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RESEARCH

Supercooling Capacity and Cold Hardiness of Band-Winged Grasshopper eggs (Orthoptera: Acrididae)

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ABSTRACT. The band-winged grasshopper, *Oedaleus asiaticus* Bei-Bienko, is one of the most dominant and economically important grasshopper species in the steppe grasslands and farming-pastoral ecotone in northern China. It is a univoltine species and overwinters as eggs in soil. The cold hardiness of its eggs was examined in the laboratory. Water content in soil significantly affected the supercooling points (SCPs), water content and fat content of prediapause eggs. With the increase of water content in soil, the SCP, and water content of prediapause eggs rose whereas the fat content declined. There was a significant relationship between the SCP and water content or fat content of prediapause eggs. The SCPs of prediapause and diapause eggs varied from −7.6 to −28.4°C and the SCPs of eggs 30 d after oviposition could be divided into two groups. The means of high SCP group (−11.0 to −11.9°C) were much higher than those of low SCP group (−21.8 to −21.9°C), and the majority belonged to the latter (90.48–93.33%). The SCPs of prediapause eggs and early-diapause eggs 30 d after oviposition were significantly higher than those of deep-diapause eggs 60 d after oviposition. The survival rates of diapause eggs were significantly different among different temperature treatments. The survival rate was higher than 88% at greater than −20°C and declined significantly to 57% at −25°C, and suddenly dropped to zero at −30°C. The lower lethal temperature (Ltemp50) for 12 h exposure was −25.3°C and the lower lethal time (Ltime50) at −20°C was 32.8 d. As the mean SCPs of diapause eggs were similar to their Ltemp50, the SCP of eggs can be considered as a good indicator of cold hardiness for *O. asiaticus* and that this grasshopper is a freeze-intolerant insect.

Key Words: supercooling point, lethal temperature, lethal time, fat content, water content

The band-winged grasshopper, *Oedaleus asiaticus* Bei-Bienko, is one of the most dominant and economically important grasshopper species in the steppe grasslands and farming-pastoral ecotone in northern China and often require chemical control during outbreaks (Kang and Chen 1994). This species prefers overgrazed steppes and xerophytous habitats, and therefore, was suggested as indicator species for steppe deterioration in typical steppe zone in Inner Mongolia (Kang and Chen 1995). Especially into 21st century, this grasshopper species has had more frequent outbreaks and occasionally developed into migratory swarms in Inner Mongolia (Cease et al. 2010). The swarms migrated into many cities from early to middle July in 2002 (Jiang et al. 2003).The grasshopper is a univoltine species and overwinters as eggs in soil. The eggs are laid in August and begin to hatch the early to mid June (Hao and Kang 2004a, Zhou et al. 2012). Although there have been some ecological studies on this pest, including food selection (Li et al. 1983), migration (Jiang et al. 2003), postdiapause development at different temperatures (Hao and Kang 2004b) or photoperiods (Chen et al. 2009), migratory polyphenism (Cease et al. 2010), population dynamics (Zhou et al. 2012), and genetic diversity (Li et al. 2010, Han et al. 2013), there has been no study on the cryo-biology of this species.

Many strategies have been developed by insects to survive adverse environmental conditions. Cold hardiness is a common strategy of insects to survive cold winters and is affected by many environmental factors such as food, temperature, and humidity (Sømme 1999). Cold hardiness is defined as the capacity of a species to survive long or short term exposure to low temperature (Lee 1991). The supercooling point (SCP) is the temperature at which an insect's internal fluid freezes. The SCP may be used as a comparative cold hardiness indicator in different physiological stages of an insect (Hodková and Hodek 2004), although some researchers argue that the SCP is not a reliable index of cold hardiness because some insects die before their bodies freeze (Bale 1996). In temperate climatic zones, the majority of insects undergo a greater risk of chilling injury and death than freezing injury and death (Bale 2002). Therefore, besides the SCP, lower lethal temperature, and lower lethal time are now used as popular indices of cold hardiness (Watanabe 2002, Jing and Kang 2003, Hao and Kang 2004b, Berkvens et al. 2010, Bűrgi and Mills 2010, Hiiesaar et al. 2011, Ju et al. 2011, Manrique et al. 2012, Morey et al. 2012, Woodman 2012). Even though there are many economically important insects in Orthoptera, only several species such as *Locusta migratoria* (Jing and Kang 2003, Wang and Kang 2003), *Myrmeleotettix palpalis*, *Aeropedellus varigatus minutus*, and *Dasyhippus barbipes* (Block et al. 1995), *Chorthippus fallax* (Hao and Kang 2004b), *Chortoicetes terminifera* (Woodman 2010), and *Austracris guttulosa* (Woodman 2012) have been studied for their supercooling ability and cold hardiness.

