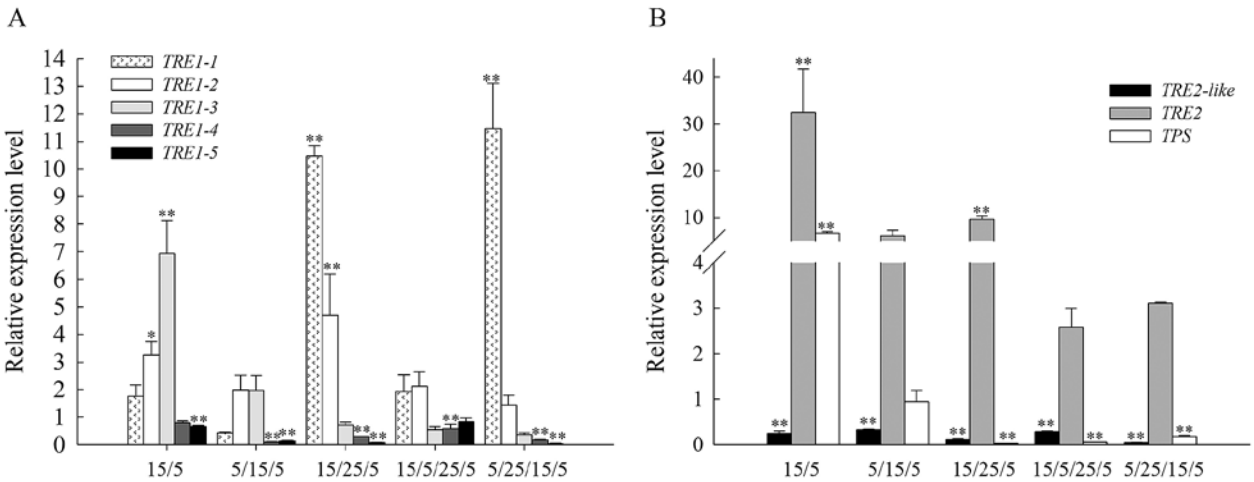

Fig. 5. Changes in the relative expression level of genes related to trehalose metabolism induced by continuous low temperature. **(A)** Five types of *TRE1* genes, and **(B)** two types of *TRE2* and *TPS* genes. Each bar represents the mean (+ SD) of three samples. Asterisks show significant differences between control and experimental treatments (***P* < 0.01; **P* < 0.05).

(*P* = 0.002), and 5/25/15/5 (*P* = 0.006), the expression was very low, and showed a significant reduction compared with the control treatment (Fig. 5B).

4 Discussion

The water contained within insects can be divided into two types, namely combined and free water. Free water readily freezes when temperatures drop below 0°C, and if insects do not excrete free water during cold winters, they are likely to freeze to death. With a decrease of free water, there are concomitant increases in the concentrations of sugars, salts, and other substances in the somatic cell protoplasm, and subsequently there is a decrease in the freezing points of the liquids within cells (Nedvěd et al. 1998, Holmstrup et al. 1999). The phenomenon that enables insects to avoid freeze under temperature conditions below 0°C is referred to as supercooling. The SCP is an important indicator of insect cold resistance. It is well known that overwintering populations of *H. axyridis* have a durable cold tolerance, and that –16°C is the lower lethal temperature for ladybeetles (Watanabe 2002), and Berkvens et al. (2010) measured a SCP value of –16.5°C in an overwintering population of ladybeetles in the field. In the present study, it was observed that the SCPs of the larvae in the 15/25/5 and 5/25/15/5 groups decreased significantly to –17.1 and –17.8°C, respectively (Fig. 1A). Similarly, Wu et al. (2016) observed a significant decrease in the SCP of *H. axyridis* in response to a short period of low temperature stress. In *Cydia pomonella* (Lepidoptera: Tortricidae), the supercooling point of the final instar larvae decreased from approximately –15.3°C during summer to –26.3°C during winter (Rozsypal et al. 2013). Studies on overwintering and experimental populations of adult *H. axyridis* have revealed that the SCP and freezing point of the overwintering population were significantly lower than those of the experimental population, whereas the average water content did not differ significantly between adults in the two populations (Wang et al. 2017b). Furthermore, in an examination of the cold responses of *H. axyridis*, Du et al. (2014) found that the water content decreased with an increase of refrigeration time, although the difference was not significant. These findings are consistent with the results obtained for some of the groups examined in the present study (Table 3), and collectively, they indicate that the resistance of *H. axyridis* to low temperature stress can mainly be attributed to a reduction in the SCP.

