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The effect of temperature on the diapause and cold hardiness of *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae)

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Key words. *Dendrolimus tabulaeformis*, acclimation, de-acclimation, cold hardiness, supercooling point, low molecular weight sugars

Abstract. Pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu, is a major pine pest in North China. The larvae enter diapause in the third or fourth instar before winter. Supercooling points (SCP) and cold hardiness of the diapausing larvae were investigated and compared in non-acclimated, acclimated and de-acclimated larvae. A bimodal frequency distribution was observed with a break point of –14°C in the SCP. Larvae in the low group (LG, SCP ≤ –14°C) were more cold tolerant with lower lethal temperatures than those in the high group (HG, SCP > –14°C). This bimodality occurred in three patterns, LG (> 60% of individuals in LG), LG–HG (< 60% of individuals in LG and HG) and HG (> 60% of individuals in HG), in response to cold acclimation and de-acclimation. The cold hardiness was ranked as: LG > LG–HG > HG pattern. Cold hardiness was enhanced by an increase in concentrations of trehalose, galactose, glucose and mannose in the haemolymph as well as by decrease in metabolism after cold acclimation, but was lost after de-acclimation. Loss of cold hardiness was correlated with decrease in sugars and increase in metabolic rate. In conclusion, the species is a chill tolerant insect, adopting the strategy of depressing SCP through accumulation of low molecular weight sugars in the haemolymph, concomitant with metabolic depression.

INTRODUCTION

Few temperate insects are able to avoid exposure to low environmental temperatures during winter, so the capacity to cold-harden is required for temperate insects to survive overwintering (Lee, 1989). Two major strategies, freeze-intolerance (or freeze-avoidance) and freeze-tolerance, are adopted by most overwintering insects (Baust & Rojas, 1985; Storey & Storey, 1988; Lee, 1989, 1991). Freeze-tolerant insects withstand the formation of internal ice and maintain a high supercooling point (SCP) through the production of ice nucleators (Storey & Storey, 1988). Freeze-avoiding insects die upon freezing, but they often avoid lethal tissue freezing by lowering the SCP due to the evacuation of food residues in the gut and/or accumulation of sugars and polyols (Barson, 1974; Rickards et al., 1987; Pullin & Bale, 1989; Bale & Pullin, 1991). Recently, a third strategy of cryoprotective dehydration has been reported (Holmstrup et al., 2002; Sinclair et al., 2003b), which might be adopted by some soil invertebrates (Holmstrup et al., 2002).

Cold hardiness or cold tolerance refers to the capacity of an organism to survive exposure to low temperature (Lee, 1989). Many temperate insects stop feeding and enter diapause before winter, reducing the loss of their reserves by lowering their metabolism and enhancing their cold hardiness (Denlinger, 1985, 1991; Tauber et al., 1986; Leather et al., 1993; Fields et al., 1998). Cold hardiness of insects can be improved through processes of cold acclimation (Salt, 1961) and rapid cold-hardening (Chen et al., 1987; Lee et al., 1987, 2006). For most cold acclimated insects the concentrations of low molecular weight polyols, sugars or amino acids increase during the acclimation period (Storey & Storey, 1988; Lee & Denlinger, 1991; Fields et al., 1998). As temperatures fluctuate, acclimation allows organisms to persist under conditions that would otherwise be lethal (Hoffmann, 1995). Insects increase their survival at lethal temperatures after pre-exposure to low (but non-lethal) temperatures, e.g., *Cryptolestes ferrugineus* (Fields et al., 1998).

Pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu (Lepidoptera: Lasiocampidae), a major pine pest in the North China (Hou, 1987; Chen, 1990), enters diapause in the third or fourth instar in response to short day-length in fall. Diapausing larvae stop feeding and mostly overwinter on or under the soil surface or leaf litter of pine trees (Li & Gia, 1989; Gia & Li, 1991). Although previous studies examined the diapause characteristics of *D. tabulaeformis*, e.g. diapause induction (Li & Gia, 1989; Gia & Li, 1991), little is known about the mechanisms of cold hardiness and the overwintering strategy (Han et al., 2005). The supercooling capacity is increased and the water content decreased in diapausing larvae. A mean SCP of –13.2°C was reported for diapausing larvae in the early stages of this species (Han et al., 2005). However, the minimum temperature in most of northern China is below –14°C in winter. Hence, diapausing larvae of *D.*

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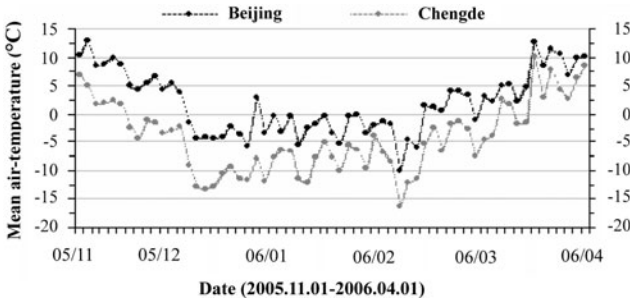

Fig. 1. Mean air-temperature in Beijing (39°N, 116°E) and Chengde (41°N, 117°E) during 1 November, 2005 to 1 April, 2006.

tabulaeformis may have adopted the strategy of freeze-tolerance or freeze-avoidance to survive in winter.

