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Effect of cooling rates on the cold hardiness and cryoprotectant profiles of locust eggs ☆

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Abstract

To examine the relationship between cooling rate and cold hardiness in eggs of the migratory locust, *Locusta migratoria*, the survival rates and cryoprotectant levels of three embryonic developmental stages were measured at different cooling rates (from 0.05 to 0.8 °C min^{-1}) in acclimated and non-acclimated eggs. Egg survival rate increased with decreasing cooling rate. The concentration of cryoprotectants (myo-inositol, trehalose, mannitol, glycerol, and sorbitol) increased in non-acclimated eggs, but varied significantly in response to different cooling rates in acclimated eggs. The acclimation process (5 °C for 3 days) did not increase eggs resistance to quick cooling ("plunge" cooling and 0.8 °C min^{-1}). Earlier stage embryos were much more sensitive than later stage embryos to the same cooling rates. Time spent at subzero temperatures also had a strong influence on egg survival.

Keywords: Migratory locust; Cold hardiness; Cooling rates; Cryoprotectant; Rapid cold hardening

Low temperature is the major adverse environmental factor that insects must overcome in temperate and cold regions. Its impact depends on the rate, intensity, and duration, of temperature change [49]. Insects have evolved a series of strategies to survive cold conditions. In the last few decades, groundbreaking research has been conducted on many aspects of insect cold hardiness [6,26,42,44,46,52–54]. Based on their responses to freezing, insects are commonly categorized as either freeze tolerant or freeze intolerant [26,46]. Bale [1] proposed further sub-divisions of opportunistic, chill-susceptible, chill-tolerant, freeze avoiding, and freeze tolerant [1].

Besides high mortality, low temperature can also cause cold injury, which is comprised of nonfreezing and freezing injuries [26]. Nonfreezing injury usually is divided into two categories: (1) direct

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chilling injury, a consequence of the rapid cooling involved in phase transitions in membrane lipids [37]; and (2) indirect chilling injury, resulting from long-term cold exposure [33]. Freezing injury leads to cellular dehydration, hypertonicity of the cytoplasm, mechanical injury of cells, recrystallization, etc [13,26]. To minimize these injuries, insects have evolved a series of behavioral and physiological strategies, such as seeking shelter, dormancy (diapause), changing the fluidity of cell membranes and accumulating sugars, polyols, antifreeze proteins, and amino acids. One of the most important physiological adaptations to low temperatures is to the ability to increase the concentration of low molecular weight organic solutions that function as cryoprotectants. These are accumulated either at high concentrations as extracellular ice to prevent intracellular volume from falling below a critical minimum, or, in low concentrations as membrane protectants that stabilize membrane bilayer structure [45]. These cryoprotectants can also lower the supercooling point in some species [8,17,27].

Because rapid freezing and thawing severely damages cells, cooling rate has an important effect on survival [31]. The relationship between cooling rate and larval and adult survival has been documented in some insect species [22,31,40]. However, to date, there has been no definitive evidence to suggest that the same relationship between cooling and survival existing in insect eggs, nor has the effect of cooling rate on cryoprotectant secretion been elucidated. Whereas mammalian embryos develop in a thermally stable environment, insect embryos are sometimes subject to large temperature fluctuations. In some insects, two major obstacles limit the cold hardiness of eggs: (1) embryos have shells (chorion) and vitelline membranes, which prevent the loss of water and uptake of cryoprotectants; and (2) their embryos are extremely sensitive to chilling so that supercooling from −5 to −20 °C is lethal [14,30]. However, many insects overwinter in the egg stage, attaining cold hardiness by diapause or acclimation at prechilling temperature [18,48].