In addition to a reduction in the free water content in overwintering larvae, glycerol, trehalose, and proline are also maintained at relatively high concentrations, as they play an important role in stabilizing the structure of proteins and biofilms during cell freezing-related dehydration (Rudolph et al. 1986, Carpenter & Crowe 1988, Lee 1991, Koštál et al. 2011b, Koštál et al. 2012, Su et al. 2017, Pathak et al. 2018). With the exception of the 5/15/5 group, for which no significant increase in glycerol content was observed, the findings of the present study are consistent with these previous observations (Fig. 2). Xu et al. (2018) found that glycerol accumulates in *Apis cerana cerana* and *Apis mellifera ligustica* (Hymenoptera: Apidae) after low temperature treatment, which is similar to the results of the present study. Surprisingly, *Dryophytes chrysoscelis* (formerly *Hyla chrysoscelis*) has been found to express an aquaporin 9 (AQP9)-like protein that promotes glycerol permeability, thereby facilitating the accumulation of this compound as a type of cryoprotectant (Stogsdill et al. 2017). In the present study, it

was also found that the overwintering larvae consumed fat at low temperatures to provide an energy source (Fig. 2B). Fat is the main energy source of insects, and fatty acids have been found play an important role in wintering insects. For example, in *Diaphania pyloalis* Walker (Lepidoptera: Crambidae) and *Hyphantria cunea* Drury (Lepidoptera: Arctiinae), fats are transformed to free and bound fatty acids during overwintering, with a portion of the free fatty acids being converted to bound fatty acids to enhance cold resistance (Chen et al. 2005, Kong 2008).

With respect to changes in the content of carbohydrates, it was found in the present study that the content of three types of carbohydrate (trehalose, glucose and glycogen) were significantly up-regulated, in the 15/25/5 group, whereas trehalose and glycogen were significantly increased in the 15/5/25/5 group (Fig. 3). From this perspective, it is assumed that *H. axyridis* larvae in the 15/25/5 and 15/5/25/5 groups were more resistant to cold than larvae in the other treatment groups. Previously, it has been demonstrated that the trehalose content of *H. axyridis* begins to increase significantly at 5°C (Shi et al. 2016). Similar increases in the accumulation of carbohydrates have also been noted in other insects exposed to low temperatures. For example, in a cold environment, the trehalose and proline content significant increases in the larvae of *Drosophila melanogaster* (Diptera: Drosophilidae) and *C. pomonella*, thereby enhancing their adaptability to low temperatures (Koštál et al. 2011a, Rozsypal et al. 2013). Similarly, under conditions of cold acclimation, *Gryllus veletis* (Orthoptera: Gryllidae) can accumulate inositol, proline, and trehalose in their hemolymph and fat body, which have antifreeze properties (Toxopeus et al. 2019). Xu et al. (2018) examined the effects of temperature on the cold resistance index of *A. c. cerana* and *A. m. ligustica*, and accordingly found that the glucose content in both these bees decreased significantly at 0°C, whereas *A. m. ligustica* accumulated glucose at 10°C. These results indicate that under low temperature stress, insects will accumulate trehalose or glycogen to resist severe cold, whereas glucose is generally used as an intermediate metabolite and its content remains relatively stable. In *Drosophila*, mutants lacking glycogen synthase and glycogen phosphorylase show growth defects and death, thereby indicating that glycogen plays a key role in larval development (Yamada et al. 2019). Moreover, glycogen, as an energy reserve, although not important for health and longevity under nutrient-sufficient conditions, can become important under conditions of energy stress (Yamada et al. 2018, Yamada et al. 2019). In this regard, it has been reported that wintering insects accumulate sugar alcohol as two types, namely, trehalose and glycogen (Hayakawa & Chino 1982), indicating that an important relationship exists between carbohydrates and insect cold resistance. Furthermore, it has been found that carbohydrate accumulation in insects differs in response to different treatments. For example, *Aedes albopictus* (Diptera: Culicidae) have been observed to accumulate glycerol during cold exposure and glucose during the recovery from cold exposure (Zhang et al. 2019). Kojić et al. (2018) examined the levels of polysaccharides (glycerol, sorbitol, and inositol) and sugars (trehalose, fructose, and glucose) during the diapause phase of *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) and found that glycerol and trehalose are the most abundant cryoprotective compounds in diapause larvae. However, it was found in the present study that the glycerin, fat, and carbohydrate content and TRE activity in 5/15/5 group larvae did not differ significantly from those in the control larvae. It is suspect that this difference could be attributable to the fact that compared with the 15/5, 15/25/5 and 15/5/25/5 groups, the 5/15/5 larvae were initially stored at 5°C and the pre-processing time was shorter than that used for 5/25/15/5 group larvae.

