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L-proline feeding for augmented freeze tolerance of *Camponotus japonicus* Mayr

Mengjia Dou [a,b,f,1], Yazhou Li [a,c,1], Ziqiao Sun [d], Lei Li [a,f,*], Wei Rao [a,e,f,*]

[a] CAS Key Laboratory of Cryogenics, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, China
[b] School of Engineering Science, University of Chinese Academy of Sciences, Beijing 100049, China
[c] School of Energy and Power Engineering, Beihang University, Beijing 100191, China
[d] Beijing Engineering Research Center of Sustainable Energy and Buildings, Beijing University of Civil Engineering and Architecture, Beijing 100044, China
[e] School of Future Technology, University of Chinese Academy of Sciences, Beijing 100049, China
[f] Beijing Key Laboratory of Cryo-Biomedical Engineering, Beijing 100190, China

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ABSTRACT

The successful cryopreservation of organs is a strong and widespread demand around the world but faces great challenges. The mechanisms of cold tolerance of organisms in nature inspirit researchers to find new solutions for these challenges. Especially, the thermal, mechanical, biological and biophysical changes during the regulation of freezing tolerance process should be studied and coordinated to improve the cryopreservation technique and quality of complex organs. Here the cold tolerance of the Japanese carpenter ants, *Camponotus japonicus* Mayr, was greatly improved by using optimal protocols and feeding on L-proline-augmented diets for 5 days. When cooling to −27.66 °C, the survival rate of frozen ants increased from 37.50% ± 1.73% to 83.88% ± 3.67%. Profiling of metabolites identified the concentration of whole-body L-proline of ants increased from 1.78 to 4.64 ng g^{-1} after 5-day feeding. High L-proline level, together with a low rate of osmotically active water and osmotically inactive water facilitated the prevention of cryoinjury. More importantly, gene analysis showed that the expression of ribosome genes was significantly up-regulated and played an important role in manipulating freezing tolerance. To the best of our knowledge, this is the first study to link genetic variation to the enhancement of ants' cold tolerance by feeding exogenous cryoprotective compound. It is worth noting that the findings provide the theoretical and technical foundation for the cryopreservation of more complex tissues, organs, and living organisms.

1. Introduction

Cryopreservation is the most effective way for long-term storage of biomaterials because the low temperature can dramatically inhibit biological and chemical reactions. However, it is still hard to achieve long-term cryopreservation and successful recovery of complex tissues, organs, and even live organisms. The challenges are not only the mechanical injury for the uncontrollable growth of ice crystal but also the complexity of maintaining different cells structure and function [1,2]. Therefore, it is important to understand the mechanisms of low-temperature effect on biomaterials and optimize the freezing/warming protocols for minimizing thermo-mechanical stress in different tissue and organ systems [1].

In nature, the ability of cold tolerance of different organisms is significantly different. The most famous freeze-tolerant organisms, *Panagrolaimus davidi*, are reported to stand 82% body water converting into internal ice [3]. From this point of view, we can learn that studying the cold tolerance mechanisms from the natural cold-tolerant organisms will inspire our thoughts for the cryopreservation. Cold-tolerant organisms usually take two strategies to response cold stimulation: freeze tolerance and freeze avoidance. Freeze-tolerant species can withstand freezing of their body fluids, while freeze-avoidant ones avoid ice formation to maintain organismal integrity. These cold-tolerant organisms have developed different effective ways to adapt to the low temperature, such as the synthesis and accumulation of cryoprotectants (CPAs), cryoprotective dehydration, and regulation of genes expression. CPAs cause cellular dehydration before cooling, reduce ice crystals and protect biological tissues from freezing damage [4]. Cryoprotective dehydration depresses the freezing point of body fluid by minimizing ice content, which contributes to reducing shrinkage in the

* Corresponding authors.
E-mail addresses: lileilei@mail.ipc.ac.cn (L. Li), weirao@mail.ipc.ac.cn (W. Rao).
[1] These authors contributed equally to this work.

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unfrozen cells [5,6]. When facing cold threatens, the expression of genes encoding heat shock protein, ferritin homologue, aquaporins, antioxidants and so on, is identified to up-regulate [4].

Inspired by nature, scientists have explored and applied biocompatible and high-efficiency CPAs for cells cryopreservation [7–10]. The concept of loading exogenous CPAs on organisms for freeze tolerance enhancement has been performed on larvae. L-proline is associated with freezing tolerance in some insects [11,12] and is an ideal candidate for natural cryoprotectant. It can penetrate into cells [13,14], lower the freezing point, reduce cell acidity, regulate cell redox potential, and stabilize macromolecules (nucleic acids, nucleoprotein complexes, protein, biological membranes) [4,15–18]. L-proline has been used to cryopreserve some cultured plant cells [14] and mammalian cells [19]. Koštál et al. [11] studied the freeze-tolerant ability of *Chymomyza costata* and found that diapause was an essential and sufficient condition, and L-proline was a prominent metabolite in survival larvae. Then they converted *Drosophila melanogaster*, a chill susceptible fruit fly, into a freeze-tolerant organism by the shutdown of the larval development and serving L-proline-augmented diets [12]. However, to explore the approach of cryopreservation for tissues even organisms, using fruit flies as the model organisms is far from enough. In recent years, ants aroused scientists' research interest due to their unique habits and social behaviors [20]. However, to the best of our knowledge, there are little reports about the conversion of cold tolerance of ants by artificial methods. In our paper, the Japanese carpenter ants, *Camponotus japonicus* Mayr (Hymenoptera: Formicidae), were used as the model organism. This kind of ants is non-freeze-tolerant organisms [21]. The size of worker ants varies from 6 to 15 mm, much larger than the larvae of *D. melanogaster* (<2.5 mm). *D. melanogaster* has only four pairs of chromosomes, whereas the *C. japonicus* Mayr has 13 pairs of chromosomes [22]. Compared to the larvae of *D. melanogaster*, *C. japonicus* Mayr is more complicated in physical structure (such as size) and biological behavior (such as the act of movement). Therefore, regulating cold tolerance response of *C. japonicus* Mayr will be more valuable for cryopreservation applications.

In this study, we first converted the freeze-sensitive ants into freeze-tolerant species with an L-proline-augmented diet and froze them with optimal protocols. The temperature distribution in the ant body during freezing/rewarming processes was analyzed by infrared thermal imaging. The content and form of water in the ants, together with the freezing point of ants' body fluid were measured to reveal the cryoprotective mechanism of L-proline. With amino acids metabolism analysis, we found that the whole-body L-proline contents of ants were increased from 1.78 to 4.64 ng g^{-1}. Besides, genes analysis indicated that differentially expressed genes including genes of heat shock proteins and antioxidants induced by L-proline-augment also contributed to the enhancement of freeze tolerance.