The migratory locust, *Locusta migratoria* L., has a remarkably broad geographic distribution from tropical to temperate zones [50]. It has 1–4 generations per year and overwinters as eggs in the soil. Agricultural crops in China have suffered serious damage from this pest [20]. In Hebei and Shandong Provinces in northern China, the migratory locust has two generations annually, the so-called "summer locust" and "autumn locust." The eggs of "autumn locusts" are laid in October and overwinter in diapause in the soil until the following April or May when the embryos begin to develop and hatch [29]. Diapause usually occurs during the balstokinesis stage (mid-stage) of locust embryogenesis [3]. The temperature in Hebei Province in winter fluctuates from 0 to −15 °C, and the average minimum temperature in January is −9.0 °C. Although the fact that eggs are laid under soil affords them some protection from extreme cold, especially from quick cooling, the ability to withstand cold is nonetheless essential for eggs to survive the north China winter. Geographical and seasonal variation in the cold hardiness of the migratory locust's eggs has been documented in detail. The fall population has been found to have a significantly lower $Ltemp_{50}$ than the summer and post-winter populations [18,19]. Furthermore the cold hardiness of first instar hoppers can be enhanced by rapid cold acclimation [51]. However, the relationship between cooling rate and cold hardiness of eggs has not been sufficiently investigated.

This paper describes the level of cold tolerance of locust eggs, and associated changes in quantity of low molecular weight cryoprotectants, in response to different cooling rates. Furthermore, we attempt to answer the following questions: (1) Is there a relationship between the concentration of accumulated cryoprotectants and egg survival at different cooling rates? (2) Does the acclimation process enhance eggs' resistance to rapid cooling rates? And (3) Do eggs at different developmental stages differ in their ability to develop cold hardiness in response to different cooling rates? Finally, we attempt to determine if eggs accumulate variable cryoprotectant concentrations at different cooling rates.

Materials and methods

Insects

Adult migratory locusts were collected in April 2003 from Huanghua County (38′25° N, 117′20°

E), Hebei province, China. The population was propagated for seven to eight generations in a laboratory. Groups of 150–200 hatchlings were reared in wood-framed cages ($50 \times 70 \times 80$ cm) in the laboratory under a long-day photoperiod (14 h light/10 h darkness cycle) at 30 ± 1 °C. Both nymphs and adults were fed wheat seedlings and wheat bran. Sterilized sand was provided as the oviposition medium. Eggs were collected daily to ensure an even stage of development. The egg pods were incubated at 30 °C in sterilized sand, and individual eggs separated from egg pods when required. Water content of the sand was kept at 10%.

Supercooling point determination

To determine the probable influence of cold hardening on the eggs' supercooling point (SCP), 30 healthy acclimated and non-acclimated mid-stage eggs were subjected to SCP measurement. The eggs were fixed to a thermocouple that was linked to an automatic recorder (uR100, Model 4152, Yologama Electrical, Seoul, Korea). The SCP was indicated on the recorder by a sudden spike in the temperature of the thermocouple. Details were described by Jing and Kang [19].

Experimental design

Locust embryonic development is divided into three stages: anatrepsis (early-stage), balstokinesis (mid-stage), and katatrepsis (late-stage) [18]. Two, seven, and 12-day-old eggs incubated at 30 °C were regarded as early-, mid-, and late-stage, respectively [18]. To examine the difference between developmental stages and the effect of acclimation vs. non-acclimation, we designed a 3×2 experimental matrix incorporating the three developmental stages with or without acclimation, as follows: early-stage acclimated (EA), mid-stage acclimated (MA), late-stage acclimation (LA), early-stage non-acclimated (ENA), mid-stage non-acclimated (MNA), and late-stage non-acclimated (LNA), respectively. Eggs were acclimated by pre-chilling for 3 days [18].

Two sets of experiments were performed to examine the effects of different cooling rates on egg cold hardiness. In the first experiment, each group of 40 eggs was transferred into plastic tubes, the temperature of which was controlled by a programmable refrigerated bath (Polyscience, USA). Eggs were cooled from 30 to −10 °C at 0.8, 0.4, 0.2, 0.1, 0.05 °C min^{-1} or to −10 °C directly ("plunge" treatment), then held at −10 °C for 10 h (this threshold was pre-determined to result in approximately 90% mortality). Eggs were then re-warmed to 30 °C at 0.1 °C min^{-1} and transferred to a 30 °C environmental chamber. The number of hatched or dead eggs was counted daily.