In order to elucidate the overwintering strategy of *D. tabulaeformis* we have examined: (1) Larval mortality in response to different temperature acclimations, (2) Effects of cold acclimation and de-acclimation on the survival (or 50% and 90% lethal time, LT_{50} and LT_{90}) at –14°C, (3) Changes in the concentration of low molecular weight sugars (i.e. trehalose) in the haemolymph, and (4) The effect of temperature acclimation and de-acclimation on larval metabolic rate.

MATERIAL AND METHODS

Insect

Diapausing larvae of the pine caterpillar, *D. tabulaeformis*, were collected on November 10, 2005 in a forest of Chinese pine, *Pinus tabulaeformis* Carr., in Chengde (CD; 41°N,117°E), Hebei province. They were placed in plastic-net screen cages (60 × 60 × 60 cm) and transported by bus (without air-control operating) to Beijing (BJ; 39°N, 116°E) in three hours. At that time, the air-temperature is 5–10°C in Beijing and Chengde (see Fig. 1 for climatic data). In a laboratory with a window (2 m × 1.5 m) opening to the outside (40–60% relative humidity at that time), the diapausing larvae were kept in 800 ml glass-beakers covered by cotton cloth and plastic film (Han et al., 2005). After 1–2 h, treatments were transferred into various temperatures in 10 min, where light was excluded (Han et al., 2005; Huang et al., 2005).

Acclimation at different temperatures

Thirty to forty diapausing larvae were kept at 27, 18, 5, 0 and –4°C ± 1°C for 3, 8, 11, 13, 15, 19, 21, 30, 40 or 45 d to determine mortality (Table 1). Treatments were kept in the dark with 50–60% RH. After determination of mortality larvae were not used again. As a control, diapausing larvae were kept under a 2cm-layer of garden soil with leaf litter in the field for various numbers of days, from November, 2005 to March, 2006 in Beijing (BJ; 39°N, 116°E) (see Fig. 1 for climatic data) as the non-acclimated group. Each treatment was replicated two to four times.

De-acclimation at 18 and 27°C

De-acclimation of insects has been defined as maintenance at a constant low temperature for a period of time followed by a transfer to another temperature above biological zero for a period of time (Šlachta et al., 2002). In order to examine the influence of de-acclimation on the cold hardiness of diapausing larvae, thirty to forty individuals acclimated at 0°C for 30 or 40 d were transferred to 18°C and 27°C for 2 days in darkness (Table 1). Each de-acclimation treatment was replicated two to four times.

Exposure experiments at –14°C

The 40 d-acclimated or de-acclimated diapausing larvae (Table 1) were directly exposed to the lethal temperature of –14°C for 0.5, 1.5, 3, 5, 10, 24 or 48 h in the dark, and then transferred to conditions of 27°C and 15.5L : 8.5D for 24 h (Han et al., 2005). The numbers of live and dead larvae were recorded. Dead larvae were determined as those with no movement and exhibiting loose body segments (Goto et al., 2001). Recorded larvae were not reused, and larvae that died during acclimation were not included in the –14°C exposure test. Each exposure period was replicated at least two times.

Determination of supercooling points (SCP)

Each larva was externally dried using filter paper, fixed with thermocouples connected to individual automatic temperature recorders (uR100, Model 4152, Yologama Electrical Co, Seoul, Korea), and placed into a Styrofoam tube (5 cm length, 1 cm diameter) (Han et al., 2005). The thermocouple with the larva was placed inside an insulating Styrofoam box in the chamber to ensure that the cooling rate was about 1°C/min for recording the SCP. The lowest temperature reached before an exothermic event occurred due to release of latent heat was regarded as the SCP (Zhao & Kang, 2000). In determination, the tested larvae kept survival if drawn out from the freezing chamber once the SCP recorded, or they would be killed by the body-freezing

TABLE 1. Experimental scheme for acclimation (AC) and de-acclimation (DA) of diapausing larvae of *D. tabulaeformis*. The larvae were collected in the field and then transferred into different acclimation regimens (N = 30–40/treatment). Samples analyzed for supercooling points (SCP) analysis and lower lethal temperature of –14°C are indicated.

