The effect of low temperatures on diapause and non-diapause larvae of the pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu (Lepidoptera: Lasiocampidae)

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Abstract
Some effects of low temperatures on diapause and non-diapause larvae of the pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu (Lepidoptera: Lasiocampidae), were investigated in laboratory experiments. The results indicated that diapause larvae had higher survival rates than non-diapause larvae at earlier stages of exposure to the low temperatures (-10, -17 and $-21°C$). In general, the larvae lives were shorter when they were exposed to the lower temperatures. Exposure to chilling at $0°C$ (cold pulse) in advance increased the survival rate of larvae when they were exposed to $-10°C$, and the survival rate increased with the length of cold pulse (doubled at 50 min). No larvae could survive after the 50th day of daily exposure to $-10°C$ for 1 h followed by exposure to $27\pm1°C$ for 23 h. The water contents of diapause and non-diapause larvae differed significantly, and the super-cooling points (SCPs) of non-diapause larvae were significantly higher than those of diapause larvae.

Key words: *Dendrolimus tabulaeformis*; cold-hardiness; super-cooling; diapause; non-diapause

INTRODUCTION

One of the key factors in the longevity of insects is their ability to survive in a wide range of environmental conditions. Cold is one of the major environmental obstacles that insects must overcome to survive in temperate and arctic climates (Fields et al., 1998). In such climatic regions, insects that do not migrate must tolerate long periods of low temperatures or develop abilities to survive prolonged exposure to freezing (below zero) temperatures (Rickard et al., 1987; Naeemullah et al., 1999).

Insects can be grouped into two general categories regarding their responses to cold temperatures: freeze-tolerant and freeze-intolerant species (Baust and Rojas, 1985; Storey and Storey, 1988; Lee and Denlinger, 1991). To avoid freezing, freeze-intolerant species develop some adaptations such as lowering super-cooling points (SCPs) (Baust and Rojas, 1985; Naeemullah et al., 1999),

which are maintained during the winter through the production of anti-freeze agents (Storey and Storey, 1988). The super-cooling point is the lowest temperature caused by the heat of crystallisation, representing the limit of low-temperature tolerance, above which cold injuries and death may also occur (Bale, 1987). Freeze-tolerant species have higher super-cooling points and freezing can occur between cells when they are exposed to temperatures below super-cooling points, but they still survive (Lee and Denlinger, 1991).

Many insects in the temperate zones enter diapause to survive seasonal environmental stresses (Denlinger, 1985, 1991; Tauber et al., 1986; Leather et al., 1993; Fields et al., 1998). Although the relationship between winter diapause and cold hardiness—the physical and metabolic adjustments that enhance survival at low temperature—has been investigated in several studies (Denlinger, 1991; Goto et al., 1998), it is not fully understood and remains a controversial topic in insect cryobi-

ology (Hodkova and Hodek, 1997). Cold-hardiness usually deals with the most extreme results of cold stress, cold-induced mortality, and it is evaluated after return to favourable conditions (Salt, 1961; Sømme, 1982).

The pine caterpillar, *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae), is a serious pest of pine trees in North China. It has one or two generations per year in the forests in Beijing (Tsai, 1959; Chen, 1990). Previous works have shown that the pine caterpillar enters diapause as the third and fourth instar larvae after the first instar larvae are exposed to short day lengths (9L : 15D), and they begin to over-winter before low temperatures arrive (Li and Gia, 1989; Gia and Li, 1991). They leave the tree and stay on the soil around the pine trees. The humus consisting of leaves and grass cover the bodies of the caterpillars. Because of the relatively long hatching period from July to August, some of the larvae receive long day lengths and enter non-diapause while others receive short day lengths and enter diapause. Hence, two over-wintering (diapausing and non-diapausing) larval states occur. During the winter, low temperatures freeze the larvae and normal temperatures thaw the frozen larvae, and this can occur repeatedly. After the winter, the population of non-diapause larvae decrease as compared to diapause larvae (Chen, 1990), but different years resulted in different ratios.

To our best knowledge, no information exists regarding the cold tolerance of diapause and non-diapause larvae of *D. tabulaeformis*. This study aimed to investigate the effects of low temperatures on both diapause and non-diapause larvae as well as the effect of the freezing-thawing cycle on diapause larvae, which occur dominantly in the natural population. We also analysed the super-cooling points and water content of the diapause and non-diapause larvae of *D. tabulaeformis*.