In the second experiment, to eliminate the potential confounding effect of different lengths of exposure to subzero temperatures, eggs were exposed to subzero temperatures for the same period of time. In the first experiment, the group of eggs cooled to −10 °C at 0.05 °C min^{-1} spent 800 min below 0 °C (of which 600 min was at −10 °C), whereas the group cooled at 0.8 °C min^{-1} was only exposed to subzero temperatures for 612.5 min. In the second experiment, we equalized the amount of time spent at potentially injurious subzero temperatures by cooling each group of eggs at a uniform rate from 0 to −10 °C. Groups of 40 ENA, MNA, and LNA eggs were first cooled at 0.8, 0.4, 0.2, 0.1 or 0.05 °C min^{-1} to 0 °C, or plunged to 0 °C, respectively, after which they were cooled at the same rate, i.e., 0.1 °C min^{-1}, to −10 °C. Subsequent treatment steps were the same as in the first experiment. In each experimental group, five replicates of 40 eggs each were used.

Measurement of low molecular weight sugars and polyols

Groups of EA, ENA, MA, MNA, LA, and LNA eggs that had been cooled to −10 °C in the first experiment were immediately frozen with liquid nitrogen and stored at −70 °C until analysis. Low molecular weight cryoprotectants were measured by capillary gas chromatography as their *o*-methyloxime trimethylsilyl (TMS) derivatives [24]. Each group of eggs was weighed and homogenized with 0.4 ml of 70% (v/v) ethanol containing 25 μg dulcitol (an internal standard) in an Eppendorf tube that had been rinsed with 0.2 μl of 70% ethanol. After centrifugation at 10,000*g* for 5 min, the supernatant was removed and the process

repeated. The pooled supernatants were evaporated until dry under a stream of nitrogen at 40 °C. Twenty-five microliter of dimethylformamide and 25 μl *o*-methylhydroxylamine in pyridine (200 mg ml^{-1}) were added to the residue for oximation, then heated at 70 °C for 15 min. Silylation was accomplished by adding 75 μl dimethylformamide and 30 μl trimethylsilylimidazol to the reaction mixture which was further heated to 80 °C for 15 min. After re-extraction of the desired derivatives into isooctane, using 2 × 75 μl of the solvent, a 1-μl aliquot was injected into an injection port of a gas chromatograph (Pye Unicam 204). Separation and quantification of sugars and polyols were achieved on a 25 m × 0.25 mm i.d. BP-5 silica capillary column. The temperature program was: 3 min at 120 °C then 12 °C min^{-1} to 280 °C for 40 min. Identity of the revealed components was established against authentic standards. Each experimental group contained three replicates (8 eggs per replicate).

Statistical analysis

Hatching success of the various treatments was compared to the corrected percentage of hatched eggs of a control group of eggs that had been incubated at normal temperature. Differences between treatments were compared either by *t* test (for comparison of two means), or by one-way analysis of variance (ANOVA) followed by a Tukey's test for multiple comparisons. The relationship between cooling rate and survival rate was simulated by linear regression analysis. Treatment differences were considered significant at $P < 0.05$. Values are reported as means ± SE. Data were analyzed using SPSS 11.0 software.

Results

Effects of cooling rate on cold hardiness

Supercooling point

There was no significant variation in the SCP values of MNA ($F_{5,179} = 1.698$, $P = 0.137$) and MA ($F_{5,179} = 0.379$, $P = 0.862$) eggs (Fig. 1).

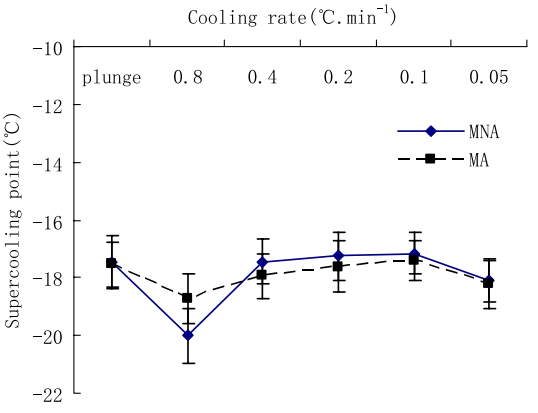

Fig. 1. Supercooling points of migratory locust eggs at different cooling rates. MNA, mid-stage non-acclimated, MA, mid-stage acclimated.