Treatments	Transfer ' Number of days (d)	Abbreviations
Non-acclimated	Field (CD) [a] → Field (BJ) [a] for 3, 8, 11, 13, 15[b], 19, 21, 30[b], 40[c], 45 d	NA (field)
Acclimated	Field (CD) → –4°C for 3, 8, 11, 13, 15[b], 19, 21, 30[b], 40[c], 45 d	AC(–4)°C
	Field (CD) → 0°C for 3, 8, 11, 13, 15[b], 19, 21, 30[b], 40[c], 45 d	AC0°C
	Field (CD) → 5°C for 3, 8, 11, 13, 15[b], 19, 21, 30[b], 40[c], 45 d	AC5°C
	Field (CD) → 18°C for 3, 8, 11, 13, 15, 19, 21, 30, 40, 45 d	AC18°C
	Field (CD) → 27°C for 3, 8, 11, 13, 15, 19, 21, 30, 40, 45 d	AC27°C
De-acclimated	Field (CD) → 0°C (for 30 [b] or 40 d [c]) ' 18°C (for 2 d)	DA18°C
	Field (CD) → 0°C (for 30 [b] or 40 d [c]) ' 27°C (for 2 d) [b]	DA27°C

[a] CD – Chengde (41°N), BJ – Beijing (39°N); [b] SCP analysis; [c] Exposure to –14°C.

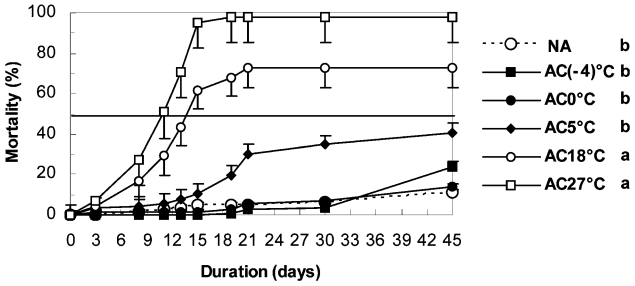

Fig. 2. Corrected mortality (mean ± SE) in the diapausing larvae of *D. tabulaeformis* after various days in the field (NA) and during different acclimations (AC). Different lower case letters represent a significant difference in total mortality after 45 days (ANOVA: F = 33.286, d.f. = 5, 9, P < 0.001, followed by Tukey's HSD at P < 0.05).

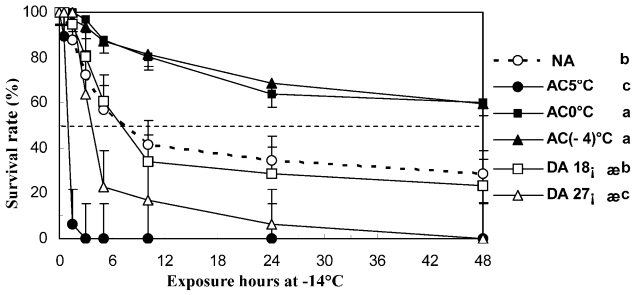

Fig. 3. Survival rate (mean ± SE) in non-acclimated (NA), acclimated (AC) and de-acclimated (DA) diapausing larvae of *D. tabulaeformis* during exposure to –14°C for 48 h. Different lower case letters represent significant differences in survival after 48 h (ANOVA: F = 93.271, d.f. = 5, 12, P < 0.001, followed by Tukey's HSD at P < 0.05).

incidence if persisting in the chamber beyond fifteen seconds or so after the SCP recorded. The live larvae were examined to determine concentrations of low molecular weight sugars in their haemolymph and analyzed based on their SCP status: low group (LG, SCP ≤ –14°C) or high group (HG, SCP > –14°C).

Low molecular weight sugar measurements

Five to eight healthy individuals of diapausing larvae, with three replicates, were used for the extraction of larval haemolymph. Larval haemolymph was collected using a capillary glass tube after removing one or two pro-legs (Han et al., 2005). Exudates were centrifuged at 2,500 g for 10 min at 4°C, and then supernatants were subjected to high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) by using a Dionex ICS-2500 ion chromatograph equipped with a Carbopac PA-1 analytical column and a Carbopac PA-1 guard column. Carbohydrates were eluted at a flow rate of 1.0 ml per min at 1,400 psi with 100 mM NaOH for 35 min (Liu et al., 2005). Carbohydrates were quantified using authentic standard sugars (Sigma, a company).