2. Materials and methods

2.1. Ants and feeding

Foraging workers (females) of the Japanese carpenter ants were caught from ant colonies located in Guiyang, Guizhou Province, China. Then these ants were cultured using artificial diets in the plastic boxes, which were put into dark cabinets at the room temperature (20–25 °C). We prepared both liquid and solid feed (Table S1 online). The basic component of the feed was hydromel, which was made by honey and purified water with the weight ratio of 1:2. The agar powder was added to the hydromel to make a solid feed, and the L-proline (Beijing Chemical Reagents Company, China) was added to prepare an L-proline-augmented feed. For each group of about 50 ants, the liquid feed was served with 1 g for four times per day, and the solid feed was served with 1 g for twice per day.

2.2. The maximum amount of L-proline-augmented diets

The feeding ants were divided into five groups, Pro-0, Pro-10, Pro-20, Pro-30, Pro-40 groups, the liquid feed and solid feed with L-proline in concentrations ranging from 0 to 40 mg g^{-1} compound diets were served. The numbers of live ants in Pro-0, Pro-10, Pro-20, Pro-30, Pro-40 groups were counted every 2 days. The survival rate was further calculated as the percentage of the number of live ants to that of the ants before feeding.

2.3. Freezing & rewarming protocols optimization and cold tolerance assay

To optimize freezing and rewarming procedures, we studied the effect of lowest temperature, cooling rate, freezing time, and rewarming temperature on the survival rate of the ants without feeding L-proline-augmented diets. The freeze dryer (AdVantage 2.0, SP Scientific, USA) was used to freeze and rewarm the ants under predesigned programs. To achieve more accurate environmental temperature change around ants, the thermocouples were stuck at the bottom of the plastic boxes, and the data was collected by the data acquisition system (34902A, Agilent Technologies, USA).

In order to test the proper lowest freezing temperature, the temperature program started at room temperature (R.T., 25 °C) and comprised of 6 steps: (i) cooling to 10 °C in 15 min; (ii) keeping at 10 °C for 10 min; (iii) cooling to the lowest temperature (set 4 groups, −1, −20, −25, −40 °C, respectively) in 15 min; (iv) keeping at the lowest temperature for 60 min; (v) rewarming to 18 °C in 20 min; (vi) keeping at 18 °C in the incubator for 24 h.

To seek the best cooling rate, we set the lowest temperature at −25 °C and tried two different cooling rates, in other words, the time required in step (iii) is set as 15, 60 min, respectively.

In order to find the suitable freezing time, we set the lowest temperature at −25 °C and the cooling time of 15 min, the duration in step (iv) was set as 0.5, 1, 5, 24 h, respectively.

We tested 18 and 25 °C respectively, in step (v) for the best rewarming temperature.

Finally, the cold tolerance of ants feeding with different L-proline-augmented diets for 5 days was tested using the optimal freezing protocol. The survival rates in all the above experiments were calculated as the percentage of the number of the live ants after cryopreservation to that of ants before freezing.

2.4. Infrared thermal analysis based on cryo-stage system

The ants with different L-proline concentration feed were fixed on the disc of the BCS196 biological cryo-stage system (Linkam Scientific Instrument, England) by double-sided tapes. The temperature of the cryo-stage was controlled by software Linksys 32X and the rate was set as 2 °C min^{-1}. The thermal infrared images were captured by an infrared camera (SC620, FLIR Systems Inc., USA).

2.5. Differential scanning calorimetry analysis

To test the ratio of osmotically active water to osmotically inactive water, related thermal analysis were conducted by a differential scanning calorimetry (DSC 200 F3 calorimeter, Netzsch, Germany) [11,12,23,24]. Each experimental ant was sealed in a 40 μL aluminum pan and subjected to a program as follows: (i) heating to 30 °C at a rate of 10 °C min^{-1} and holding for 1 min; (ii) cooling to 20 °C at a rate of 20 °C min^{-1} and holding for

1 min; (iii) cooling to −5 °C at a rate of 10 °C min^{-1} and holding for 1 min; (iv) cooling to −35 °C at a rate of 1 °C min^{-1} and holding for 1 min; (v) heating to 20 °C at a rate of 20 °C min^{-1} and holding for 2 min; (vi) ending up the process at the protected temperature of 40 °C.

All the ants froze at their supercooling point (SCP) temperature. The thermal curves were analyzed by using the thermal analysis software (Proteus Analysis, Netzsch, Germany).

The amount of frozen, osmotically active (OA) water was calculated by using the following equation:

$$m_{OA} = m_w \times H/\Delta H. \quad (1)$$

m_w was the total water mass of the ants and ΔH was calculated by using the equation [25] as follows:

$$\Delta H = -2.05(T_s - 273.15) - 334.5. \quad (2)$$

The enthalpy of melting, H, and the supercooling temperature, T_s, were measured by the DSC and calculated from the thermal analysis software. The value of 334.5 J g^{-1} is the freeze exothermic enthalpy of water.

To get water content of each group of ants, several ants from the specified group were measured as the total weight (m_t). The weighted ants were dried in a vacuum dry oven at 80 °C for two days and then weighted for the dry weight (m_d) (Dataset S1 online). The water mass (m_w) was calculated by substracting the m_d from m_t. The water content of each group of ants was the ratio of m_w to m_t. For each tested ant in the DSC measurement, m_w was calculated through water content multiplied the m_t of the ant. The amount of unfrozen, osmotically inactive (OI) water was determined by subtracting the OA water from the m_w.

In order to confirm whether the ants' body fluid was frozen under the optimal freezing and rewarming protocol, we measured the freezing point with DSC by using the following programs: (i) heating to 30 °C at a rate of 10 °C min^{-1} and holding for 5 min; (ii) cooling to 10 °C at a rate of 10 °C min^{-1} and holding for 5 min; (iii) cooling to −40 °C at a rate of 1.75 °C min^{-1} and holding for 5 min; (iv) heating to 18 °C at a rate of 2 °C min^{-1} and holding for 5 min; (v) ending up the process at the protected temperature of 40 °C. The freezing temperature T_f was measured by DSC and calculated using the thermal analysis software.

2.6. Metabolomic profiling

We performed the targeted metabolomics analysis of animal amino acids for the ants fed with different L-proline-augmented diets (groups of Pro-0, 10, 20, 30). The different parts (head, thorax, and abdomen) of the ants in groups of Pro-0 and Pro-30 were also studied. After 5 days feeding, the tissue homogenate of 10 samples in each group (10 ants of group Pro-0, 10, 20 and 30; 10 heads, 10 thoraxes, and 10 abdomens of group Pro-0, and 30, respectively) was prepared. 500 μL of methanol and 1 mL of water were added to the (60 ± 10) mg of tissue homogenate. Then the solutions were sonicated for 60 min, centrifugated for 10 min, and diluted with DI-water for 50 times. Finally, the supernatant was used for the following mass analysis.