Survival at different cooling rates

The slowest cooling rate resulted in the highest egg survival rate in all six treatment groups (Fig. 2), and, whether acclimated or not, mid- and late-stage eggs always had much higher survival than early-stage eggs.

Non-acclimated eggs displayed significant differences in survival at different cooling rates (ENA: $F_{5,29} = 23.147$, $P < 0.001$; MNA: $F_{5,29} = 87.425$, $P < 0.001$; LNA: $F_{5,29} = 46.609$, $P < 0.001$; Fig. 2A). Eggs cooled at 0.05, 0.1, 0.2 °C min^{-1} had significantly higher survival than those that were "plunge" cooled or cooled at 0.8 °C min^{-1}. However, similar survival was observed following acclimation (5 °C for 3 days) (EA: $F_{5,29} = 26.754$, $P < 0.001$; MA: $F_{5,29} = 158.768$, $P < 0.001$; LA: $F_{5,29} = 44.515$, $P < 0.001$; Fig. 2B). At relatively fast cooling rates ("plunge" cooling and 0.8 °C min^{-1}) there was no significant difference in survival between non-acclimated and acclimated eggs (mid-stage: "plunge," $t = 0.937$, $P = 0.376$, 0.8 °C min^{-1}, $t = 1.676$, $P = 0.132$; late-stage: "plunge," $t = 1.608$, $P = 0.146$, 0.8 °C min^{-1}, $t = 5.188$, $P < 0.001$). Earlier stage embryos were considerably more cold sensitive than later stage embryos at the same cooling rates (relative survival rate: ENA < MNA < LNA, EA < MA < LA). Despite this, slow cooling (0.1 and 0.05 °C min^{-1}) significantly increased the survival of early-stage

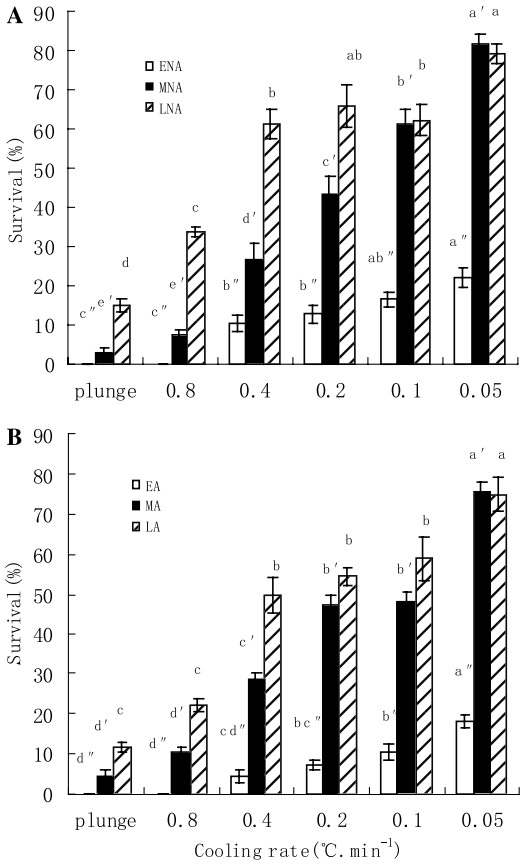

Fig. 2. Survival of (A) non-acclimated, and (B) acclimated, migratory locust eggs subjected to different cooling rates after 10 h exposure to −10 °C. ENA, early-stage non-acclimated; MNA, mid-stage non-acclimated; and LNA, late-stage non-acclimated. EA, early-stage acclimated; MA, mid-stage acclimated, and LA, late-stage acclimated.

embryos compared to quick cooling (0.8 °C min^{-1} and "plunge" cooling) (Fig. 2A). The survival rates of late- and mid-stage embryos were similar after slow cooling, but the former were more resistant to quick cooling than the latter.