Respiration measurements

Oxygen uptake was measured in a Gilson Differential Respirometer (Gilson, 1963) using methods adapted from Daniel & Smith (1994), Guedes et al. (2003) and Gao et al. (2008). A series of 13 ml flasks was used for each measurement, with each flask containing 1 to 2 diapausing larvae that had been dried using filter paper. Larvae were allowed to adapt to the flask environment for five to 10 min at 20 ± 1°C and a small filter paper wick with 0.30 ml alkali solution (10% KOH) was placed in the centre of the flask for CO_2 absorption. The changes in volume of gas represented oxygen uptake, which was read by manometric adjustments with a micrometer scale. Readings were taken every 10 min over 30 min and the last barometric pressure readings were used to convert the respirometer volume changes to standard temperature and pressure conditions (Daniel & Smith, 1994). Before each measurement larvae were weighed using an electronic balance (sensitivity: 0.1 mg, Sartorius, R200 D.A.G., Göttingen, Gernamy). The respiration rate was calculated as the amount of O_2 uptake per fresh weight per hour (µl O_2 / mg / h) (Daniel & Smith, 1994; Guedes et al., 2003; Gao et al., 2008). Each measurement was replicated six times.

Statistical analysis

Distribution of SCP is often bimodal (Spicer & Gaston, 1999). Cannon & Block (1988) discussed the separation of bimodal SCP distributions into high (freeze at higher subzero temperatures) and low (freeze at lower subzero temperatures) groups. However, the breakpoints in bimodal distributions are often determined arbitrarily, depending on the supercooling characteristics of the species studied and visual assessment of a histogram for an obvious break (Block & Sømme, 1982; Worland & Convey, 2001; Sinclair et al., 2003b; Chen & Kang, 2005). The mean SCP of the control group (NA, see Table 1) in the study was –13.5°C ± 0.73 (Mean ± SE). Using a break point of –14°C, individuals were classed into a Low group (LG, SCP ≤ –14C) or High group (HG, SCP > –14C). Three patterns of frequency distribution of SCP were defined as LG pattern (> 60% of individuals in LG), LG–HG pattern (< 60% of individuals in LG and HG) and HG pattern (> 60% of individuals in HG).

All survival or mortality data were expressed as the corrected percentage of diapausing larvae using two calculations

Corrected percentage mortality =

$$\frac{\text{Survival of control-survival of treatment}}{\text{Survival of control}} \times 100$$

Corrected percentage survival = 1 – Corrected percentage mortality

adapted from Zhao & Kang (2000). T-test of mean values and one-way analysis of variance (ANOVA) was used for data analysis, and Tukeys Honest Significant Difference test procedure was used to make multiple comparisons. The amount of time (95% fiducial limits) necessary to achieve 50% and 90% mortality (LT_{50} and LT_{90}) in a given time period was estimated by Probit analysis (see Wang & Kang, 2005) using SPSS software. The percentage data were arcsine transformed before analysis, and untransformed data were presented.

RESULTS

Mortality of diapausing larvae acclimated at different temperatures

Significant differences in mortality rates were observed after acclimation treatments for 45 days (ANOVA: F = 33.286, d.f. = 5,9, P < 0.001) (Fig. 2). Acclimation at high temperatures, such as 18°C (AC18°C) and 27°C (AC27°C), had a significantly higher total mortality after 45 days than the NA group. The time that it took to kill 50% (LT_{50}) or 90% (LT_{90}) was 10.6 d and 14.4 d respectively in AC27°C, and the LT_{50} was 14.5 d in AC18°C, all significantly higher than other groups. Although no significant differences were observed among the NA, AC(–4)°C (acclimated at –4°C), AC0°C (acclimated at 0°C) and AC5°C (acclimated at 5°C) groups after 45

