

Supercooling capacity and cold hardiness of the eggs of the grasshopper *Chorthippus fallax* (Orthoptera: Acrididae)

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Abstract. Cold tolerance of the eggs of the grasshopper, *Chorthippus fallax* (Zubovskiy), was examined in the laboratory. Egg supercooling points varied from -6°C to -32.4°C and could be divided into two groups. The supercooling points of the higher SCP group ranged from -6°C to -14°C and those of lower SCP group from -21.8°C to -32.4°C . Although low temperature acclimation could slightly decrease the supercooling points of eggs, the effect was not significant for all embryonic developmental stages or acclimation periods. The supercooling capacity was obviously different between pre-diapause, diapause and post-diapause embryonic stages. The mean supercooling points of pre-diapause and diapause eggs were similar; $-28.8 \pm 1.6^{\circ}\text{C}$ and $-30.7 \pm 1.0^{\circ}\text{C}$ for non-acclimated eggs and $-29.5 \pm 1.3^{\circ}\text{C}$ and $-31.18 \pm 0.8^{\circ}\text{C}$ for acclimated eggs respectively. However, the mean supercooling points of post-diapause eggs were significantly higher; $-12.9 \pm 5.6^{\circ}\text{C}$ for non-acclimated and $-13.5 \pm 4.5^{\circ}\text{C}$ for acclimated eggs respectively. The survival rates of diapause eggs at $> -25^{\circ}\text{C}$ were not significantly different from that at 25°C , but survival rates at $< -30^{\circ}\text{C}$ decreased significantly. The lethal temperature ($L_{temp_{50}}$) for a 12 hrs exposure was -30.1°C , and the lethal time ($L_{time_{50}}$) at -25°C was 44 days. Since the SCPs of diapause eggs was similar to their $L_{temp_{50}}$, we may consider the supercooling capacity of such eggs is a good indicator of their cold hardiness and the species is a true freeze avoiding insect. Based on the analysis of local winter temperature data, pre-diapause and diapause, low SCP eggs can safely survive severe winters, but not the post-diapause, high SCP eggs. The importance of the overwintering strategy and the relationship between diapause and cold hardiness of this species is discussed.

INTRODUCTION

Many strategies have been developed by insects to survive adverse environmental conditions. The egg stage is the principal overwintering stage in many insects and other terrestrial arthropods, and considerable interest has been shown by entomologists in the cold resistance of eggs (Sømme, 1982, 2000; Gehrken, 1989; Hanson & Craig, 1994; Block et al., 1995; Strathdee et al., 1995; Shintani & Ishikawa, 1999; Jing & Kang, 2003). Cold hardiness is defined as the capacity of a species to survive long or short term exposure to low temperature (Lee, 1991). This capacity is influenced by many factors, including developmental stage, genetic potential, season, duration of exposure and nutritional status. Insects are commonly classified into two main categories: freeze intolerant and freeze tolerant (Storey & Storey, 1988; Lee, 1991). The supercooling point (SCP) is considered an indicator of the lower lethal temperature, but many researchers argue that the SCP is not a reliable index of cold hardiness since some insects die before their body freezes (Bale, 1993, 1996). Neither the lower lethal temperature of freeze tolerant insects, nor the supercooling capacity of freeze intolerant insects should be considered the only measure of the cold hardiness of a given species (Nedvěd, 2000a). In addition, the ability to survive prolonged periods of cold exposure must be evaluated at temperatures both above and below the freezing point of the insects' body fluids (Sømme, 1996).

Cold hardiness has been studied in many insects and mites (Lee, 1991; Nedvěd, 2000b; Sømme, 2000; Bale, 2002). However, apart from several papers documenting the SCPs of *Locusta migratoria* (Lozina-Lozinskii, 1974; Jing & Kang, 2003), *Myrmeleotettix palpalis*, *Aeropedellus varigatus minutus* and *Dasyhippus barbipes* (Block et al., 1995), and *Hemideina maori* (Ramløv et al. 1992; Ramløv, 1999), there are few available data on cold hardiness of Orthoptera, especially the Acrididae.