MATERIALS AND METHODS

Experimental culture. The larvae of *D. tabulaeformis* were collected from a pine forest in the suburbs of Beijing, the People's Republic of China. The larvae were allowed to form cocoons and emerge in a growth chamber kept at 27 ± 1°C, 60–80% RH under a 15L (light) : 9D (dark) photoperiod. After the adults oviposited, the second-generation eggs were collected and placed in cli-

mate chambers (LRH-250-GS, Harbin Production) equipped with eight fluorescent 30 W tubes controlled by an automatic time switch and maintained at 27 ± 1°C and 60–80% RH. The larvae were fed fresh pine needles of *Pinus tabulaeformis* carr. collected from the suburbs of Beijing. Only newly excuvial individuals of the fourth instars were used throughout the experiments.

Induction of diapause and non-diapause larvae. Larvae of *D. tabulaeformis* can develop and become pupa under a 15L : 9D photoperiod at 27°C; however, they enter diapause as third and fourth instars if they are grown under a 9L : 15D photoperiod at 27°C (Li and Gia, 1989; Gia and Li, 1991). The diapause larvae cease feeding and are easily recognisable by their small size and yellow body colour when compared with non-diapause larvae. In this experiment, newly hatched larvae were reared in two separate growth chambers (both at 27 ± 1°C, 60–80% RH), one under a 9L : 15D photoperiod and the other under a 15L : 9D photoperiod to obtain diapause and non-diapause larvae, respectively. All of the newly exuvial fourth instar diapause and non-diapause larvae were exposed to the temperature of 10°C without light for 30 d to carry out the adaptation. This method simulates the period when larvae leave the trees, go to the soil and cover themselves with organic debris before cold temperatures arrive.

Survival at low temperatures. According to the history records of Beijing temperatures, the lowest day atmospheric temperature reaches −20°C. Therefore, we set three different temperatures that were -10 ± 1, -17 ± 1 and -21 ± 1°C. Thirty diapause and 30 non-diapause fourth instar larvae were each separately placed in plastic boxes (12 cm×17 cm×7 cm). Eighteen boxes were placed in refrigerators to expose the larvae to each designed temperature. We took out three boxes, each exposed to a different temperature, after every observation period. Table 1 summarises the temperatures and the exposure periods used in the experiment. After exposure to designated temperatures and periods, larvae were transferred to 27 ± 1°C conditions in a growth chamber for 24 h. The number of alive and dead larvae was recorded. Larvae showing no movement and loose body segments were assumed to be dead (Goto et al., 2001b).

Determination of super-cooling points (SCPs). Twenty-six diapause and 25 non-diapause fourth

Table 1. Exposure periods (h and min) and temperatures (°C) to which larvae were exposed

Temperatures	Duration of exposure periods					
	1	2	3	4	5	6
−10±1°C/h	10	20	30	40	50	60
−17±1°C/h	0.5	1	1.5	2	2.5	3
−21±1°C/min	10	15	17.5	20	22.5	25

instar larvae were fixed with thermocouples connected to individual automatic temperature recorders (uR100, Model 4152, Yologama Electrical Co, Seoul, Korea). The larvae were then put into a Styrofoam tube (5 cm length, 1 cm diameter) and the tubes were put into a freezing chamber with a cooling rate of 1°C/min to record the SCPs (Chen and Kang, 2002). The SCP was defined as the temperature initiating spontaneous freezing as indicated by the liberation of latent heat.

Body weight and water content determination. Two larvae groups, with 30 larva for diapause and 45 larva for non-diapause, were weighed individually using an electronic balance (sensitivity: 0.1 mg, Sartorius, R200D.A.G., Göttingen, Germany), kept at 65°C for 24 h in an oven (Costanzo et al., 1997) and reweighed to obtain the dry weight. The water contents were expressed as the percentage of loss of body mass after being dried. The fresh body weight was also measured.

Effect of short-term chilling at 0°C (cold pulse) on survival of diapause larvae when exposured to low temperatures (−10°C). In forests, after exposure to low temperatures, populations of non-diapause larvae decrease and the populations of diapause larvae become dominant and endure further exposure to low temperatures (Chen, 1990). Therefore, we chose diapause larvae to examine the effects of low temperatures on this species.