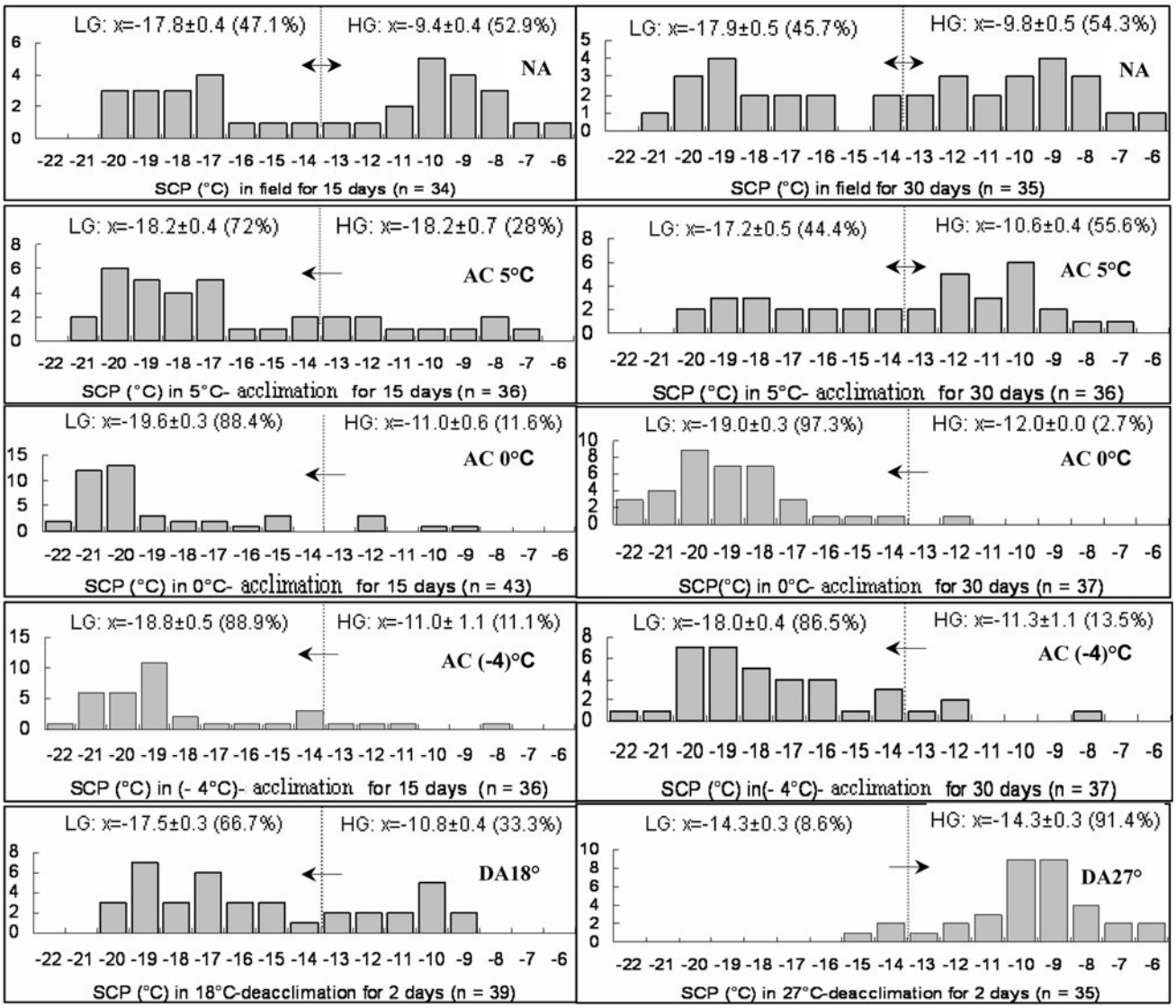

Fig. 4. Bimodal frequency distribution of SCP in diapausing larvae of *D. tabulaeformis* showing differences between non-acclimated (NA), acclimated (AC) and de-acclimated (DA) diapausing larvae. The break point is designated at –14°C between High group (HG; SCP > –14°C) and Low group (LG; SCP ≤ –14°C). The number of individuals tested (n), mean value of SCP (mean ± SE) and the percentage in HG or LG are indicated in the figure. Acclimation/de-acclimation shows a shift in the bimodal pattern as indicated by arrows; LG (> 60% of individuals in LG, left arrow), LG-HG (< 60% of individuals in LG and HG, dual-direction arrow) and HG pattern (> 60% of individuals in HG, right arrow).

days, an increase in mortality was observed in AC5°C (Fig. 2). The LT_{50} in NA, AC(–4)°C, AC0°C and AC5°C were all beyond 45 days.

Survival of diapausing larvae at –14°C

The corrected survivorship showed some significant differences between NA, AC(–4)°C, AC0°C, AC5°C, DA18°C and DA27°C groups exposed to the temperature of –14°C for 48 h (ANOVA: $F = 93.271$, d.f. = 5, 12, $P < 0.001$) (Fig. 3). Survival in AC(–4)°C and AC0°C groups was significantly higher than in other groups, and their LT_{50} values were both above 48 h. The DA18°C group had the similar survival to the NA group. The LT_{50} was 7.6 h and 8.4 h respectively in DA18°C and NA group. The DA27°C and AC5°C had the lowest survival when exposed to –14°C. The LT_{50} and LT_{90} were only 4.1 h and 12.7 h respectively in DA27°C. However, in the case of AC5°C, the LT_{90} was below 1.5 h (Fig. 3).