The amino acids of different samples were quantitatively determined by liquid chromatography (LC-20AXR, Shimazu, Japan) coupled with mass spectrometry (Otrap5500, AB SCIEX, America) [15]. 1 μL of the supernatant of each sample was injected into liquid chromatograph and separated by UPLC HSS T3 capillary column (1.8 μm, 2.1 mm × 150 mm) at 50 °C and the flow rate of 0.3 mL min^{-1} using gradient elution. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 95% acetonitrile in water (containing 0.1% formic acid and 1 mmol L^{-1} ammonium formate). The gradient elution program was processed in 95% A for 1 min then 6 min, 50% A for 2 min, 95% A for 3 min. Mass spectrometry was performed by using a quadrupole tandem mass spectrometer equipped with an ESI electrospray ion source. The source temperature was 150 °C and the capillary voltage was 2.0 kV with a positive ion acquisition mode. High purity nitrogen was provided as desolvation gas at 600 °C and the flow of 1000 Bar, and cone gas at the flow of 150 L h^{-1}. Liquid quality system was controlled by Analyst mass spectrometry data acquisition software (SCIEX). Multiquant data analysis software and Skyline quantitative analysis software were used to further analyze and obtain quantitative results. The metabolites were identified against relevant standards and subjected to quantitative analysis by using an internal standard calibration method. All chemical reagent was purchased from Sigma-Aldrich and Fisher Scientific.

2.7. Gene analysis

The *de novo* transcriptome sequencing analysis was performed on the ants of groups Pro-0 and Pro-30. Firstly, the total RNA samples were tested from the following 4 aspects to make sure the qualification: (i) RNA degradation and contamination was detected on 1% agarose gels; (ii) RNA purity was affirmed by spectrophotometer (NanoPhotometerIMPLEN, CA, USA); (iii) RNA concentration was measured by Qubit RNA Assay Kit and Flurometer (Qubit 2.0, Life Technologies, CA, USA); and (iiii) RNA integrity was determined applying the RNA Nano 6000 Assay Kit based on the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then the mRNA was purified from 1.5 μg of RNA from each sample using poly-T oligo-attached magnetic beads. Using mRNA fragmentation as a template, first strand cDNA was synthesized by adding random hexamers primer and M-MuLV Reverse Transcriptase (RNase H). Then the DNA Polymerase I and RNase H were added to synthesized second strand cDNA and exonuclease/polymerase were added to convert remaining overhangs into blunt ends. After adenylation of 3′ ends of DNA fragments, hybridization was prepared by ligating NEBNext Adaptor with hairpin loop structure. AMPure XP system (Beckman Coulter, Beverly, USA) was used to purify the library fragments for selecting cDNA fragments. At last, PCR amplification was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. After PCR products were purified, library quality was assessed with the Agilent Bioanalyzer 2100 system. According to the manufacturer's instructions, the clustering was performed by TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and cBot Cluster Generation System. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated [26,27].

Raw data were processed by in-house perl scripts to get clean data with high quality. Then transcriptome and transcription assembly were performed. Single nucleotide polymorphisms, insertion-deletion, simple sequence repeats, and gene expression levels were progressed in sequence. The differential expression analysis was performed using the DESeq (2010) R package [28]. The P-adjusted < 0.05 & $|\log_2(\text{fold change})| > 1$ was set as the threshold for significantly differential expression. Gene Ontology (GO), together with Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed genes were performed by the GOseq R packages and KOBAS software, respectively [29,30].

3. Results and discussion

3.1. The maximum amount of L-proline-augmented diets

For exploring the effect of L-proline-augmented diet on the freeze tolerance of ants, we first screened a proper concentration and feeding days. Five groups of ants were fed by five different

L-proline concentration diets for up to 9 days. The survival rate decreased with the increasing of feeding days, especially at the Pro-40 group (Fig. S1 online). Though L-proline is nontoxic [19], its metabolite ammonia shows dose-dependent toxicity to organisms, primarily neurological impairment [31]. It is confirmed that the concentration of L-proline should be no more than 30 mg g^{-1} to ensure a high survival rate of ants. Moreover, the survival rate of various L-proline concentration groups showed a statistically significant difference after 5 days.

3.2. Optimal freezing and rewarming procedure

We studied the effect of lowest temperature, cooling rate, freezing time, and rewarming temperature on the survival rates of the ants to explore the optimized freezing and rewarming procedure.

Four different lowest temperatures were preset through instrument input system, which were −1, −20, −25, and −40 °C, respectively. In order to monitor the temperature of the experimental ants, the actual temperatures were measured by thermocouples after reaching thermal equilibrium, which were −3.42, −21.46, −27.66, and −42.48 °C, respectively. When the actual temperature was −3.42 °C, we found that survival rate of ants was as high as 96.39% ± 1.94%, and even when temperature reduced to −27.66 °C, there were still 37.50% ± 1.73% alive ants (Fig. S2a online). However, when the temperature reached − 42.48 °C, the survival rate was zero. These results indicate that *C. japonicus* Mayr can tolerate slight low-temperature stress when they are not frozen (such as −3.42 °C). Further DSC data showed that the freezing point of ants of the group with feeding Pro-30 was −26.5 °C (Fig. 1a). It is found that once the phase change occurs, the survival rate will decrease largely with the decreasing of the lowest temperature (see Fig. S2a (online), from −21.46 to −27.66 °C). So we chose −27.66 °C as the lowest temperature to make sure ants were at freezing state. According to the experimental results, we set the lowest temperature of the freezer as −25 °C for the subsequent experimental treatment, which applied for improving the survival rates.