Separate sets of 30 fourth instar diapause larvae were placed in plastic boxes (12 cm×17 cm×7 cm) which was put into a refrigerator kept at 0°C to give cold pulses at different times of 0, 10, 20, 30, 40, 50 and 60 min. This was done with three replicates (boxes) and a total of 90 larvae were used for each time period. After exposure to 0°C for the designated time, larvae were transferred to another refrigerator kept at −10°C and left there for 25 h. Then, the larvae were transferred into a growth chamber at 27±1°C and fed fresh pine needles. The survival rate of diapause larvae at low temperature (−10°C) after exposure to chilling at 0°C for different time periods was calculated by making a comparison with live larvae capable of moving and feeding.

Effects of low temperature cycles between −10 and 27°C on diapause larvae. A set of 30 fourth instar diapause larvae were placed in a plastic box (12 cm×17 cm×7 cm) which was put into a refrigerator kept at −10°C and left 1 h. Following this time period, the larvae were transferred immediately to a growth chamber kept at 27±1°C and left there for 23 h. This was repeated every day until all larvae died. A total of three replicate sets (90 diapause larvae) were observed. The survival rates were recorded as the number of dead larvae every 10 d. The mortality rate for a specific time period was obtained by counting the number of dead larvae for that period and comparing it to the total number of larvae at the beginning. Larvae showing no normal movement were judged to be dead (Goto et al., 2001a).

Statistical analyses. Data were analysed by *t*-test and one- or two-way analysis of variance followed by Tukey's multi-range test (SAS Institute, 1996). The results were expressed as mean±SE and considered significantly different at $p < 0.05$.

RESULTS

Survival at low temperatures

The results indicated that both diapause and non-diapause larvae did not survive more than 60 h if they were exposed to a temperature of −10°C (Fig. 1), and they did not survive longer than 3 h and 30 min if they were exposed to −17°C (Fig. 2) and −21°C (Fig. 3), respectively. The survival rates of diapause larvae were significantly higher than those of non-diapause larvae: up to 20 h when exposed to −10°C, up to 1 h at −17°C, and up to 15 min at −21°C ($p < 0.05$). The survival rates of larvae declined more drastically between the first and second exposure periods than the other periods when they were exposed to −10°C (Fig. 1) and −17°C (Fig. 2). The same trend was exhibited between the third and fourth periods when the larvae were exposed to −21°C (Fig. 3).

SCPs, body weight and water content of diapause and non-diapause larvae

The body weight of diapause larvae did not differ from those of non-diapause larvae, but the water contents and super-cooling points (SCPs) of diapause larvae were significantly lower than those of the non-diapause larvae ($p < 0.05$) (Table 2).

Survival rate of diapause larvae at −10°C after short-term chilling at 0°C

All of the diapause larvae that were exposed to 0°C before they were exposed to −10°C (Fig. 4) survived longer than the control larvae (i.e., those that were not exposed to 0°C before they were ex-

posed to −10°C). The length of exposure to chilling at 0°C affected the survival rates, and as the exposure period to 0°C was prolonged, the survival rate of larvae increased. As short as 50 min of exposure to 0°C doubled the rate of survival at −10°C without 0°C exposures. No differences occurred in survival rates when larvae were exposed to 0°C for longer than 40 min.

Effect of low-temperature cycle on diapause larvae

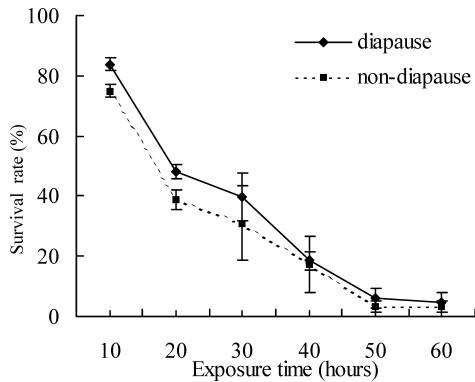No larvae could survive after the 50th day of

Fig. 1.   Survival rate (mean±SE) in diapause and non-diapause larvae of *D. tabulaeformis* exposed to −10°C.
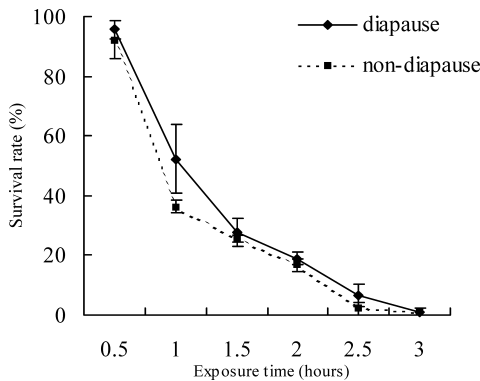