The grasshopper, *Chorthippus fallax* (Zubovskiy), causes visible grass loss in China's Inner Mongolian steppe region (Li & Kang, 1991; Lockwood et al., 1994). The species is univoltine and overwinters as eggs in the soil. The eggs are laid in mid/late September and hatch the following July, therefore, *C. fallax* can be considered a late hatching species (Kang & Chen, 1994a, b). We have observed that the embryos of grasshopper eggs collected from local fields in the mid-November were in stage 9–13 (according to the standard of Van Horn, 1966), and maintenance at a constant 25°C indicated that the grasshopper eggs entered diapause in embryonic stage 18–19 (unpublished data). Consequently the grasshopper overwinters as eggs in the pre-diapause embryonic stage. These overwintering eggs will experience a prolonged period of low temperature in winter. Since the cold hardiness of *C. fallax* eggs is critical to determine their survival, it has a major influence on population dynamics in following seasons. Although there have been some ecological studies on *C. fallax*, including food selection,

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TABLE 1. Mean, standard deviation and range of SCPs of non-acclimated and acclimated pre-diapause eggs of the grasshopper *C. fallax*.

Acclimation time(day)	N	Low SCP group			High SCP group		
		Mean (°C)*	SD	Range (°C)	Mean (°C)*	SD	Range (°C)
0	22	-28.8	1.608	-30.5 – -24.1			
30	18	-28.1	2.020	-31.3 – -24.5	-7.7	1.162	-9.0 – -6.7
60	45	-29.5	1.261	-32.4 – -21.8	-10.5	2.803	-14.0 – -6.8
90	31	-28.9	3.186	-31.8 – -21.8	-7.5	1.885	-10.0 – -6.0

*Mean SCP of non-acclimated pre-diapause eggs in the low SCP group was not significantly different from that of acclimated eggs ($F = 1.719$, $df_1 = 3$, $df_2 = 101$, $P = 0.167$)

resource utilization, egg pod location and identification, and population dynamics (Li et al., 1983; Li & Chen, 1985, 1987; Liu et al., 1990; Kang & Chen, 1994a, b), there has been no comprehensive study of the cryobiology of this species.

Considering this grasshopper as a late hatching species that overwinters as pre-diapause eggs raises two questions: (1) is there a difference in cold hardiness between pre-diapause and diapause eggs? (2) can pre-diapause eggs safely overwinter and hatch in the following July? This study attempts to answer these two questions by investigating the cold hardiness and supercooling capacity of *C. fallax* eggs in different embryonic stages. This research should improve understanding of the overwintering capabilities, survival capacity and population dynamics of this grasshopper in following seasons.

MATERIALS AND METHODS

Egg origin and collection

Grasshopper eggs were obtained from adults collected at the Chinese Academy of Sciences Inner Mongolia Grassland Ecosystem Research Station (43°26'–44°08'N, 116°04'–117°05'E, and 1000–1050 m in elevation). Adult grasshoppers were maintained at $28^\circ\text{C} \pm 1^\circ\text{C}$ during the day and $20^\circ\text{C} \pm 1^\circ\text{C}$ at night with a photoperiod of 14L : 10D. The grasshoppers were fed on wild host plants collected from the field (Li & Chen, 1985). Soil from the collection area was sieved through a 2 mm mesh and placed on the bottom of cages as an oviposition substrate. The soil layer was approximately 15 cm deep, and was moistened each day. Egg pods were collected from the oviposition cages weekly and prepared for study as follows: egg pods were rinsed from soil into a sieve and placed in small plastic boxes which contained washed silica sand (40 mesh) that had been moistened to the point of being wet but without free standing water. The boxes with egg pods and sand were placed in a $25^\circ\text{C} \pm 1^\circ\text{C}$ incubator for 20 days to obtain pre-diapause eggs (embryos were in about stage 11–13), for 60 days to allow the eggs to reach the diapause stage (embryos were in stage 18–19), and some diapause eggs were placed at $0^\circ\text{C} \pm 1^\circ\text{C}$ for 90 days to terminate diapause and then transferred to $25^\circ\text{C} \pm 1^\circ\text{C}$ for 10 days to obtain post-diapause eggs (embryos were in stage 22–23).