Cooling rate plays a significant role in the successful cryopreservation of biological materials. According to the "two-factor hypotheses of freezing injury" [32], at the cellular level, the optimal cooling rate should be screened to avoid both intracellular ice formation and cellular dehydration/shrinkage. For large-scale organisms, optimal cooling rate minimizes the thermo-mechanical stress and gets structural integrity and functionality [1]. Two different cooling rates were tested in our experiments,

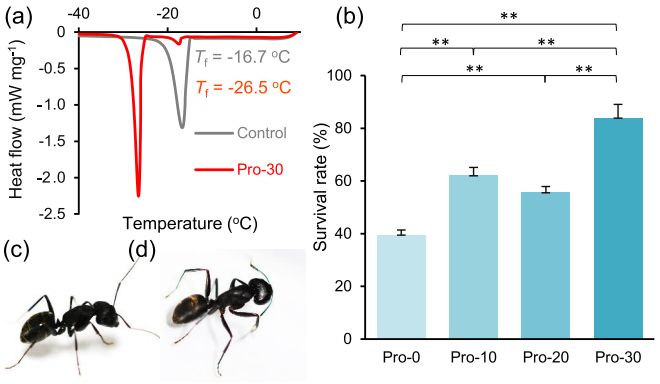


Fig. 1. (Color online) The enhanced freeze tolerance of the Japanese carpenter ants. (a) The reduction of a freezing point with the accumulation of L-proline. Control: foraging ants collected from nature. (b) The survival rate of ants in different L-proline concentration feeding groups. (c) Morphology of ants collected from nature. (d) Morphology of ants after freezing and rewarming operation. Each column represents the mean ± SD (standard deviation). **: $P < 0.01$.

which were 1.75 and 0.55 °C min^{-1} calculated by thermocouples measurement results (Fig. S3 online), respectively. Compared to 0.55 °C min^{-1} slow cooling rate, ants cooling at 1.75 °C min^{-1} achieved a higher survival rate (Fig. S2b online). The cooling rate of 0.55 °C min^{-1} is too slow for freezing, which will induce cellular dehydration and shrinkage.

According to Arrhenius equation, biological and chemical reactions are dramatically depressed with the decrease of temperature. However, living organisms can not be preserved in a static low-temperature state indefinitely, which means that the freezing duration is also important to apply cryopreservation for organisms. Four different freezing durations were tested, which were 0.5, 1, 5 and 24 h, respectively. The survival rate decreased with the increase of the freezing time, from 68.89% ± 3.74%, 37.50% ± 1.73%, and 14.44% ± 1.94%, down to 1.11% ± 0.28%, which were shown in Fig. S2c (online). At high subzero temperature, cellular activity still slowly progressed. It is assumed that the harmful metabolic intermediates, by-products, reactive oxygen species, and oxidative damage to macromolecules accumulate for the less flexible enzymes in low temperature. Reduced protein function truncates cellular response to stressors. In addition, the loss of membrane ion balance appears at low temperature [4,33]. Additionally, the recrystallization intensifies over time and causes mechanically damage to cells and tissues. To make sure the effect of cold tolerance regulation experiment, 1 h is chosen considering survival rate and biological response.

Two different rewarming temperatures were tested, which were 18 and 25 °C. The survival rate of 18 °C group (37.50% ± 1.73%) was higher than that of the 25 °C group (7.22% ± 1.00%), which was shown in Fig. S2d (online). Because the rewarming time of ants from −25 to 18 or 25 °C is the same, the rewarming rates are different in these two rewarming processes. So we inferred that the rewarming rate at the 18 °C group might be more in favor of surviving for ants, causing less recrystallization and structural and physiological damage. Another reason may be due to that rewarming at 18 °C for 24 h is propitious to the physiological recovery of ants (restore homeostasis and repair damage). We noticed that ants were not active immediately post-thaw, while after keeping at 18 °C for more than 1 h, part of ants showed the signs of recovery. It indicated that the recovery of ants after freezing and rewarming operation needs time.

Considering the results above, we set the freezing and rewarming procedure as cooling the temperature to −27.66 °C with the cooling rate of 1.75 °C min^{-1}, keeping at −25 °C for 1 h, and then rewarming to 18 °C for 24 h.

3.3. L-proline-augmented diets improve ants cold tolerance

Based on the results of the maximum screening amount of L-proline-augmented diets experiment and the optimal freezing-rewarming protocol, we fed the ants of Pro-0, Pro-10, Pro-20, Pro-30 groups for 5 days, and then froze them to −27.66 °C with the cooling rate of 1.75 °C min^{-1} and kept for 1 h, and then rewarmed them to 18 °C and kept for 24 h. Then we counted both the live and dead ants and calculated the survival rate of the ants in each group, shown in Fig. 1b. We noted that the cold tolerance of ants enhanced slightly (39.44% ± 1.38% vs. 37.50% ± 1.73%) after 5 days' acclimation in dark environment (Figs. 1b, S2 (online)). The worker ants of *C. japonicus* Mayr stay in the dark nest to spend cold winter. With our acclimation conduction for ants, the continuous dark environment may be a signal for the ants to prepare for the cold threats. However, the slight enhancement of cold tolerance only assists the ants to go through the relatively warm winter in the nest. The survival rate of the ants increased from 39.44% ± 1.38% to 61.94% ± 2.27% after acclimation with 10 mg L-proline diets. While the survival rate slightly decreased from Pro-

10 to Pro-20 group (55.55% ± 1.68%), they are still both higher than the Pro-0 group. However, there was no significant difference between these two groups. The survival rate of the ants increased to 83.88% ± 3.67% as the L-proline concentration increased to 30 mg g^{-1}. Our results revealed that the survival rate of ants significantly increased with the increase of the L-proline concentration in the range of 0–30 mg g^{-1} ($P < 0.01$). Notably, the survival rate in Pro-30 group is significantly different from that in the Pro-10 and the Pro-20 group, indicating feeding 30 mg g^{-1} L-proline is the most effective for improving cold tolerance of worker ants. Moreover, the morphology of ants showed no difference with the ants without any experimental processing after cryopreservation (Fig. 1c, d). In addition, most live ants could spontaneously crawl and take food normally after freezing (Movie S1, S2, S3 online).

3.4. Visualization of temperature changes and distribution in ants body

We recorded the infrared thermal images of the ants in different L-proline concentration groups during the cooling and rewarming process to reveal the temperature changes and distribution of the ants visually (Figs. 2a, b and S4a (online)). The infrared thermal images (Figs. 2a, and S4a (online)) revealed that the cold energy of cryo-stage transferred from the surface of the ants' body to the inside of ants' body during the cooling process. While during the initial warming process, ants not only obtained heat from the cryo-stage but also absorbed heat from surroundings. So the body temperature of ants was higher than the disc temperature in infrared thermal images (Figs. 2b, and S4a (online)). Using the software of the infrared camera, we calculated the average temperature of the total body (Figs. 2c, and S4b (online)) and different body parts (Figs. 2d, e, and S4c, d (online)). At the same recording time and the same cooling rate, the ants without L-proline-augmented feed showed the fastest cooling rate to the target temperature (Figs. 2c and S4b (online)). Moreover, with the increase of L-proline concentrations, the inhibition on the cooling rate of the ants gradually enhanced, which revealed that L-proline could delay the cooling process of the body fluids of the ants. In addition, the head of the ants in Pro-30 group cooled more slowly than abdomen, while the cooling rate of head was faster than that of abdomen in ant of Pro-0 group (Figs. 2d, e, and S4c and d (online)) demonstrating that slow cooling rate in head may play an important role in brain protection and be beneficial for surviving.