We assessed 1) the effect of soil humidity on the SCP of *O. asiaticus* eggs, 2) the relationship between the SCP and water and fat content in the eggs, 3) the difference in the SCP between prediapause and diapause eggs, 4) lower lethal time after exposure of diapause eggs to constant subzero temperatures, and 5) lower lethal temperature after exposure of diapause eggs to different subzero temperature for a constant period of time.

Materials and Methods

Egg Origin and Collection. Grasshopper eggs were obtained from adults collected on Gegentala Grasslands of Inner Mongolia (41°46N, 111°49E, and 1,423 m in elevation) in early August, 2012. Adult grasshoppers were reared with corn leaves in cages in a laboratory. Soil from the collection location was sieved through a 2-mm mesh and placed on the bottom of cages as an oviposition substrate. The soil layer was 15-cm deep and moistened every 2 d. To obtain even development of eggs, newly laid eggs were collected daily. Egg pods were placed in small plastic cups which contained the sand-soil mixture with a predesigned water content. The cups were placed in a 25°C ± 1°C incubator for 10 d to obtain prediapause eggs and for 60 d to allow the eggs to reach the diapause stage (Hao and Kang 2004b). At the same time, some eggs were dissected to determine the embryonic development stages based on morphological characteristics.

Preparation of the Sand-Soil Mixture with Different Water Contents. After the soil from the collection location of adult grasshoppers and silica sand had been dried for 6 h at 180°C in an oven, the soil and sand were mixed at 1:4 (weight/weight). Water was added into the soil-sand mixture. The mixtures were prepared with the tested water content at 4, 7, 10, and 13%.

Measurement of SCP. The SCPs of eggs at different developmental stages and water content treatments were measured. Each egg was weighed and then fixed with a plastic tape to the tip of a thermocouple attached to a multichannel automatic recorder (TOPTEST TP9024, Top Electric Co., Shenzhen, China). The thermocouple with the egg was placed inside an insulating Styrofoam box in a freezing chamber to ensure that the egg's temperature decreased at a cooling rate of about 1°C min^{-1}. The SCP was identified as the lowest temperature reached before an abrupt spike in temperature.

Determination of Lower Lethal Temperature and Lower Lethal Time. In addition to being killed by freezing at the SCP, an insect can also die at temperature above the SCP. This is caused by chill injury. Chill injury can be characterized by the Ltemp50 (direct chilling injury, i.e., the temperature at which 50% of the test individuals die during a certain time) and the Ltime50 (indirect chilling injury, i.e., the time required to kill 50% of the population at a certain lower temperature) (Berkvens et al. 2010). For each of 11 treatments, 4 replicates of 30 diapause eggs were exposed to −25 ± 0.5°C for 0.5, 1, 2, 4, 8, 16 d and −35, −30, −25, −20, and −15 ± 0.5°C for 12 h. At the same time, additional batches of eggs were kept at 0 ± 1 °C for 90 d, which ended diapause. After treatment, the eggs were kept in a dark chamber at 25 ± 1°C. Egg death and hatching were observed and recorded daily for 2 mo. The eggs which became flaccid, brown, or moldy were considered dead whereas those which were cream-colored and turgid were considered live (Fisher 1997).

Measurement of Water and Fat Contents in Eggs. After each egg was weighed with an electronic balance (Sartorius BP211D, definition = 0.01 mg), it was placed into a drying oven at 60°C for 72 h to achieve constant mass (DW). After drying, the eggs were individually reweighing to determine mass loss as a proxy for water content.

After dry weight was determined, seven eggs from the same treatment were mixed and ground. A mixture of chloroform and methanol in the ratio of 2:1 was added and the material homogenated on ice. The supernatant was removed after centrifugation at 2,600× *g* for 10 min and recentrifuged once. The residue was dried in an oven at 60°C for 72 h to achieve constant mass (LDW). Absolute (mg/egg) and relative fat content (%) were determined as [(DW-LDW)/7] and [(DW-LDW)/fresh weight] × 100, respectively (Colinet et al., 2007). Three replicates were made for each treatment.