Acclimation

The grasshopper eggs at pre-diapause, diapause and post-diapause embryonic stages were kept at $5^\circ\text{C} \pm 1^\circ\text{C}$ for 0, 30, 60 and 90 days. The SCPs of the eggs were measured on batches of 15–25 eggs from each treatment group (2 eggs were taken from each egg pod).

Determination of supercooling point (SCP)

The SCPs of eggs at different developmental stages (pre-diapause, diapause and post-diapause) and from different low temperature acclimation treatments were measured. After the surface of each egg was dried with filter paper, individual eggs were fixed with plastic tape to the tip of a thermocouple which was linked to a recorder (uR100, Model 4152, Yologama Elect. Co., Seoul, Korea). The thermocouple with the egg was placed inside an insulating styrofoam box in a freezer to ensure that the cooling rate was about 1°C min^{-1} . The lowest temperature reached before an exothermic event occurred due to release of latent heat was regarded as the supercooling point of the eggs (Zhao & Kang, 2000; Chen & Kang, 2002).

Determination of lethal temperature and lethal time

To evaluate survival at low temperature, for each of 12 treatments, 6 replicates of 20 diapause eggs were exposed to $-25^\circ\text{C} \pm 0.5^\circ\text{C}$ for 0.5, 1, 2, 4, 8, 16 days and to -15°C , -20°C , -25°C , -30°C , -35°C and $-40^\circ\text{C} \pm 0.5^\circ\text{C}$ for 12 h. Additional batches of eggs were kept at $0^\circ\text{C} \pm 1^\circ\text{C}$ for 90 days, which terminated diapause. After treatment, eggs were removed and kept in dark chambers at $25^\circ\text{C} \pm 1^\circ\text{C}$. Egg mortality and hatching success were recorded daily for 60 days. Eggs that became flaccid, brown or moldy were considered dead whereas those that were cream-colored and turgid were considered alive (Fisher, 1997).

Statistical methods

Multi-way Analysis of variance (ANOVA) (SPSS 10.0 Software) was used to determine the effects of constant low temperatures and exposure duration on supercooling points and hatching rate. The Tukey Honest Significant Difference (HSD) test was used to detect significant differences between means. The form of the relationship between survival rate and time or temperature of exposure was determined by Weibull function or Probit analysis (see Kalushkov & Nedvĕd, 2000). The exposure duration that resulted in 50% survival ($L_{time_{50}}$), or the exposure temperature that resulted in 50% survival ($L_{temp_{50}}$), was estimated.

RESULTS

Supercooling capacity and the effects of low temperature acclimation

The SCPs of 117 non-acclimated and acclimated pre-diapause eggs at 5°C varied from -6°C to -32.4°C . The frequency distribution of SCPs was significantly bimodal, indicating the existence of a low (SCP $< -15^\circ\text{C}$) and a high SCP group (SCP $> -15^\circ\text{C}$) (Table 1). Although the mean SCP of non-acclimated pre-diapause eggs in the low SCP group was not significantly different from that of acclimated eggs ($F = 1.719$, $df_1 = 3$, $df_2 = 101$, $P = 0.167$), there was a tendency for the number of eggs with

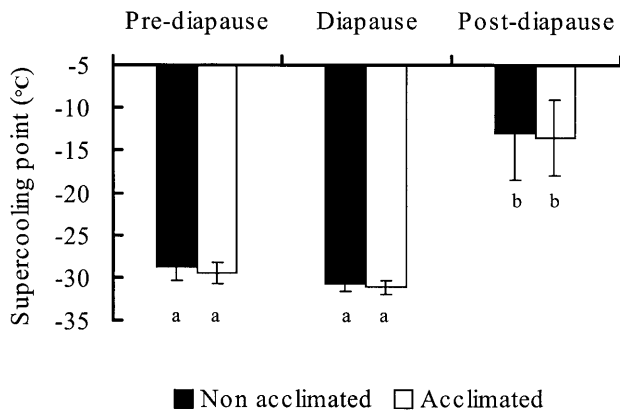


Fig. 1. SCPs of *C. fallax* eggs in different stages of embryonic development, non acclimation and acclimation at 5°C for 60 days. Bars (mean ± SD) with the same letter below are not significantly different.

SCPs < -30°C to increase with extended duration of acclimation.

