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Cold tolerance and *myo*-inositol accumulation in overwintering adults of a lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Abstract. I investigated the seasonal changes of cold tolerance and polyol content in adults of *Harmonia axyridis* to elucidate their overwintering strategy. Adults decreased their supercooling point and lower lethal temperature only during the winter. Although the seasonal trends for both values were almost consistent, there seemed to be considerable mortality, without being frozen, at -20°C in mid-winter. The pattern for seasonal change in tolerance at moderately low temperatures differed among the temperatures exposed: the survival time at -5°C peaked in winter, but the time at 5 or 0°C peaked in autumn. Because both autumn and winter adults were completely paralyzed only at -5°C and survived much longer at 0°C than at 5°C, the survival time at -5°C indicates the degree of chilling tolerance, whereas the time at 5 or 0°C seems to show starvation tolerance. This beetle accumulated a relatively large amount of *myo*-inositol during winter. *Myo*-inositol content synchronized seasonally with supercooling capacity, the lower lethal temperature and the chilling tolerance, suggesting that *myo*-inositol may play some role in the control of cold tolerance in this beetle.

INTRODUCTION

Acquisition of cold hardiness is one of the essential physiological adaptations for winter survival in insect species distributed in the temperate, frigid and polar regions (Denlinger, 1991). Insect cold hardiness has been evaluated by various indices and methods, for example, by calculating the supercooling point (SCP), the lower lethal thresholds for exposure temperature and time, together with the seasonal patterns and concentrations of cryoprotectants such as polyols and sugars (Bale, 1991).

Harmonia axyridis Pallas is one of the most common aphidophagous lady beetles in Japan, and the genetic variation in elytral pattern has been studied in detail (Komai, 1956; Laboratory of Insects, University of Tamagawa, 1975). This beetle produces two to four generations annually in Japan. In late autumn, adults often swarm for a while, and then move to their overwintering site, such as crevices in rock or concrete objects (Obata, 1986). The adults of this species enter reproductive diapause before winter, decrease their respiration rate and accumulate glycogen and lipids (Sakurai & Chujo, 1977; Sakurai et al., 1992). However, it remains unknown whether this beetle has any physiological mechanism of cold tolerance to endure low winter temperatures. Such information would also be important for using this beetle as a biological control agent against various aphid pests. In this study, I investigated seasonal changes of SCP, lower lethal thresholds for exposure temperature and time and polyol content in this beetle to elucidate the cold tolerance strategy.

MATERIALS AND METHODS

Insects

Adults of *H. axyridis* swarmed on and around the walls of my institute in Tsukuba (36.0 °N) between late October and early December 1998 or between early and mid December 1999. Each of 50 to 60 pairs of males and females was put together into a plastic container (upper diameter 11.3 cm, bottom diameter 9.8 cm, height 7.2 cm) with folded filter papers and a bottle of water with a cotton plug. Each container was kept throughout winter in open shade overhung by a concrete wall in a net house. These beetles were regarded as overwintering adults. In early March, they were transferred to a bigger wood-framed cage (16 × 40 × 40 cm) covered with nylon mesh because they began to move around and mate.

Winter ambient temperature

Temperatures at the artificial overwintering site mentioned above were recorded every 30 min by using a thermorecorder (RT-10, Tabai Espec, Osaka) from November 1997 to March 1998.

Winter survival

Thirty pairs of male and female adults swarming on 10 November 1997 were placed in the plastic container under the outdoor conditions mentioned above. On 6 March when the adults started mating, they were transferred to the bigger wood-framed cage mentioned above. The survival was checked every 2 to 3 day until the end of March.

Measurements of SCP

The SCP of adults collected or taken from outdoors between October 1997 and April 1998 was determined by the method of Watanabe & Tanaka (1997). The tip of a copper-constantan thermocouple was attached to the dorsal abdomen of an adult with rubber bond. The cooling rate was around 1°C/min.