Statistics. Data analysis was carried out using SPSS13.0 (SPSS, Chicago, USA). Least significant difference (LSD) post hoc tests were used to compare the different treatments. The percentage data, i.e., water content, fat content, and egg survival, were analyzed after application of an arcsine square root transformation. The relationship between survival rate and time or temperature of exposure was analyzed by Weibull function or Probit analysis, and Ltime50 and Ltemp50 were estimated (Hao and Kang 2004b).

Results

SCP, Water Content, and Fat Content of Prediapause Eggs in Soil with Different Water Content. Water content in soil significantly affected the SCPs, water content, and relative fat content of prediapause eggs (Table 1). With the increase of water content in soil, the SCP, and water content of prediapause eggs rose whereas relative fat content declined. The SCP in soil at 4% was significantly lower than that at 10 or 13%. The water content of eggs in soil at 4% was significantly lower than that at 7, 10, or 13%, while relative fat content was significantly higher. Correlation analysis showed that there was a significant relationship between the SCP and water content ($R^2 = 0.9821$, $F = 109.49$, $P = 0.0090$) or relative fat content of prediapause eggs ($R^2 = 0.9786$, $F = 91.31$, $P = 0.0108$) but no significant relationship with absolute fat content ($R^2 = 0.3396$, $F = 1.03$, $P = 0.4172$).

SCP, Water Content, and Fat Content of Eggs in Different Developmental Stages. The mean SCPs of eggs in different developmental stages were from −20.75 to −24.86°C (ranged from −7.6 to −28.4°C) (Tables 2 and 3). The SCPs could be divided into two groups: a high SCP group (SCP > −15°C) and a low SCP group (SCP < −15°C), 30 d after oviposition. The means of high SCP group (−11.0 to −11.9°C) were much higher than those of low SCP group (−21.8 to −21.9°C), and the majority belonged to the latter (90.58–93.3%). However, there was only low SCP group in eggs 60 d after oviposition.

The developmental stages significantly affected the SCP, water content and fat content of eggs (SCP: $F_{(5,173)}$=9.43, $P = 0.0001$; water content: $F_{(5,173)}$=48.55, $P < 0.0001$; relative fat content: $F_{(5,12)}$=67.51, $P < 0.0001$; absolute fat content: $F_{(5,12)}$=10.569, $P = 0.0005$). With the embryonic development, the SCP and water content of eggs declined whereas the fat content increased. The SCPs of prediapause eggs 15 d and early-diapause eggs 30 d after oviposition were significantly higher than those of deep-diapause eggs 60 d after oviposition (Table 3). Correlation analysis showed that there was a significant relationship between the SCP and fat content of eggs (relative: $R^2 = 0.8728$, $F = 27.44$, $P = 0.0063$; absolute: $R^2 = 0.8706$, $F = 21.91$, $P = 0.0066$). Although there was a tendency for the SCP to decline with decreasing water content in eggs, there was no significant relationship between them at $P = 0.05$ ($R^2 = 0.6216$, $P = 0.0624$, $F = 6.57$).

Lower Lethal Temperature. Figure 1 shows the relationship between exposure to different constant lower temperatures for 12 h and survival rates of diapause eggs. The survival rates of diapause eggs were significantly different among different temperature treatments ($F_{(5,24)}$=549.08, $P < 0.0001$). The survival rate was higher (>88%) at greater than −20°C and declined significantly to 57% at −25°C, and suddenly dropped to zero at −30°C. The sigmoid increase in survival rate with increasing exposure temperature was well described by Weibull function: $P = 1-\exp(-((t+33.00)/8.93)^{\wedge}2.51)$ (t: temperature; $F = 132.25$, $P = 0.0012$, $R^2 = 0.9888$). The Ltemp10, Ltemp50, and Ltemp90 values of diapause eggs exposed to low temperature for 12 h were estimated as −29.4, −25.3, and −20.6°C, respectively, and the lowest temperature for diapause egg survival was about −30°C.