Fig. 2.   Survival rate (mean±SE) in diapause and non-diapause larvae of *D. tabulaeformis* exposed to −17°C.
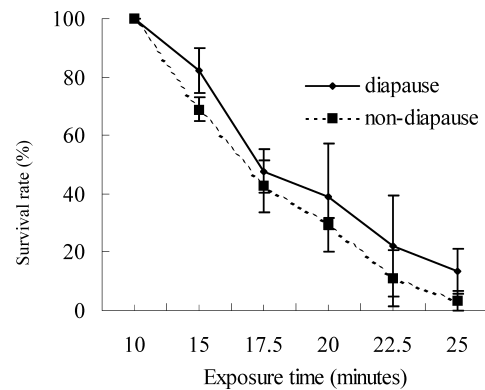

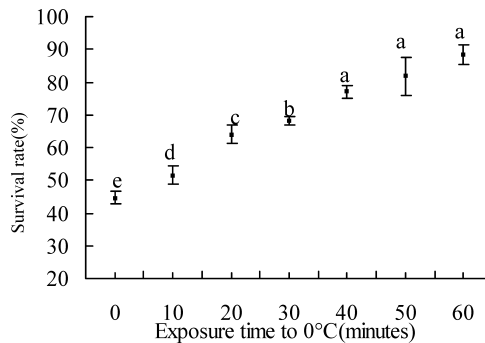Fig. 3.   Survival rate (mean±SE) in diapause and non-diapause larvae of *D. tabulaeformis* exposed to −21°C.

Fig. 4.   Effect of the duration of chilling at 0°C before exposure to −10°C on the survival rate (mean±SE) in diapause larvae of *D. tabulaeformis* (values with different letters indicate significant differences ($p < 0.05$) in survival rates in time).

Table 2.   Water content, body weight and super-cooling points (SCP) (mean±SE) of diapause and non-diapause larvae of *D. tabulaeformis* (The values in parentheses indicate the number of larvae examined. Within a column, values indicated by different letters are significantly different at $p < 0.05$)

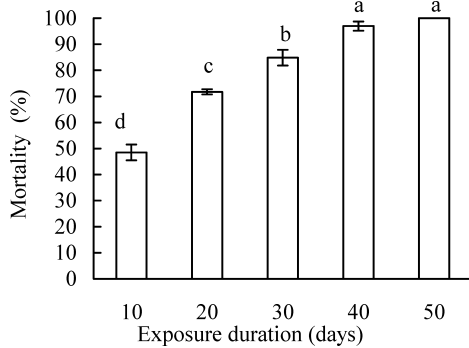| Types	Body weight (g)	Water content (%)	SCP (°C)
L : D=9 : 15 Diapause	0.0852±0.0067 (30) a	79.50±1.34 (30) b	−13.16±0.20 (26) b
L : D=15 : 9 Non-diapause	0.0868±0.0081 (45) a	83.19±0.60 (45) a	−6.09±0.92 (25) a

Fig. 5. The mortality rate (mean±SE) in diapause larvae of *D. tabulaeformis* after 1-h cold exposure to −10°C and 23-h exposure to 27°C (values with different letters indicate significant differences ($p<0.05$) in mortalities in time).

daily exposure to −10°C for 1 h followed by exposure to 27±1°C for 23 h (Fig. 5). The mortality of larvae increased until the 40th day of exposure. No difference occurred in the mortalities of larvae afterwards because most of the larvae had already died.

DISCUSSION

The larvae of *D. tabulaeformis* responded differently to low temperatures, and the length of cold exposure affected their responses. In general, the larvae died more quickly as the temperature decreased. The survival rate of the diapause larvae was higher than that of the non-diapause larvae after they were exposed to −10, −17 and −21°C at earlier stages of all exposures (Figs. 1, 2 and 3). According to the results of this study, the diapause larvae were more cold-tolerant to the three low temperatures than the non-diapause larvae, especially at the beginning of the low temperatures trial. In general, the survival rates declined more drastically in the earlier periods of exposure. Exposure of the larvae to 0°C even for a few minutes strengthened their cold tolerance to lower temperatures.