Lower lethal temperature

Twenty males or females collected in the field on October 25 1997, or taken from outdoors on 15 December 1997, 15 February 1998 and 1 April were put together into a Petri dish (diameter 9 cm, height 2 cm). The dish, containing moistened tissue papers, was placed in the plastic container described above. The container was exposed to -5, -10, -15 or -20°C for 1 day, and then gradually rewarmed to 20°C. It was estimated that the adults were alive when they normally walked 2 days after

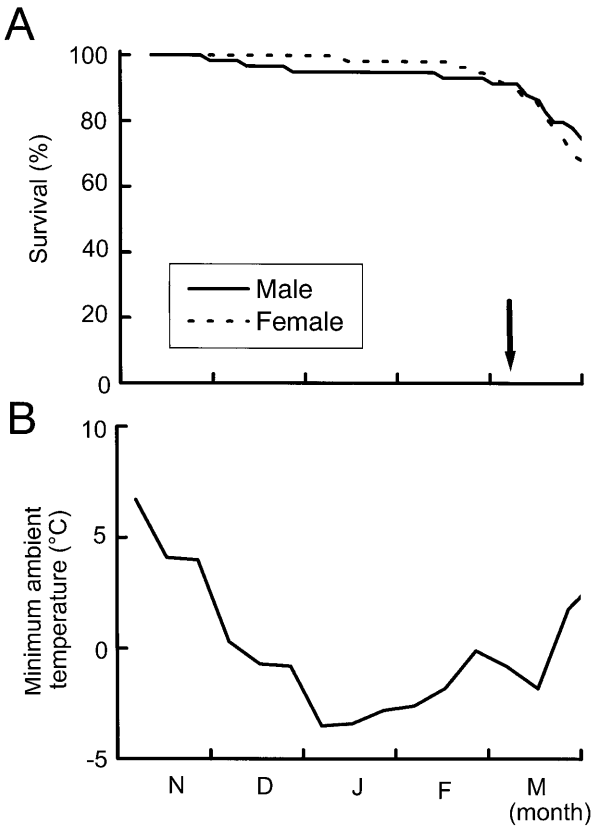

Fig.1 Seasonal changes of survival rate in *H. axyridis* adults (A, N = 30 ♂♂ ♀♀, duplicated) and the 10-day-minimum temperatures at the artificial hibernation site (B). The date when first mating was observed is shown by an arrow in Fig.1A.

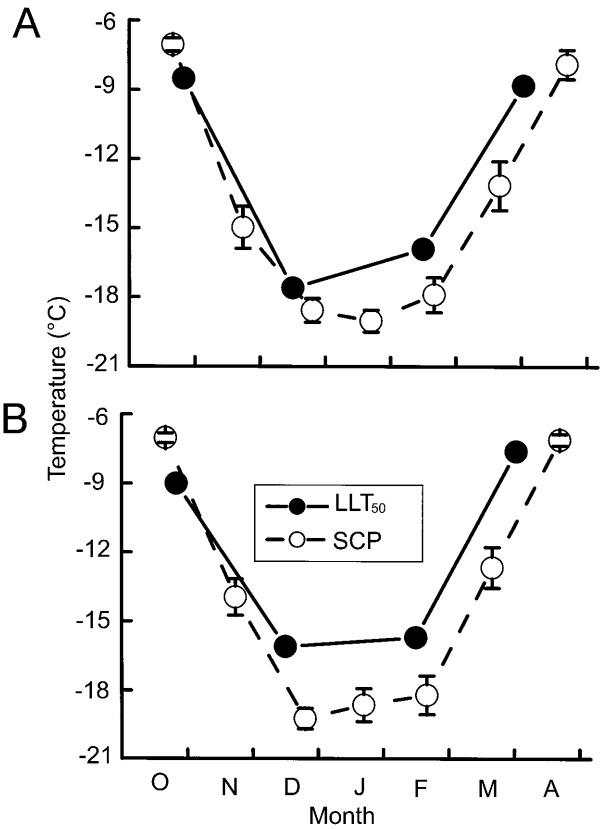

Fig.2 Seasonal change of supercooling point (SCP, dotted line with open symbols) and 50% lower lethal temperature (LLT_{50}, solid line with closed symbols) in male (A) and female (B) *H. axyridis* collected in the field or taken from outdoors. Each point shows mean ± SE. N = 15 to 20 ♂♂ ♀♀ (SCP) and 20 ♂♂♀♀ (LLT_{50}).

the low temperature treatment. The median lower lethal temperature (LLT_{50}) was calculated from the mortality of male or female adults by probit analysis.