3.5. Metabolomic profiling

In order to explore whether the L-proline had accumulated in ants, we analyzed the content of L-proline in the ants by using liquid chromatography-mass spectrometry (LC–MS, Fig. 3a). The targeted amino acids analysis suggested that rearing the ants on L-proline-augmented diets promoted the significant accumulation

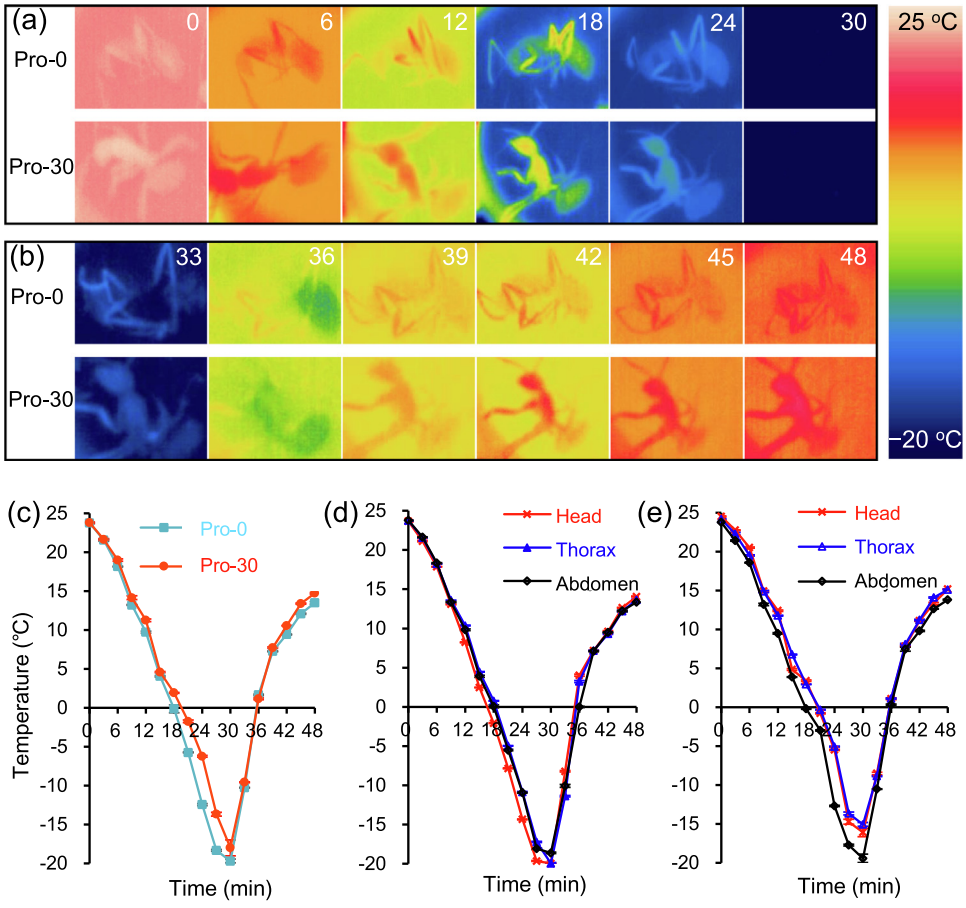


Fig. 2. (Color online) The temperature changes and distribution in the body of ants during cooling and rewarming process detected by infrared analysis based on the cryo-stage system. (a) Infrared thermal images of ants during the cooling process at the rate of 2 °C min^{-1}. (b) Infrared thermal images of ants at the rewarming rate of 2 °C min^{-1}. The white font in (a) and (b) means the time of ants operated by cryo-stage from 25 °C, the unit is minute. (c) The average temperature of ants fed with Pro-0 and Pro-30 diets during the cooling and rewarming processes. (d) The average temperature in different parts of ant body feeding Pro-0 diets. (e) The average temperature in different parts of ant body feeding Pro-30 diets.

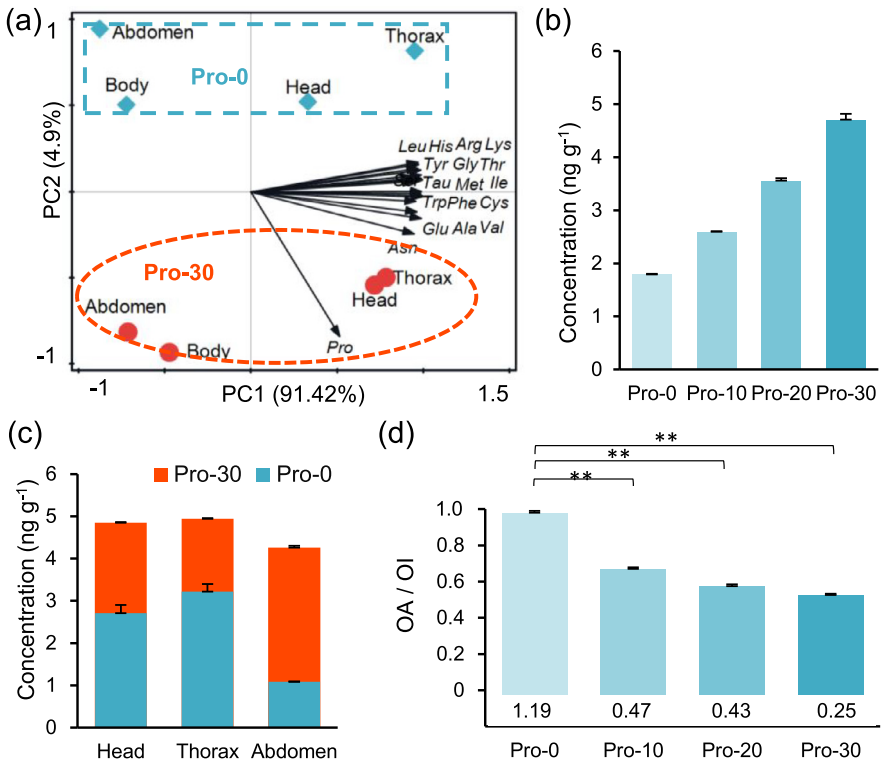


Fig. 3. (Color online) The biophysics changes of ants with feeding L-proline-augmented diets. (a) Principal component analysis of amino acid metabolomic changes (eigenvectors) of *Camponotus japonicus* Mayr with different concentration L-proline feeding (Pro-0: blue; Pro-30: jacinth) using Canoco v5.0 for Windows. PC1 explained 91.42% of variance and PC2 showed 4.9%. (b) Changes of L-proline levels in ants with different concentration L-proline feeding. (c) Accumulation of L-proline in different body parts (head, thorax, and abdomen) of ants feeding Pro-0 and Pro-30. Each column represents the mean ± SD. **: $P < 0.01$. (d) The gradual decrease of the OA/OI (OA: osmotically active water; OI: osmotically inactive water) ratio with the increase of feeding L-proline concentration. The data below the four columns are OA (Unit: mg mg^{-1} DM, DM: dry mass).