Effects of egg developmental stage on SCP

Differences in SCP and the effects of low temperature acclimation on the SCP of pre-diapause, diapause and post-diapause eggs were examined. The SCPs of eggs in pre-diapause and diapause conditions were similar, but significantly lower than that of post-diapause eggs ($F_{(2, 128)} = 55.26, P < 0.0001$). Though low temperature acclimation could slightly decrease the SCP of eggs from $-28.8 \pm 1.6^\circ\text{C}$ to $-29.5 \pm 1.3^\circ\text{C}$ in pre-diapause eggs, $-30.7 \pm 1.0^\circ\text{C}$ to $-31.1 \pm 0.8^\circ$ in diapause eggs, and from $-12.88 \pm 5.58^\circ\text{C}$ to $-13.53 \pm 4.52^\circ\text{C}$ in post-diapause eggs, the effect of acclimation was not markedly different ($F_{(1, 128)} = 0.313, P = 0.577$), and there was no significant statistical interaction between acclimation and developmental stage ($F_{(2, 128)} = 1.728, P = 0.182$). Regardless of whether eggs had been acclimated or not, diapause eggs had the lowest SCPs (Fig. 1).

Low lethal temperature

The survival rates of diapause eggs exposed to different low constant temperatures for 12 hrs were determined. Survival rates of eggs at -25°C or higher were not significantly

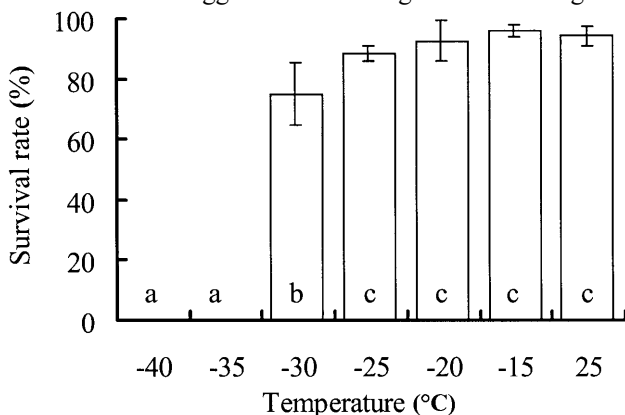


Fig. 2. Survival rate of diapause eggs of *C. fallax* exposed to different low constant temperatures for 12 hours. Bars (mean ± SD) with the same letter below are not significantly different.

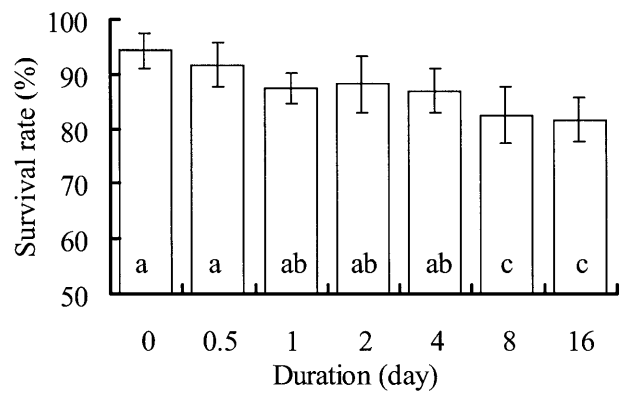


Fig. 3. Survival rate of diapause eggs of *C. fallax* exposed to -25°C for different lengths of time. Bars (mean ± SD) with the same letter below are not significantly different.

cantly different, nonetheless survival rate declined significantly at -30°C and suddenly dropped to zero at -35°C (Fig. 2). The response curve was an asymmetric sigmoid, so that the Weibull function was used to describe the relationship between survival and exposure time. The Weibull function was $P = 1 - \exp(-(t + 35.008)^2/18.035)$ (t : temperature, Pearson goodness of fit Chi-square was significant, $df = 34, P < 0.0001, R^2 = 0.946$). The $L_{temp_{10}}, L_{temp_{50}}$ and $L_{temp_{90}}$ values of diapause eggs exposed to low temperatures for 12 hrs were estimated as -33.6°C, -31.5°C and -28.6°C respectively, and the lowest temperature for egg survival was about -35°C.