Survival at calibrated subzero temperatures

The time eggs spent at subzero temperatures had a significant effect on their survival. After calibration, survival of MNA eggs increased following "plunge" cooling (*t* test, $t = 5.512$, $P = 0.001$), 0.8 ($t = 3.151$, $P = 0.014$), 0.4 ($t = 3.463$, $P = 0.009$) and 0.2 °C min^{-1} ($t = 3.842$, $P = 0.005$). The survival of

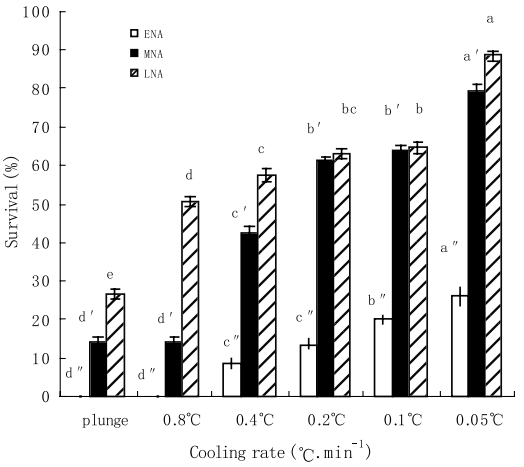

Fig. 3. Survival of non-acclimated eggs of the migratory locust subject to different cooling rates after 10 h exposure to −10 °C (time spent at subzero temperature standardized).

LNA eggs also increased following "plunge" cooling ($t = 5.512$, $P = 0.001$), 0.8 ($t = 9.504$, $P < 0.001$) and 0.05 °C min^{-1} ($t = 3.05$, $P = 0.016$), but there was no significant change in the survival of ENA eggs (Fig. 2 and Fig. 3).

Levels of low molecular weight sugars and polyols at different cooling rates

Glycerol, glucose, fructose, myo-inositol, trehalose, mannitol and sorbitol were identified as free sugars and sugar alcohols in all three stages of egg development. There were significant increases in the amounts of myo-inositol, trehalose, mannitol, glycerol and sorbitol at different cooling rates.

In non-acclimated eggs, concentrations of all five cryoprotectants increased with decreasing cooling rate. The most remarkable increases were in mannitol and sorbitol, which rose from 0.2 to 0.05 °C min^{-1}, and whose concentration in LNA eggs increased 9-fold (from 10.4 to 100.2 μg/g; $F_{5,53} = 399.05$, $P < 0.001$; Fig. 4A) and 11-fold (from 14.1 to 155.2 μg/g; $F_{5,53} = 445.93$, $P < 0.001$; Fig. 4A) at 0.05 °C min^{-1}, respectively. Concentrations of these substances were not statistically different to those in the control group at 0.8 °C min^{-1}. Other substances that showed significant differences in abundance at different cooling