The *TRE1-1, TRE1-2, TRE1-3*, and *TRE1-4* genes of *H. axyridis* are involved in trehalose metabolism, among which *TRE1-4* plays the most important role in the cooling process (Shi et al. 2016). In insects, TRE2 is an exogenous transmembrane enzyme involved in numerous physiological processes, including flight, reproduction, development, and midgut digestion (Wegener et al. 2010). When exposed to low-temperature stress, larvae can modify the metabolism of trehalose by regulating changes in TRE activities, and the relative expression levels of *TRE* and *TPS*. In theory, increases in expression of the *TRE* gene and activity of TRE promote the conversion of trehalose to glucose, and there is a corresponding decrease in the expression of *TPS*, thereby contributing to a reduction in trehalose content (Shi et al. 2017). However, the results obtained for groups 15/25/5, 15/5/25/5, and 5/25/15/5 in the present study would appear to be inconsistent with this scenario, which could be explained in terms of four factors. Firstly, given that the different groups were stored under different conditions, the time points for determining the content of carbohydrates were not uniform. Secondly, in the three groups of larvae subjected to a temperature of 25°C during storage, large amounts of antifreeze substances may have been synthesized. When the specimens were subsequently placed under low-temperature stress at 5°C for 24 h, these substances were consumed as an energy source. Therefore, it was theoretically reasonable to measure the expression level of *TPS* (Fig. 5B). Thirdly, it is conceivable that alternative metabolic regulators or pathways are involved in these processes, which were not taken account of in the present study. Fourthly, we examined the expression of only a single *TPS* gene, and there may have been differences in other types of *TPS*. For example, *TPS1* and *TPS2* are present in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae) (Yang et al. 2017), and the TPS and TPP domains are present in the *TPS* gene (Wang et al. 2017c). In the third-instar of *Anisakis simplex* (Nematoda: Anisakidae) subjected to low (0°C) and high (45°C) temperatures, it was found that the tissue content of trehalose was dependent on the activity of the TPS and TPP enzymes on the second day of incubation, and that TPS and TPP are more active at 45°C and 0°C, respectively. Furthermore,

changes in TPP activity have been shown to be consistent with changes in the transcriptional level of the *TPP* gene and trehalose levels (Łopieńska-Biernat et al. 2019).

In conclusion, in the present study, it was found that the larvae of *H. axyridis* exposed to a succession of temperatures, including a low temperature of 5°C, survived by reducing the SCP, accumulating carbohydrates and glycerol, and consuming fat. Furthermore, it was determined that a temperature sequence of 15, 25, and 5°C was the optimal storage temperature combination. It is believed that these data will provide a valuable theoretical basis for developing procedures for the low-temperature storage and large-scale production of *H. axyridis*.

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