Lethal time

The survival rates of diapause eggs exposed to -25°C for different periods are shown in Fig. 3. Survival rates of diapause eggs at -25°C decreased slowly with increasing duration of exposure to low temperature. But there was no significant difference between 0 d and exposures of 0.5, 1, 2, and 4 ds. Only the survival rates of eggs exposed for 8 and 16 ds were significantly less than those exposed for shorter lengths of time. The dose response curve was not sigmoid, so the Probit function was used to describe their relationship. The function was $\ln(p/(1-p)) = 2.2224 + 0.05089t$ (t : time, Pearson goodness of fit Chi-square was significant, $df = 40$ and $P = 0.0197, R^2 = 0.832$). Probit analysis of egg survival vs exposure time indicates that the $L_{time_{10}}, L_{time_{50}}$ and $L_{time_{90}}$ values of diapause eggs at -25°C were about 87 d, 44 d and 0.5 d respectively.

DISCUSSION

Supercooling and survival

The results of this study show that the SCPs of pre-diapause eggs could be divided into low and high SCP groups (Table 1). The mean SCPs of eggs were approximately -29°C for the low group (range from -21.8°C to 32.4°C), and -8°C for the high group (range from -6°C to -14°C). The percentage of eggs in low group was 88%. A substantial number of individual eggs froze at temperature several degrees lower than the mean value, and some eggs had SCPs as low as -32°. This result was similar to

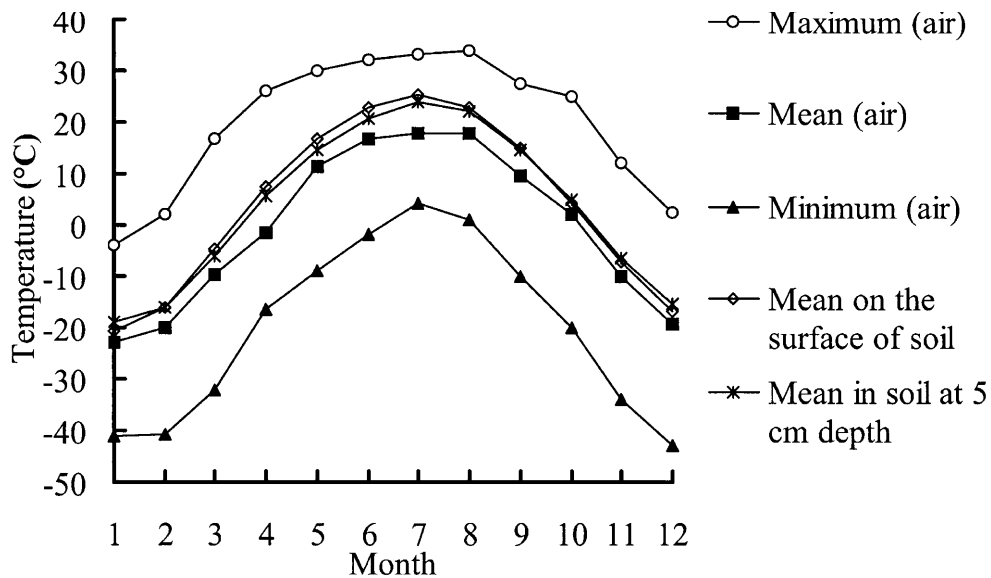


Fig. 4. Maximum, mean and minimum air temperatures at 150 cm height, soil surface and at 5 cm below the soil surface recorded at the Inner Mongolia Grassland Ecosystem Research Station during 1970–2000.

those recorded in *M. palpalis*, *A. variegatus minutus* and *D. barbipes* (early hatching grasshoppers also found in the Inner Mongolian steppe) (Block et al., 1995). It appears that there are two types of overwintering eggs in grasshoppers, some eggs die at relatively high subzero temperatures while others do not.