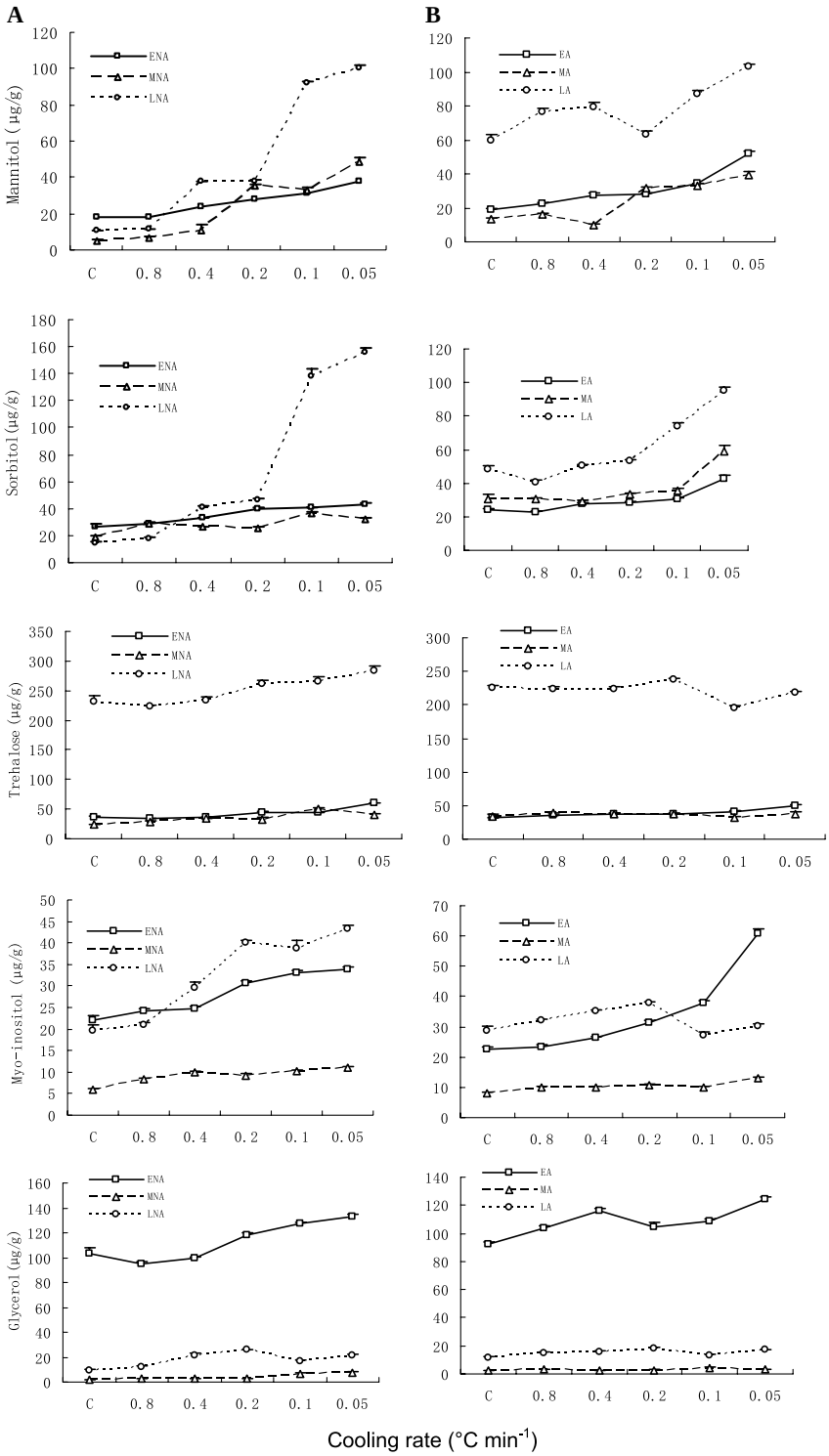

Fig. 4. Effect of cooling rate on concentrations of mannitol, sorbitol, trehalose, myo-inositol and glycerol in migratory locust eggs. (A) non-acclimated, (B) acclimated ($n = 3$, each group contained 8 eggs). Eggs as control (indicated as C in the x-axis) were incubated at 30 °C.

rates were trehalose (ENA: $F_{5,53}=44.639$, $P<0.001$; MNA: $F_{5,53}=27.007$, $P<0.001$; LNA: $F_{5,53}=12.09$, $P<0.001$; Fig. 4A) and glycerol (ENA: $F_{5,53}=43.94$, $P<0.001$; MNA: $F_{5,53}=54.858$, $P<0.001$; Fig. 4A). Although the content of myo-inositol increased with decreasing cooling rate (ENA: $F_{5,53}=34.36$, $P<0.001$; MNA: $F_{5,53}=32.21$, $P<0.001$; LNA: $F_{5,53}=64.77$, $P<0.001$), this trend was not stronglylinear (Fig. 4A).