Survival time at moderately low temperatures

Fifteen or twenty males or females collected between November 1997 and April 1998, or between December 1998 and March 1999 were put together into a Petri dish with moistened tissue papers in the plastic container, and then transferred to an incubator at 5 or -5°C. Another Petri dish containing beetles was buried in crushed ice in a styrene foam container (38 × 26 × 23 cm). This was kept in a refrigerator (ca. 4°C) to avoid melting the ice. By doing so, temperatures inside the dishes were maintained at ca. 0°C and near 100% relative humidity. To check whether the adults were alive or not, the dishes were taken out from the incubator or ice every week, and warmed up gradually to 20°C (ca. 0.26°C/min). After removing dead individuals, dishes containing the survivors were again cooled as mentioned above. This procedure was repeated until all the individuals died. Thus, the adults were exposed to cyclic warming, 3 to 4h, every week in the course of this experiment.

Polyol and sugar determination

Adults collected or taken from outdoors between September 1997 and April 1998 were homogenized individually with 4 ml of 80% ethanol and 100 μg of erythritol added as an internal standard. Low molecular weight carbohydrates and polyols were detected by high performance liquid chromatography (HPLC) as described by Watanabe & Tanaka (1999).

Measurements of dry body weight and water content

The adults collected in the field on 2 December 1998, 17 January 1999 and 15 March were individually dried in an oven at 60°C for 2 days. Water content was calculated from the difference between before and after the thermal treatment.

RESULTS

Winter survival and ambient temperatures

More than 90% of male and female beetles survived until the end of February (Fig. 1A). On and after 6 March when mating behaviors were first observed, the survival rate decreased rapidly in both sexes.

Seasonal change of the minimum ambient temperatures was shown in Fig. 1B. The temperatures often dropped below 0°C between mid December and mid February. The lowest recorded ambient temperature was -3.5°C in early January.

Seasonal change of SCP and lower lethal temperature

Both SCP and LLT_{50} of adults changed seasonally (Fig. 2). Adults that were swarming on October had a relatively high SCP (ca. -7°C) in both sexes. The SCP lowered rapidly in November, reached a lowest level between December and the following February (ca. -18°C), and raised again from March onwards (-7 to -8°C in April). LLT_{50} value was relatively high (ca. -9°C) in October, but