There was a close correlation between SCP and both lethal temperature and lethal time. The SCPs of diapause eggs were similar to their lethal temperature. The survival rates of diapause eggs of *C. fallax* at $> -25^{\circ}\text{C}$ and at 25°C (control treatment) were not significantly different, while survival rates at $< -30^{\circ}\text{C}$ were significantly decreased (Fig. 2). The $L_{temp_{50}}$ for 12 hrs was -31.5°C , and the mean SCP of diapause eggs was -30.7°C for non-acclimated and -31.1°C for acclimated eggs. Previous studies have demonstrated that the SCP is not a suitable index for cold hardiness of many species as it does not take into account mortality at sub-zero temperatures above the insect's supercooling point (Milonas & Savopoulou, 1999; Nedvěď, 2000a; Bale, 2002). However, a good correlation between survival at low temperature and SCP in several species has also been reported, and in these species it is suggested that the SCP is a reliable indicator of low temperature tolerance (Lee & Denlinger, 1985; Nedvěď et al., 1995; Hodková & Hodek, 1997). Even though slight mortality of diapause eggs was observed after prolonged exposure at sub-zero temperature ($L_{time_{50}}$ at -25°C was about 44 days), this may not have been caused by low temperature injury but by dehydration (Renault et al., 2002). Thus the supercooling capacity of grasshopper eggs might indeed be a convenient indicator of their cold hardiness.

Cold hardiness strategies of insects are often divided into two types with respect to their cold tolerance and overwintering strategies. Freeze-tolerant insects survive freezing; they can tolerate temperatures below their SCP. Freeze intolerant insects freeze and die at their SCPs. The

freeze intolerance strategy is further divided into four more classes: freeze avoidance, chill tolerance, chill susceptibility, and opportunistic survival (Bale, 1993, 1996). The freeze avoidance strategy is similar to the freeze intolerance, but with a strict definition. Bale (1993) cited only two species, the moths *Epiblema scudderiana* and *Epirrita autumnata*, as examples of freeze avoidance that survive exposures "as long as the minimum winter temperatures remain above the SCP". Considering the close correlation of SCP and lower lethal temperature, as well as very slight increase of mortality with time at temperature just above SCP (lethal time at -25°C), diapause eggs of *C. fallax* can be classified as freeze avoiding species (Bale, 1996) or the cold injury class DOC (Nedvěď, 2000b). *Chorthippus fallax* may represent the first experimental example of true freeze avoiding insect, while the previous examples, the moths *E. scudderiana* and *E. autumnata*, were suggested as freeze avoiding only according to ambient air temperature measurements (Virtanen et al., 1998; Nedvěď, personal communication).

Diapause and acclimation

Diapause is a genetically programmed process that enables insects to tolerate predictably unfavorable conditions, such as extreme temperatures, drought or limited food supply. There is conflicting evidence as to the existence of a relationship between diapause and cold hardiness (Denlinger, 1991; Pullin, 1996; Košťál & Šimek, 1995; Košťál et al., 2001). In some insects species, supercooling capacity or cold hardiness in the diapause stage is higher than that of the non-diapause stage, suggesting that cold hardiness is likely to be an integral part of diapause (Hodková & Hodek, 1994; Han et al., 1995; Milonas & Savopoulou, 1999; Watanabe & Tanaka, 1999; Jo & Kim, 2001; Šlachta et al., 2002). While in other species cold hardiness can occur completely independently of diapause (Salt, 1961; Ring, 1972). The eggs of *C. fallax*, however, differ from the above mentioned pattern since

the SCPs of pre-diapause and diapause eggs are similar, but significantly lower than that of post-diapause eggs.

By comparing the distribution of SCPs and lethal temperature data, it is evident that the SCPs of the lower SCP group of eggs lie within the $Ltemp_{10}$ and $Ltemp_{90}$ values of sample groups. Analysis of local winter climate conditions (Fig. 4) suggests that pre-diapause and diapause eggs in the lower SCP group, but not post-diapause eggs in higher SCP group, could safely survive the lowest winter temperatures. Nonetheless, if spring or early summer temperatures are lower than -8°C , the post-diapause eggs would suffer severe cold injury and high mortality.

It was reported that acclimation to low temperature can raise the supercooling capacity and cold tolerance of organisms (Fields et al., 1998). Our results also indicate that acclimation to low temperature can slightly increase the supercooling capacity of both pre-diapause and diapause eggs. The slow decline of temperature in autumn may also have acclimation effects and enhance the cold hardiness of *C. fallax* eggs with respect to the coming winter.

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