In acclimated eggs, levels in the five cryoprotectants were different at different cooling rates. With decreasing cooling rate, the concentration of mannitol and sorbitol increased in all developmental stages (mannitol: EA, $F_{5,53}=211.68$, $P<0.001$; MA, $F_{5,53}=140.39$, $P<0.001$; LA, $F_{5,53}=40.99$, $P<0.001$; sorbitol: EA, $F_{5,53}=42.6$, $P<0.001$; MA, $F_{5,53}=21.65$, $P<0.001$; LA, $F_{5,53}=161.13$, $P<0.001$; Fig. 4B), however, trehalose, glycerol and myo-inositol increased only in early-stage embryos (trehalose: $F_{5,53}=73.5$, $P<0.001$; glycerol: $F_{5,53}=28.4$, $P<0.001$; myo-inositol: $F_{5,53}=283.99$, $P<0.001$; Fig. 4B).

The developmental process also had noticeable impact on the accumulation of cryoprotectants, especially trehalose and glycerol. The concentration of trehalose was much higher in late-stage than early-stage embryos, with a 6–7-fold increase in LNA and LA eggs, respectively (36 to 231 μg/g and from 31.5 to 224.6 μg/g, respectively), while the content of glycerol was much higher in early-stage than late-stage embryos, with an 11–8-fold increase in ENA and EA eggs, respectively(from 9.5 to 103.6 μg/g, and from 11.7 to 92.3 μg/g, respectively) (Fig. 4).

Discussion

Eggs subject to different cooling rates exhibited no significant variation in SCPs despite their different degrees of cold hardiness. This result is consistent with the findings of Jing and Kang [19] on eggs from four locust populations collected from tropical to temperate regions of China. Thus, the SCP value cannot be used to estimate the minimum temperature threshold of survival, or as an index of the cold hardiness of locust eggs. Locusts are similar in this respect to the wheat stem sawfly, *Cephus cinctus* [40], however, in some insect species, such as *Melasoma collaris*, a marked depression of SCP has been observed at slower cooling rates [11]. In some species SCPs decrease with the accumulation of cryoprotectants [8,16,28], but not in others [34]. It has been demonstrated that only when high concentrations (in the order of a Mol) of sugars or polyols are accumulated do they collectively depress SCPs [53].

The survival of eggs at low temperature is a reliable index of cold tolerance in locusts [18,19]. Our results indicate that cooling at approximate natural rates, such as 0.1 and 0.05 °C min^{-1}, greatly increased the survival at low temperature of all the three developmental stages. There was a significant linear relationship between cooling rate and survival in different developmental stages. This was negatively correlated with the cooling rate in both non-acclimated (ENA: $r^2=0.936$, $P=0.007$; MNA: $r^2=0.863$, $P=0.022$; LNA: $r^2=0.871$, $P=0.021$) and acclimated eggs (EA: $r^2=0.767$, $P=0.052$; MA: $r^2=0.822$, $P=0.034$; LA: $r^2=0.922$, $P=0.009$). Similar results have been found in adults of *Drosophila melanogaster* [22] and nymphs of *Sitobion avenae* [36], the survival of which at low temperatures increased with decreasing cooling rate. A thermoperiodic cycle has been found to have the same effect as slow cooling [23]. However, in "moderately freeze tolerant species" survival does not increase with slower cooling rates [41].

We found that mid and late-stage embryos were much more tolerant to cold than early-stage embryos, especially when exposed to −10 °C for 10 h. This result probably reflects adaptation to the climatic conditions of north of China. In this region, the eggs of "autumn locusts" are laid in the soil in early October [3,29], and overwinter in the balstokinesis stage (mid-stage) over a sub-zero winter for several months before developing at katatrepsis (late-stage) after warm acclimation in late spring and early summer [19]. This suggests that it is the synchronization of embryonic development and cold hardiness that allows locust eggs to safely overwinter in temperate regions [29].

Organisms that are exposed to sub-lethal low temperatures for a short period will often show a marked increase in their resistance to more-extreme temperatures [4]. This rapid cold harden-

ing (RCH) is induced by exposure to moderately low temperature within minutes or hours, but its underlying mechanism remains unclear [27]. Our data indicated that locust eggs have the capacity for RCH, which was induced by slow cooling. Eggs that were cooled at a slower rate spent a longer period at acclimation temperature (0–10 °C) and so had longer to adjust their physiology to survive even colder conditions (−10 °C). Similar observations have been made in first instar hoppers of the migratory locust [51], *D. melanogaster* [22] and *S. avenae* [36]. Thermoperiod also can induce RCH, as has been observed in *D. melanogaster* [23].