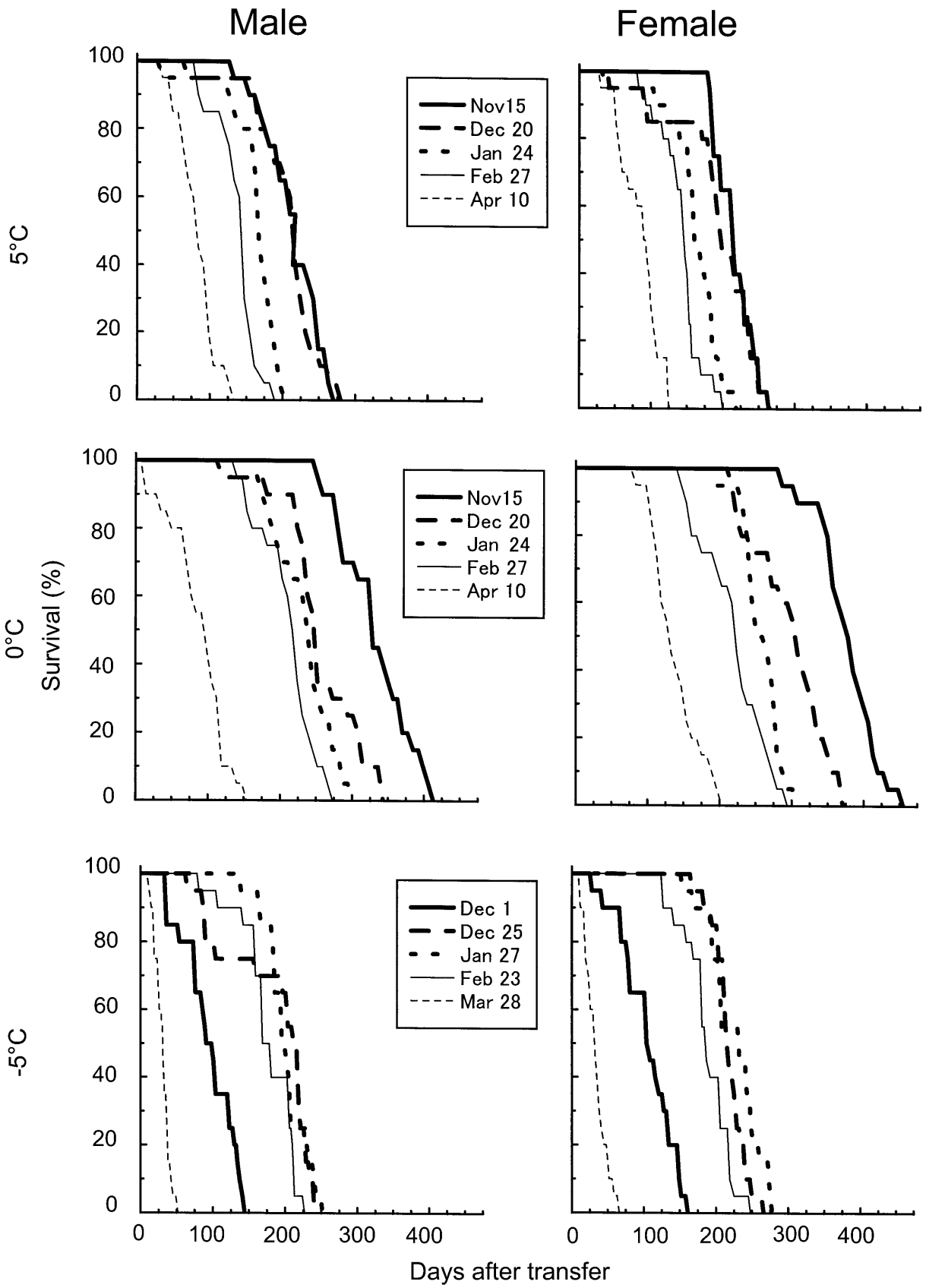

Fig. 3 Seasonal change of survival time at moderately low (non-freezing) temperatures (5, 0, -5°C) in male and female adult *H. axyridis*. Each point shows mean. N = 20 ♂♂♀♀.

lowered to less than -16°C in December and the following February. It rose again in overwintered adults collected in April. Seasonal changes of SCP and LLT_{50} were almost consistent, but a proportion of winter adults

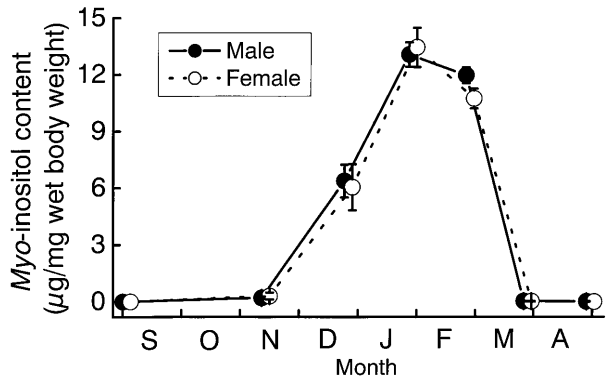

Fig. 4 Seasonal change of *myo*-inositol content in male and female adults of *H. axyridis*. Males, solid line with closed symbols; females, dotted line with open symbols. Each point shows mean ± SE. N = 5 ♂♂♀♀.

appeared to die without being frozen at -20°C; more than 30% of the individuals were not frozen at -20°C from the data of SCP, but none of them survived after exposure to -20°C for 1 day.