of L-proline from 1.78 to 4.64 ng g^{-1} (Fig. 3b, Dataset S2 (online)), and the accumulation was positively correlated with the survival rate of ants (Fig. 1b and 3b, Dataset S2 (online)). Additionally, we noticed that the contents of other types of amino acids also increased (Fig. 3a, Dataset S2 (online)), especially asparagine, which increased nearly 18 times. This phenomenon may owe to the conversion among amino acids. According to the principal component analysis, we found that the concentration of alanine, asparagine, glutamic acid, and valine increased, which was related to the L-proline accumulation (Fig. 3a). Among these increasing amino acids, alanine was reported to accumulate in some freeze-tolerant insects [4,34].

In addition, the accumulation of L-proline was also supported by the targeted amino acids analysis of different body parts of ants. The results showed that L-proline distributed among all parts of the body in Pro-0 group. With L-proline feeding (Pro-30 group), the accumulation of L-proline in the three parts was all improved and L-proline accumulated more prominently in the abdomen than the other two parts (Fig. 3c). This probably is because both the temporary storage (crop) and absorption (midgut) of amino acids happen in the abdomen. The accumulation of L-proline in the head was more prominent than in the thorax. This is consistent with the results observed by the infrared camera and further explained the slower cooling rate of head owing to the more accumulation of L-proline.

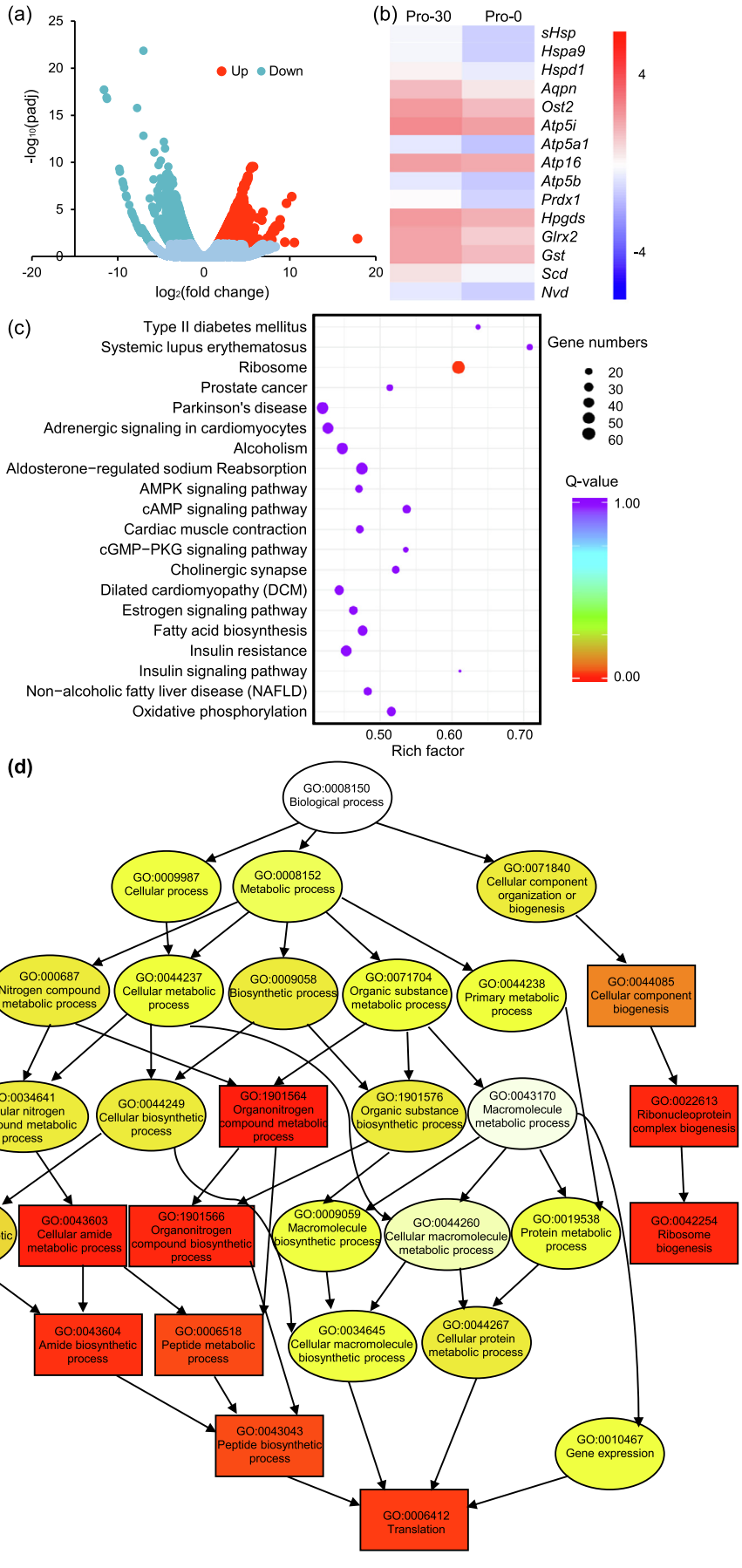
3.6. L-proline-augmented diets promote dehydration

The transition of water-ice is a great obstacle in the cryopreservation of hydrated tissues and organs, which causes terrible mechanical injury. The location, form (free or bound), quantity, and dissolved solutes of water are all influential for the survival rate of complex organisms. Cryoprotective dehydration is one strategy for the winter survival of some cold-tolerant organisms. The high rate of water loss contributed to the depression of body fluid freezing point for the increase of solutes concentration.

We found the L-proline-augmented diet acclimation resulted in the decrease of water content in ants' body fluid, from 70.58% down to 41.75% (see Dataset S1 online), and promoted the cryoprotective dehydration of ants. We further studied the form of water in ants by using DSC thermal analysis. Firstly, the amount of osmotically active water (OA) significantly decreased from 1.19 to 0.25 mg mg^{-1} DM with the increase of the L-proline concentration (Fig. 3d, Dataset S1 (online)), which resulted in the decrease of ice crystals' number and reduced the potential damage of intracellular ice during freezing. The amount of osmotically inactive water (OI) also decreased from 1.21 to 0.46 mg mg^{-1} DM (Fig. 3d). The OA/OI ratio of different groups showed a significantly decreasing trend with the increasing of L-proline concentration, from 98.10% ± 1.02% (Pro-0), 67.04% ± 0.78% (Pro-10), 57.57% ± 0.87% (Pro-20) to 52.64% ± 0.62% (Pro-30), shown in Fig. 3d. This trend was significantly correlated with the increasing survival rate during the freezing and rewarming procedure. Our results indicated that the L-proline-augmented diet can change the water state in the body of ants and benefit to their cold hardiness.