When subject to rapid ("plunge" and 0.8 °C min^{-1}) cooling, eggs spent longer at subzero temperatures post-calibrationand their survival rate increased. However, longer exposure to subzero temperatures was also harmful to eggs. When cooled more slowly (0.05 °C min^{-1}), exposure to subzero temperature after calibration was less and survival was higher.

Comparison of non-acclimated and acclimated eggs indicates that the acclimation process does not make eggs more resistant to quick cooling. There are two possible reasons for this: (1) an acclimation period of 3 days at 5 °C may not afford sufficient protection against injurious low temperatures (a 10-day acclimation period has been found to have a significant effect on cold hardiness of eggs from the Huanghua population, therefore a longer acclimation period may be required [18]); and/or (2) exposure to −10 °C for 10 h is not the most suitable test of cold hardiness.

Our research demonstrates that myo-inositol, trehalose, mannitol, glycerol and sorbitol played important roles in eggs' ability to withstand low temperature stress. The concentration of mannitol and sorbitol increased significantly at slower cooling rates. As straight chain hexitols, mannitol and sorbitol do not readily cross membranes, which is one of the major reasons why they function as osmoprotectants in animal cells [9]. Although mannitol is the most abundant acyclic polyol in nature, it has been reported in very few insects [10,15,25,43], whereas sorbitol has been commonly identified. In many cold-hardy insect species, sorbitol is an intermediate in the conversion of glucose and fructose [32]. Its marked increase after cooling treatments in locust eggs seems to be the result of this metabolic conversion. Trehalose, the major insect blood sugar, has previously been considered a cryoprotectant [12,47]. Trehalose accumulated at a high level in late-stage locust eggs and could play an important role in improving their survival by stabilizing proteins and membrane lipids [39]. It has been demonstrated that trehalose has a non-specific stabilizing effect on protein conformation during thermal stress or hydration, and a specific stabilizing effect on the phase behavior of membrane phospholipids during dehydration; water replacement in phospholipid headgroup hydration shells [5,7].

The accumulation of cryoprotectants in eggs could increase the amount of bound water and thereby reduce ice formation. Some insects can improve their cold hardiness by excreting water [38], but this is not possible for the migratory locust because their eggs are protected by waxy material in the vitelline membrane that, while allowing gas exchange, effectively prevents the movement of water or aqueous solutes. Therefore, cryoprotectants play an important role in decreasing the potentially lethal ice content of locust eggs. Multiple cryoprotectants are thought to reduce the possible toxic effects associated with the high concentrations of single compounds required to achieve the same level of cryoprotection [2].

Our data indicate that slow cooling (0.1 or 0.05 °C min^{-1}) allows locust eggs a relatively long period at a temperature range in which they accumulate cryoprotectants. In most insect species, low temperatures are known to trigger polyol production. The trigger temperature is most often in the range between 0 and 5 °C with maximal rates of synthesis at somewhat lower temperature; 0 to −5 °C [46]. A thermoperiod can accelerate this process [25,35].

Prediction and integrated management of locust plagues largely depend on accurate monitoring of population dynamics. However, some population dynamic models have not incorporated relevant information on cold hardiness and over-wintering mortality of eggs. In particular, the effects of cooling rate on egg mortality have often been ignored [18]. Cooling rates are important in two respects: (1) the process of slow cooling acts as natural accli-

mation; and (2) a sudden temperature decline can result in high egg mortality before over-wintering. This information is important to the effective prediction and management of locust plagues.

In summary, slow cooling plays a very important role in the development of cold hardiness in locust eggs, primarily through facilitating the increase of cryoprotectants. The physiological mechanisms involved, such as the actual roles of sugar and polyols, require further investigation. Many differences in gene expression between the solitary and gregarious phases of the migratory locust have been found [21]. Because differences in cold hardness are often regulated by relative gene expression, further research on the regulation of gene expression associated with cold hardiness is required.

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