The supercooling points (SCP) and their distribution pattern

The SCP of diapausing larvae in NA groups exhibited a bimodal distribution with the break point value of –14°C. The SCP ranged from –6°C to –22°C (Fig. 4). The two NA groups had a LG–HG pattern, which also occurred in the group of AC5°C after 30 days. The LG pattern was observed in the AC(–4)°C, AC0°C, DA18°C groups as well as in the AC5°C group after 15 days. However, DA27°C group had a HG pattern (Fig. 4). The mean SCP ranged from –19.6 ± 0.3°C to –14.3 ± 0.3°C in LG, but from –12.0 ± 0.0°C to –9.4 ± 0.4°C in HG in the NA,

TABLE 2. Concentration (mean ± SE) of trehalose, galactose, glucose and mannose in the haemolymph of diapausing larvae of *D. tabulaeformis* with supercooling point frequency distribution in High group (HG; SCP > –14°C) and Low group (LG; SCP ≤ –14°C). Different lower case letters (a vs. b) represent a significant difference between concentration of trehalose versus galactose, glucose and mannose (ANOVA: F = 128.285, d.f.= 3,8, P < 0.001 for LG; F = 360.463, d.f. = 3,12, P < 0.001 for HG, followed by Tukey's HSD at P < 0.05). Different capital letters (A vs. B) represent a significant difference in concentration between HG and LG (by t-test).

Sugar	Concentration (mg / ml)		LG vs. HG		
	LG	HG	t	d.f.	Sig. (2-tailed)
Trehalose	17.6 ± 1.4 Aa	8.3 ± 0.4 Ba	7.219	5	0.001
Galactose	1.3 ± 0.1 Ab	0.0 ± 0.0 Bb	21.392	5	< 0.001
Glucose	0.6 ± 0.1 Ab	0.2 ± 0.1 Bb	12.474	5	< 0.001
Mannose	1.8 ± 0.3 Ab	0.4 ± 0.1 Bb	5.518	5	0.003

AC(–4)°C, AC0°C, AC5°C, DA18°C and DA27°C groups (Fig. 4).

Low molecular weight sugars in haemolymph of diapausing larvae

Four low molecular weight sugars (trehalose, galactose, glucose and mannose) were detected in the haemolymph of diapausing larvae of *D. tabulaeformis*. Trehalose was the major sugar detected in the haemolymph of either LG or HG individuals; concentration was 17.6 mg / ml in LG and 8.3 mg / ml in HG, both of which were significantly higher than levels of galactose, glucose and mannose (ANOVA: F = 128.285, d.f. = 3,8, P < 0.001 for HG; F = 360.463, d.f. = 3,12, P < 0.001 for LG, Table 2). The concentrations of trehalose, glucose and mannose were all significantly different between the LG and HG groups. The trend for all sugars consistently pointed to a significantly higher concentration in the haemolymph of LG individuals with lower SCPs (Table 2). Moreover, no galactose was detected in the HG group, though a higher concentration of galactose (1.3 mg / ml) was detected in the LG group.

Respiration rate in diapausing larvae acclimated or de-acclimated at different temperatures

Significant differences in O_2 uptake were detected between NA, AC(–4)°C, AC0°C, AC5°C, DA18°C and DA27°C groups (ANOVA: F = 14.45, d.f. = 5,31, P < 0.001) (Fig. 6). The DA18°C and DA27°C treatments had the highest respiration rate levels (1.0 and 1.3l / mg / h) compared with other groups. The respiration rate was significantly higher in NA group (0.8l / mg / h) than in three AC groups: AC(–4)°C, AC0°C and AC5°C. The AC(–4)°C and AC0°C groups had the same lowest rate of O_2 uptake (0.1l / mg / h) (Fig. 6).

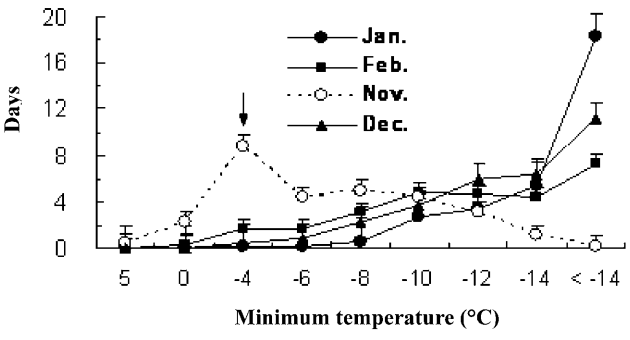

Fig. 5. Number of days (mean ± SE) of minimum air-temperature below 5°C in November (Nov.), December (Dec.), January (Jan.) and February (Feb.) in Chengde (41°N) during 1995–2006. Arrows represent the critical periods of acclimation temperatures (black arrow) and periods of lethal temperatures (grey arrow) during the diapause of larval pine caterpillar, *D. tabulaeformis*, in the field.