3.7. L-proline enhances freeze tolerance of ants

We attained the DSC curves of the ants in different groups by using the DSC measurement with the same cooling/rewarming


(a)
Up
Down
-log10(padj)
log2(fold change)
(b)
Pro-30
Pro-0
sHsp
Hspa9
Hspd1
Aqpn
Ost2
Atp5i
Atp5a1
Atp16
Atp5b
Prdx1
Hpgds
Glrx2
Gst
Scd
Nvd
(c)
Type II diabetes mellitus
Systemic lupus erythematosus
Ribosome
Prostate cancer
Parkinson's disease
Adrenergic signaling in cardiomyocytes
Alcoholism
Aldosterone−regulated sodium Reabsorption
AMPK signaling pathway
cAMP signaling pathway
Cardiac muscle contraction
cGMP−PKG signaling pathway
Cholinergic synapse
Dilated cardiomyopathy (DCM)
Estrogen signaling pathway
Fatty acid biosynthesis
Insulin resistance
Insulin signaling pathway
Non−alcoholic fatty liver disease (NAFLD)
Oxidative phosphorylation
Rich factor
Gene numbers
Q-value
(d)
GO:0008150 Biological process
GO:0009987 Cellular process
GO:0008152 Metabolic process
GO:0071840 Cellular component organization or biogenesis
GO:000687 Nitrogen compound metabolic process
GO:0044237 Cellular metabolic process
GO:0009058 Biosynthetic process
GO:0071704 Organic substance metabolic process
GO:0044238 Primary metabolic process
GO:0044085 Cellular component biogenesis
GO:0034641 Cellular nitrogen compound metabolic process
GO:0044249 Cellular biosynthetic process
GO:1901564 Organonitrogen compound metabolic process
GO:1901576 Organic substance biosynthetic process
GO:0043170 Macromolecule metabolic process
GO:0022613 Ribonucleoprotein complex biogenesis
GO:0044271 Cellular nitrogen compound biosynthetic process
GO:0043603 Cellular amide metabolic process
GO:1901566 Organonitrogen compound biosynthetic process
GO:0009059 Macromolecule biosynthetic process
GO:0044260 Cellular macromolecule metabolic process
GO:0019538 Protein metabolic process
GO:0042254 Ribosome biogenesis
GO:0043604 Amide biosynthetic process
GO:0006518 Peptide metabolic process
GO:0034645 Cellular macromolecule biosynthetic process
GO:0044267 Cellular protein metabolic process
GO:0043043 Peptide biosynthetic process
GO:0010467 Gene expression
GO:0006412 Translation

procedure as the optimal protocol. We found that the freezing point of the ants collected in nature was around −16.7 °C, while it was down to about −26.5 °C after feeding L-proline diet in Pro-30 group (Fig. 1a). This further confirmed that the accumulation of L-proline in ants inhibited crystallization and depressed the freezing point. However, the actual temperature we detected in the experiment was −27.66 °C, which is lower than the freezing point of ants in Pro-30 group. Therefore, we suggested that ants after L-proline-augmented feeding took the freeze-tolerant strategies to survive at −27.66 °C.

3.8. L-proline feeding induces gene expression responses

Based on RNA-seq technology, genome-wide analysis of gene expression was performed on the ants of Pro-0 and Pro-30 groups to identify the responsive genes and biological pathways influenced by the accumulation of L-proline (Dataset S3 online) [35]. Rearing the ants on L-proline-augmented diets, 1351 genes were up-regulated (Dataset S4 online), and 2166 genes (Dataset S5 online) were down-regulated (Fig. 4a). Among the differentially expressed genes, several heat shock protein genes were up-regulated, such as *Hsp70*, *Hsp60,* and low-molecular-weight *Hsps* (Fig. 4b, Dataset S4 online). Heat shock proteins, as molecular chaperones, play an important role in decreasing protein folding, degradation as well as transportation of cellular material when environmental stresses occur [36,37]. *Aqpn*, an aquaporin gene, was identified in up-regulated genes. *Aqpn* is beneficial for augmented cold tolerance, because the aquaporin is membrane water channels that is essential to adjust the volume and osmotic conditions of cells during the freezing process (Fig. 4b, Dataset S4 online) [38]. Two genes of desaturase that attributed to membrane restructuring were also up-regulated (Fig. 4b, Dataset S4 online). Various genes of antioxidants, including superoxide dismutase and glutathione-s-transferase, were up-regulated and contributed to decreasing the disruption of aerobic respiration caused by cold stress (Fig. 4b, Dataset S4 online) [39]. These genes were usually found to regulate during dehydration and hydration [34,40,41].

Gene Ontology (GO) enrichment analysis classified differentially expressed genes into three main categories: biological process, cellular component, and molecular function. There were 2356 up-regulated GO terms (Dataset S6 online) and 3306 down-regulated GO (Dataset S7 online). For biological process categories, "cellular metabolic process", "nitrogen compound metabolic process", and "cellular nitrogen compound metabolic process" were enriched in the up-regulated genes (Fig. S5 online). The top enriched terms of the cellular component were "intracellular", and "macromolecular complex" (Fig. S5 online). According to the GO analysis (Figs. 4d and S6 (online)), the accumulation of L-proline enhanced the metabolism associated to amino acids, including amino acid catabolism, nitrogen compound metabolic process, cellular nitrogen compound biosynthetic process, organonitrogen compound metabolic process, and peptide metabolic process. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis was further performed to

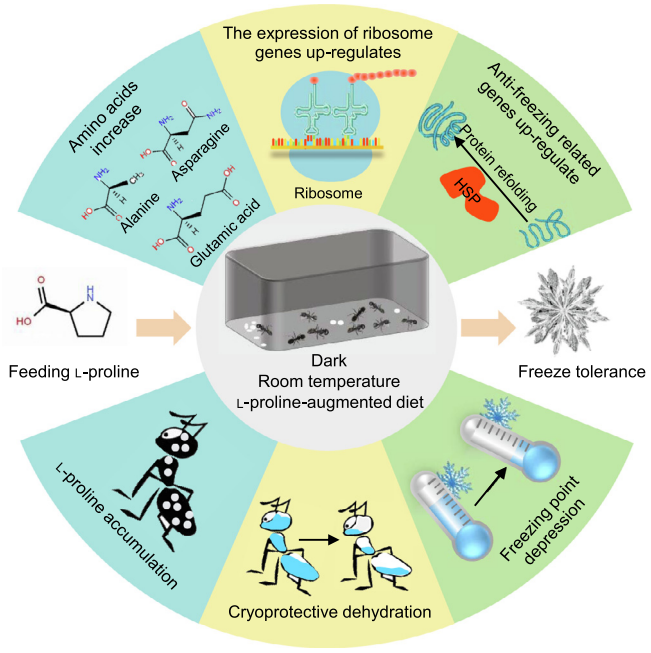


Fig. 5. (Color online) Mechanisms of improved cold tolerance of ants with interventions. HSP: heat shock protein.