Lower Lethal Time. Figure 2 shows that the survival rates of diapause eggs at −20°C decreased slowly with increasing exposure time to low temperature. The survival rates of diapause eggs were significantly different among different time treatments ($F_{(6,21)}$=13.19, $P<0.0001$). There was no significant difference between 0 and 0.5 d, and the survival rates after 1 d exposure were significantly less than that for 0 d. The Probit function was used to describe the relationship between survival rates and exposure durations to low temperature. The function was $\ln(p/(1-p)) = 2.2491-0.0685t$ (t: time; $F = 38.754$, $P < 0.0001$, $R^2 = 0.5985$). The Ltime10, Ltime50, and Ltime90 values of diapause eggs at −20°C were estimated as 64.9, 32.8, and 0.8 d, respectively.

Discussion

SCP, Water Content, and Fat Content. Many factors are involved in insect cold tolerance, mainly: cryoprotectants, ice nucleators, and water content (Clark and Worland 2008). Our results show that the water and fat content in eggs significantly influenced the SCP. The SCP declined with decreasing water content or increasing fat content in eggs. This relationship has also been confirmed in many other insect species (Worland 1996, Colinet et al. 2007, Zhao et al. 2008, Xu et al. 2009, Wang et al. 2011).

Supercooling Capacity and Survival. The SCPs of prediapause and early-diapause eggs could be divided into low and high groups whereas

Table 1. SCP, water content, and fat content of prediapause eggs in soil with different water content ($n = 22$)

Water content in soil (%)	SCP(°C)	Water content in egg (%)	Relative fat content (%)	Absolute fat content(mg/egg)
4	−22.50 ± 0.31 b	55.22 ± 0.72b	10.87 ± 0.44a	0.44 ± 0.020a
7	−20.13 ± 0.93ab	66.46 ± 1.16a	9.55 ± 0.37b	0.41 ± 0.025a
10	−19.58 ± 0.96 a	67.83 ± 0.60a	9.08 ± 0.19b	0.44 ± 0.018a
13	−19.61 ± 0.73a	69.81 ± 1.94a	8.91 ± 0.14b	0.42 ± 0.017a

Table 2. SCP of eggs in different developmental stages

Days after oviposition	N	High SCP group			Low SCP group		
		Mean (°C)	Range(°C)	Percentage	Mean (°C)	Range(°C)	Percentage(%)
1	42	−11.0 ± 1.35a	−8.3 to −13.9	9.52	−21.9 ± 0.40a	−19.7 to −23.6	90.48
15	23	−11.1 ± 3.50a	−7.6 to −14.6	8.7	−21.9 ± 0.22a	−17.2 to −24.2	91.3
30	45	−11.9 ± 1.40a	−9.2 to −13.9	6.67	−21.8 ± 0.20a	−19.7 to −23.6	93.33
60	23	—	—	—	−23.2 ± 0.46ab	−19.6 to −27.2	100
90	23	—	—	—	−23.5 ± 0.47bc	−19.2 to −27.1	100
120	23	—	—	—	−24.9 ± 0.50c	−19.2 to −28.4	100

Table 3. SCP, water content, and fat content of eggs in different developmental stages

Days after oviposition	SCP (°C)	Water content (%)	Relative fat content (%)	Absolute fat content(mg/egg)
0	−20.75 ± 0.53a	68.02 ± 0.76a	9.79 ± 0.41d	0.45 ± 0.045c
15	−20.99 ± 0.78a	60.11 ± 1.23b	10.09 ± 0.15cd	0.42 ± 0.035c
30	−21.11 ± 0.40a	51.42 ± 1.47c	10.69 ± 0.11c	0.50 ± 0.013bc
60	−23.23 ± 0.50b	50.19 ± 1.46cd	12.19 ± 0.18b	0.51 ± 0.040bc
90	−23.47 ± 0.47b	48.16 ± 0.88d	13.97 ± 0.08a	0.59 ± 0.039b
120	−24.86 ± 0.51b	47.24 ± 1.02d	13.60 ± 0.11a	0.72 ± 0.016a

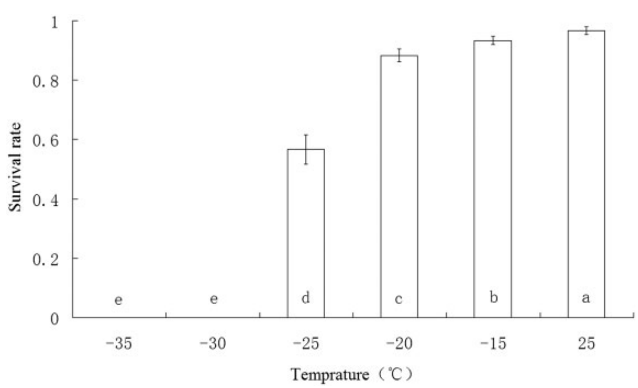

Fig. 1. Survival rate of diapause eggs of *O. asiaticus* exposed to different low temperatures for 12 h. Bars (mean ± SE) with the same letter below are not significantly different at $P = 0.05$ (LSD).