DISCUSSION

Overwintering strategy and the supercooling point (SCP)

Most overwintering insects in temperate climates are thought to be freeze-intolerant, relying on the process of supercooling to lower their supercooling points (SCPs) (Bale, 1991). The SCP is a valid measurement of the lower lethal temperature. It is correlated with the level of cold tolerance for many insect species (Worland & Convey, 2001; Klok et al., 2003; Sinclair et al., 2003a). In this study, *D. tabulaeformis* could not be freeze-tolerant as is evident from its body-freezing profile in SCP determinations. Its cold hardiness was enhanced through depression of the SCP. Basing on the original two strategies, freeze-tolerance and freeze-avoidance, Bale (1996) proposed that insects could be conveniently classified into five groups representing a continuum from the most to the least cold-hardy. They are freeze-tolerant, freeze-avoidant (only including those species in which there is little or no low-temperature mortality in the absence of freezing),

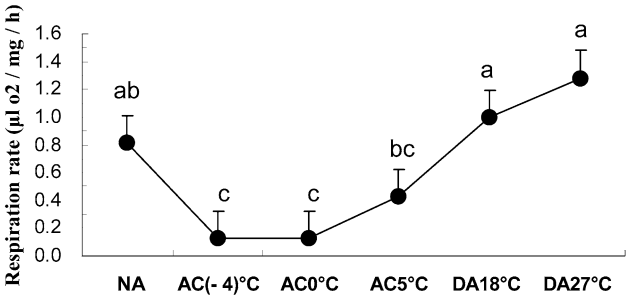

Fig. 6. Respiration rate (mean ± SE) of non-acclimated (NA), acclimated (AC) and de-acclimated (DA) larvae. Different letters represent a significant difference in respiration rate (ANOVA: F = 14.45, d.f. = 5, 31, P < 0.001; followed by Tukey's HSD at P < 0.05).

chill tolerant, chill susceptible and opportunistic survival (Bale, 1996, 2002). Our results show that *D. tabulaeformis* might belong to the chill tolerant group. It adopted the strategy of depressing SCP to enhance its cold hardiness, and could be tolerant of low temperatures (i.e. –14°C). However, mortality occurred above SCP, and became apparent with increasing periods of low temperature exposure (Fig. 3). At the time of entering diapause, the SCP of larvae was maximally depressed by 7°C compared with that of non-diapause larvae, and the survival rates were significantly higher than the latters when exposed to lower lethal temperatures (e.g. –10, –17°C etc.) for minutes or hours (Han et al., 2005). After acclimation at low temperatures in advance, such as at –4°C or 0°C for 15 to 30 days, the SCP of the diapausing larvae was significantly lowered, and at the same time the cold hardiness was increased when placed at –14°C compared with the non-acclimated larvae.

Within a population, the SCP shows substantial variation, with a frequency distribution that is often clearly bimodal (Sømme & Block, 1982; Klok & Chown, 1998; Chen & Kang, 2005; Worland et al., 2006). Such variation is attributed directly to adaptive responses to changing environmental conditions (Block, 1990), and these bimodal SCP distributions might represent a bet-hedging strategy that would allow animals to survive unexpected cold snaps (Klok & Chown, 1998). However, the reasons why the variable and/or bimodal SCP distributions occur remain poorly understood (Worland et al., 2006). In the diapausing larvae of *D. tabulaeformis*, the SCP ranged from –6.0°C to –22.0°C, and its frequency distribution was also bimodal with shift to and from LG vs. HG group influenced by cold acclimation and de-acclimation. For instance, an LG–HG pattern of the non-acclimated larvae could be changed into a LG pattern when acclimated at low temperatures, such as –4°C or 0°C for 15 to 30 days, but then would be transformed into a HG pattern when de-acclimated at high temperatures, such as at 27°C for only two days (see Fig. 4).

The SCP variable can be correlated with a variety of factors, such as starvation (Sømme & Block, 1982; Leinaas & Sømme, 1984; Leinaas & Fjellberg, 1985; Worland & Block, 1999), moulting (Worland et al., 2006) and undergoing a range of biochemical changes that include the production of cryoprotectants (Chown & Nicolson, 2004). In most insects, the levels of low molecular weight polyols, sugars or amino acids, such as glycerol, sorbitol, trehalose, sucrose, proline and alanine tend to increase when larvae undergo a cold-acclimation (Storey & Storey, 1988; Lee & Denlinger, 1991; Fields et al., 1998). However, they return to their pre-acclimation levels after a de-acclimation, as documented for trehalose and proline in *Cryptolestes ferrugineus* (Fields et al., 1998). In our results, the levels of trehalose, glucose, mannose and galactose in the haemolymph of diapausing larvae were correlated with the SCP variable as well as the degree of cold hardiness. Levels of the four low molecular sugars were all higher in LG larvae than in HG larvae. This suggests that cold acclimation increased the cold hardiness of the diapausing larvae through depressing the SCP, while de-acclimation did the reverse. The SCP variable was positively correlated to the levels of trehalose, glucose, mannose and galactose in the haemolymph, while these levels were negatively correlated to the metabolic rate. For instance, the increased rate of metabolism after de-acclimation at high temperature, such as 27°C, led to a decrease in the level of these low molecular weight sugars. Conversely, the low levels of metabolism after cold acclimation, such as at –4°C or 0°C, maintained high levels of these sugars.