understand the biochemical metabolic pathways and signal transduction pathways (Dataset S8, and S9 online). The pathway, "ribosome", was up-regulated, which was consistent with the results of GO enrichment analysis (Figs. 4c, S7 (online)). The profound up-regulated gene expression patterns caused by the increasing concentrations of L-proline in tissues of ants may also explain the maximum amount of L-proline-augmented diets and the increase of some amino acids. It was confirmed in our study that both GO, and KEGG enrichment suggested the characteristics of ammonia toxicity, such as Parkinson's disease, AMPK signaling pathway (Figs. S5 and S7, Dataset S6–S9 online) [31]. The genes expressions regulating alanine, aspartate, and glutamate metabolism were also enhanced, which was consistent with the alanine, asparagine, and glutamic acid accumulation in the metabolism analysis (Dataset S8 online).

According to the results above, we suggested that feeding ants with L-proline-augmented diets at the dark and room temperature environment could prominently improve the ability of freeze tolerance of Japanese carpenter ants. The targeted metabolomics analysis of amino acids proved L-proline accumulated in tissues. We also found that other kinds of amino acids (alanine, asparagine, glutamic acid) increase with the accumulation of L-proline according to the principal component analysis of amino acid metabolomic changes. The L-proline accumulation also contributed to the cryoprotective dehydration and the depression of the freezing point of ants. Additionally, the genes expression related to cold tolerance was up-regulated. The enrichment analysis of the differentially

Fig. 4. (Color online) Gene expression of ants under the L-proline-augmented diets feeding. (a) Volcano plot of differentially expressed genes. (b) Heatmaps of several representative up-regulated genes after feeding L-proline. Each column is an experimental condition (Pro-0, Pro-30), and each row represents a gene. The ratio of change for the differentially expressed genes is displayed in colors, in which red indicates enrichment and blue indicates depletion. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. The vertical axis represents the KEGG pathway name, and the horizontal axis is the rich factor corresponding to the pathway. The Q-value is the P-value (the statistically significant level of enrichment analysis) after multiple hypothesis test corrections and is represented by the color of the dot: the color is closer to the red which represents the smaller Q-value. The numbers of differential expressed genes contained in each pathway is represented by the size of the dot. (d) One of Gene Ontology (GO) enrichment categories: the biological process. Branches represent inclusive relationships, and the range of functions defined from top to bottom is becoming more specific in the directed acyclic graph. The depth of the color represents the degree of enrichment: the darker color shows a higher degree of enrichment. The 10 boxes are the main nodes of the directed acyclic graph representing the highest degree of enriched GO terms.

expressed genes suggested that translation was the main pathway influenced by feeding L-proline. All these mechanisms contributed to the improvement of freeze tolerance of Japanese carpenter ants (Fig. 5).

4. Conclusions

In conclusion, this study demonstrates that cold tolerance of complex organisms, *Camponotus japonicus* Mayr, which is cold sensitive and 6–15 mm long, can be enhanced to withstand frozen as low as −27.66 °C. Other than pure freezing and rewarming protocol, this conversion of cold tolerance is further achieved by simple artificial interventions which mimicks the cold response mechanisms of freeze-tolerant organisms in nature. Firstly, feeding ants with L-proline-augmented diets facilitates the accumulation of L-proline and other kinds of amino acid, such as alanine, asparagine, and glutamic acid, in tissues of ants. Secondly, the water contents in ants' body decrease with the elevation of L-proline levels. Thirdly, the freezing point of ants' body depresses owning the accumulation of L-proline and decrease of water contents. Though the freezing of ants' body fluid is significantly inhibited, this species is at the frozen states when the temperature is down to −27.66 °C. Therefore, we suggest the ants are freeze-tolerant after artificial augment of L-proline. Last but not least, the genes expression of *C. japonicus* Mayr are also regulated. The genes encoding heat shock protein, desaturase, and antioxidants are up-regulated and contribute to the enhancement of cold tolerance. The results of GO and KEGG analysis also indicate that the pathway "ribosome" was prominently up-regulated. More importantly, this enhancement of cold tolerance by simple artificial interventions is an inspirational approach to overcome the challenges of cryopreservation of tissues and organs.

Conflict of interest

The authors declare that they have no conflict of interests.

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Author contributions

Mengjia Dou, Yazhou Li and Ziqiao Sun performed the investigation. Mengjia Dou wrote the original manuscript. Lei Li and Wei Rao further edited the manuscript. Lei Li and Wei Rao supported and supervised the research. All the authors contributed to discussion.

Appendix A. Supplementary materials

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Mengjia Dou obtained her B.E. degree from University of Science and Technology Beijing, China. She is a Ph.D. candicate at the Technical Institute of Physics and Chemistry, Chinese Academy of Sciences under the guidance of Prof. Wei Rao. She mainly focuses on the cryopreservation of organisms and bioinspired cryoprotectants.

Yazhou Li received the B.E. degree in Energy and Power Engineering from the Beijing University of Civil Engineering and Architecture in 2018. He is currently pursuing the Master degree with the School of Energy and Power Engineering, Beihang University. His research interests include cryopreservation, data processing, and machine learning.

Lei Li is an associate professor at Technical Institute of Physics and Chemistry, Chinese Academy of Sciences (CAS). She received her Ph.D. degree in Biomedical Engineering from Tsinghua University in 2009. She was a visiting scholar at Massachusetts General Hospital & Harvard Medical School from 2015 to 2016. She is continually interested in lab-on-a-chip applications using microfluidics. Her recent work lies at the intersection between micro-tissue engineering, biopreservation and microfluidic platforms.

Wei Rao is a professor at the Technical Institute of Physics and Chemistry (TIPC), Chinese Academy of Sciences (CAS). She obtained her bachelor's and master's degrees at Harbin Institute of Technology in 2003 and 2006, respectively. Then she received her Ph.D. degree in Cryogenic Engineering from TIPC, CAS. Her research is highly interdisciplinary, and covers cryobiology, micro/nanomaterials, and bioheat transfer. She is dedicated to introducing frontier micro/nanotechnology into cryomedicine to achieve precise controllability of temperature distributions.