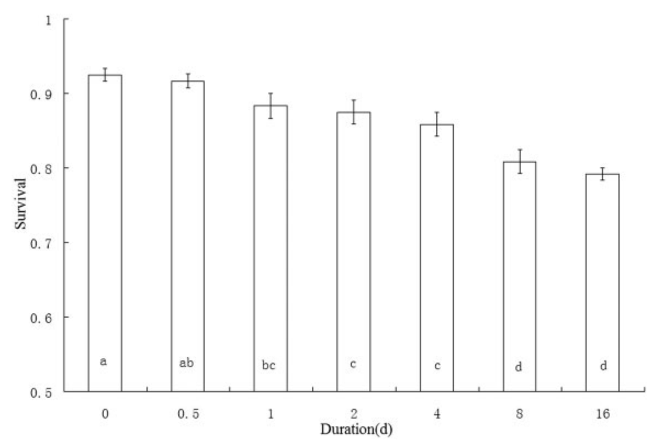

Fig. 2. Survival rate of diapause eggs of *O. asiaticus* exposed to −20°C for different duration. Bars (mean ± SE) with the same letter below are not significantly different at $P = 0.05$(LSD).

there was only a low group in deep-diapause eggs, and the percentage of eggs in low group was greater than 90%. This result was similar to those recorded in other grasshopper species such as *M. palpalis*, *A. varigatus minutus*, and *D. barbipes* (Block et al. 1995), and *C. fallax* (Hao and Kang 2004b). However, in these studies it was not demonstrates whether the SCPs of diapause eggs were bimodal.

There have been arguments about whether the SCP is a suitable indicator for cold hardiness of insects. Our results indicate that there was a close correlation between SCP and low lethal temperature, and the SCPs of diapause eggs of *O. asiaticus* were similar to their low lethal temperature. The mean SCPs of diapause eggs were from −23.23 to −24.86°C, and the Ltemp50 for 12 h was about −25.3°C. Some research has shown that the SCP is not a suitable index for cold hardiness as the mortality occurs at temperatures above the SCP (Bale 1987, 2002; Bennett and Lee 1989; Nedvěd 2000; Hiiesaar et al. 2011). However, other researchers have reported that there is a good correlation between low temperature survival and SCP in several species and suggested that the SCP is reliable as an indicator of cold hardiness (Lee and Denlinger 1985, Nedvěd et al. 1995, Hodková and Hodek 1997). Hao and Kang (2004b) obtained similar results to ours in studying the egg cold hardiness of another grasshopper *C. fallax* in Inner Mongolia, China. Therefore, the SCP of grasshopper eggs might be a good indicator of their cold hardiness. And furthermore, according to Lee (2010), if most mortality occurs at the SCP, the insect is freeze-intolerant, below the SCP it is freeze-tolerant, and above the SCP it is chill-intolerant. The SCP of diapause eggs of *O. asiaticus* is similar to the Ltemp50, and therefore, diapause eggs of *O. asiaticus* can be classified as freeze-intolerant species.

Cold Hardiness and Diapause. Diapause is a genetically programmed developmental response to changing seasons and environmental conditions, i.e., extreme temperature, drought, or limited food supply. Although cold hardiness can occur completely independently of diapause in some insect species (Salt 1961), in most insect species, diapause enhances cold stress tolerance (Denlinger 2002), and a close relationship between cold response and diapause is supported by an increasing number of studies (Bale and Hayward 2010, Morey et al. 2012). In this paper, the SCPs of diapause eggs of *O. asiaticus* were significantly lower than those of prediapause eggs, and the SCP is a reliable indicator of cold hardiness for this grasshopper. Therefore, our results also support the viewpoint that diapause enhances cold hardiness of insects.

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