Diapause is an endocrine-mediated dormancy that occurs at a specific developmental stage (Denlinger, 1985, 1991; Tauber et al., 1986), and it is essential for the maintenance of prolonged cold tolerance. Some authors have reported that cold tolerance is closely associated with diapause (Asahina, 1966; Wyatt, 1967; de Wilde, 1970; Mansingh, 1974; Tauber et al., 1986; Saunders and

Hayware, 1998), while others reported that it is independent of diapause (Salt, 1961; Baust and Miller, 1970; Nordin et al., 1984; Tanaka, 1997) although Denlinger (1991) accepted both possibilities. For example, the cold tolerance of the barnyard grass stem borer *Enosima leucotaeniella* is not associated with diapause because its diapause is terminated in November, before low temperatures occur in the winter (Goto et al., 1998). On the other hand, Tzanakakis (1959) has demonstrated that the diapause larvae of *Plodia interpunctella* are more cold-tolerant than non-diapause larvae. It is well known that the lower the rate of metabolism, the greater the cold tolerance and intensity and duration of diapause (Mansingh, 1971), like in *Plodia interpunctella* (Naeemullah et al., 1999). Our results on the SCPs and the survival rates of diapause and non-diapause larvae of *D. tabulaeformis* indicated similar trends. The SCP of diapause larvae was lower than that of non-diapause larvae. The mean SCP of diapause larvae was lower than −10°C, while that of non-diapause larvae was around −6°C. So, it can be inferred that the two states were affected by different mortality factors at the beginning of exposure to −10°C. Diapause larvae might have been killed by chilling injury, while the non-diapause counterparts might have been killed by internal tissue freezing. When exposure was prolonged to more than 20 h or the exposure temperature was decreased to −17 or −21°C, both the diapause and non-diapause larvae could have been killed by tissue freezing. When low-temperature weather arrives at the autumn or at the beginning of winter (just after the larvae leave the trees to over-winter), most of the diapause larvae can survive on the soil while most of non-diapause larvae die because of their weaker capacity to cold tolerance as compared to diapause larvae. So the diapause larvae dominate before and during the coldest season.

Naeemullah et al. (1999) reported that the diapause larvae of the Indian meal moth, *Plodia interpunctella*, reared at 25°C were more tolerant to −20°C than non-diapause larvae. Diapause larvae showed considerably higher survival rates than non-diapause larvae after exposure for 20 to 40 min, though the difference after 30-min exposure was not statistically significantly. Our experiment gave a similar result for the comparison between diapause and non-diapause larvae of this

pine caterpillar. Hanec (1966) showed that the SCP of newly hatched larvae of the forest tent caterpillar, *Malacosoma disstria* Hbn. (Lepidoptera: Lasiocampidae), was −13.4°C, and under laboratory conditions, larvae could survive at −12°C for at least 7 d. When the temperatures approached Alberta (Canada) spring conditions (−7 to −1°C), only little mortality occurred (Raske, 1975).

Some studies have shown that there is affinity between water content and the SCP of insects (Yancey et al., 1982; Lee and Denlinger, 1991; Šlachta et al., 2002). Our results showed the same trend; the water content and super-cooling point of the diapause larvae were lower than those of non-diapause larvae, while the body weight showed no difference between the two diapause states. A cold-resistant substance could have been concentrated in the diapause larvae with lower water content as compared to the non-diapause larvae.

Lee et al. (1987) showed that the survival rates of *Sarcophaga crassipalpis* that received a brief cold pulse (chilling) was higher than those that did not receive a cold pulse. They also suggested that the duration of the brief cold pulse could affect the survival rate differently. Our results also indicate that a cold pulse of 0°C can affect the survival rate of larvae when exposed to −10°C. Increasing the time of the cold pulse increased the survival rate. The survival rate of larvae that were exposed to 0°C for 50 min before they were exposed to −10°C doubled the rate of survival of the larvae without 0°C exposure under −10°C. But no significant differences occurred in the rate of survival after 40-min exposure to chilling at 0°C. Therefore, short-term chilling at 0°C resulted in protection against the cold-shock injury that occurs at temperatures above the super-cooling point.

Our results indicate that even when diapause larvae are exposed to −10°C for 1 h everyday, most of them couldn't survive for 50 d. However, this kind of low soil temperature does not occur everyday for 50 d in a year (Chinese National Weather Bureau, 2000). In Beijing, the mean temperature of 5-cm deep soil is around −4°C, and it does not exceed −10°C even in the coldest season, although the lowest air temperature can reach −15°C or lower. With protection by the humus covering, the temperature around over-wintering caterpillars' larvae would remain higher than the atmospheric temperature. In the field, the lowest soil temperature does not usually last more than 2 h. Exposure to sub-zero or zero temperatures in the field might enhance the ability of larvae to adapt to low temperatures. Therefore, exposure to different low temperatures in the winter might be one of the factors that influence the population structure of *D. tabulaeformis*.

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