Effects of acclimation and de-acclimation on cold hardiness and mortality

Organisms, such as bacteria, plants, and animals, can increase their survival to otherwise lethal stressful environmental conditions by prior exposure to non-lethal but stressful conditions. This ability to acclimate is considered to be an adaptive response to changing environmental conditions (Hofmann, 1995). The diapausing larvae of *D. tabulaeformis*, had the ability to become acclimated at low temperatures, such as at 0°C and –4°C for 15 or 30 days. The cold acclimation improved their ability to survive at –14°C, as shown by the LT_{50} in AC(–4)°C and AC0°C above 48 h, both significantly higher than the 8.4 h in NA. In the field, seasonal acclimation could increase survival in adverse conditions, such as cold shocks in winter. During 1995–2006, in North China the temperatures decreased rapidly once autumn commenced, and the minimum temperatures were always below –14°C (Fig. 5), the lower lethal temperature for the diapausing larvae. However, cold acclimation in advance at some non-lethal low temperature in autumn might improve the tolerance of the diapausing larvae to the lower lethal temperatures later in winter. Fig. 5 shows a prolonged period (several weeks) with minimum temperatures between –4°C and 0°C in November in Chengde. After undergoing this period of cold acclimation most diapausing larvae would survive the following cold shock through December to February. Consequently, the pine forest can be damaged again in spring as severely as during the preceding summer after diapause termination of *D. tabulaeformis* (unpubl. data).

Interestingly, cold acclimation was lost in a short time following de-acclimation (i.e., 27°C for two days) of diapausing larvae of *D. tabulaeformis*. Results showed that the LT_{50} and LT_{90} were decreased significantly to 4.1 h and 12.7 h in DA27°C. The loss of cryoprotectants, such as trehalose, glucose, mannose and galactose in haemolymph, because of the rapidly increasing metabolism during de-acclimation, might be a major reason for the loss of cold acclimation in the de-acclimated larvae. Similar results were also found in *Cryptolestes ferrugineus* (Fields et al., 1998). Most insects could be prevented from dying during a warm winter, but the cold acclimation would be lost during a prolonged acclimation at above zero temperature (i.e. 5°C in *D. tabulaeformis*). Moreover, the cold acclimation might be decreased during de-acclimation in warm days. So when cold shocks occurred following the warm days, the overwin-

tering insect would be killed a lot for the loss of cold hardiness, such as in the case of diapausing larvae of *D. tabulaeformis*.

Acclimation itself can be fitness decreasing (Hofmann, 1995). In the diapausing larvae of *D. tabulaeformis*, acclimation at high temperatures, such as 18°C and 27°C, increased mortality rapidly during the acclimation. It might be the consequence of a rapid loss of energy at high temperatures as metabolic rate increases. However, the acclimation at temperatures close to 0°C, such as at 5°C or –4°C was more complicated. Shorter than 15-day acclimation at 5°C can increase cold tolerance (Fig. 4), which, however, can be lost rapidly with the loss of cryoprotectants (Ring, 1982) during longer acclimation at 5°C. For instance, the LT_{90} was below 1.5 h in AC5°C after a 40 d-acclimation. Mortality did not increase significantly when acclimated at 0°C or –4°C, but chilling injury took place, especially at –4°C, during the prolonged period (e.g. above 45 d) of acclimation, because of the detrimental changes in membrane viscosity, enzyme activity or ionic concentrations (Fields et al., 1998).

In conclusion, the diapausing larvae of *D. tabulaeformis* were chill tolerant insects according to Bales system (Bale, 1996). They adopted the strategy of depressing the SCP through accumulation or production of cryoprotectants, such as trehalose, glucose, mannose, galactose, to enhance their cold hardiness. Bimodal frequency distribution of SCP was demonstrated in the diapausing larvae, but the bimodality changed into three patterns, LG, LG–HG and HG pattern, in response to cold acclimation and de-acclimation. Overall, the degree of cold hardiness could be ranked as: LG > LG–HG > HG. The level of metabolism could affect the LG–HG frequency distribution of SCPs through suppressing the levels of trehalose, glucose, mannose, and galactose. The ability of larvae to cold acclimate at non-lethal temperatures in autumn could be an adaptive strategy to avoid the cold shocks in winter. Apropos, acclimation in warm conditions or a long-term acclimation at temperatures above 0°C could increase their mortality through elevation of the lower lethal temperatures. Chill injury could also affect the diapausing larvae after a long-term cold acclimation.

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