Seasonal change of survival time at moderately low (non-freezing) temperatures

The tolerance at moderately low temperatures was evaluated by transferring beetles from outdoors to 5, 0 or -5°C and by determining how long they survived at each temperature. The seasonal pattern of tolerance differed among the temperatures the beetles were exposed to (Fig. 3). In both sexes, the survival time at 5 or 0°C was longest in adults that were swarming in November (ave. ca. 340 or 380 days at 0°C, 220 days at 5°C), and then gradually decreased as the season progressed. The adults survived significantly longer at 0°C than at 5°C at any time of the experimental period except for males in April (*t*-test, $p < 0.05$). On the other hand, the survival time at -5°C was relatively short in the adults that were swarming in early December (ca. 100 days), then it increased rapidly, reaching a maximum between the end of December to the following February (ca. 200 days), and then rapidly shortened again until the end of March (ca. 30 days). The adults were able to stand and move their legs and antennae at 5 or 0°C, whereas at -5°C, they were completely paralyzed irrespective of the date of transfer.

Seasonal changes of polyol and sugar contents

Beetles showed seasonal changes in *myo*-inositol content (Fig. 4). In mid November when the first swarming was observed, the amount of *myo*-inositol was still small (male, 0.2 ± 0.1 μg/mg wet body weight; female, 0.3 ± 0.2 μg) (mean ± SE). It increased rapidly thereafter, reached a maximum level of more than 13 μg (1.3% of the body weight) in the following January, and then declined rapidly in March (male, 0.03 ± 0.01 μg; female, 0.01 ± 0.01 μg). Although trace amounts of glucose and *scyllo*-inositol were detected (< 0.2 μg), no consistent seasonal change was observed.

Seasonal change for *myo*-inositol content (log value) correlated negatively with that for SCP (male, $r^2 = 0.90$, $p < 0.01$; female, $r^2 = 0.80$, $p < 0.05$) and that for LLT_{50} (male, $r^2 = 0.79$, not significant; female, $r^2 = 0.87$, $p < 0.05$), while correlated positively with that for chilling tolerance at -5°C (male, $r^2 = 0.98$, $p < 0.01$; female, $r^2 = 0.94$, $p < 0.01$).

Relationships between *myo*-inositol and water content

I also examined whether the increase of *myo*-inositol content per wet body weight was in part caused by water loss. The water content of overwintering adults was ca. 53% in early December (male, 53.7 ± 0.9%; female, 53.0 ± 0.9%)(n = 20 pairs, mean ± SE), 56% in mid-January when the *myo*-inositol content peaked (male, 56.1 ± 0.9%; female, 56.2 ± 0.8%)(n = 20 pairs) and 57% in late March when the *myo*-inositol had already declined (male, 57.3 ± 0.6%; female, 56.9 ± 0.8%)(n = 20 pairs). This indicated that water loss did not contribute to the elevation of *myo*-inositol content based on the wet body weight.

DISCUSSION

Many freeze-intolerant species change their supercooling ability seasonally: SCP often lowered only during winter (Sømme, 1982). This was also the typical case in adult *H. axyridis*. Although seasonal change of the SCP was almost consistent with that of the lower lethal temperature, there appeared to be considerable mortality without being frozen. Judging from the data for winter ambient temperatures in this study site (minimum, -3.5°C), low temperatures would represent little threat to overwintering adults of *H. axyridis*, and if there were, chilling injury would be more possible than freezing injury.

Survival times at moderately low (non-freezing) temperatures also varied seasonally, but the seasonal trends differed among the temperature conditions exposed; survival time at -5°C peaked in January, the coldest month, but at 5 and 0°C in autumn. One possible explanation for such different seasonal trends is that the survival time at 5 and 0°C is not a good index of the chilling tolerance for this beetle, while that at -5°C could be better. Several lines of evidence support this explanation. First, the beetles were able to move at 5 and 0°C, but were paralyzed completely at -5°C. This suggests that 5 and 0°C do not result in cold stress for the beetles in comparison with -5°C. The temperature of -5°C would result in more cold damage, especially in swarming adults (early December) which have not acquired enhanced cold tolerance yet. Second, although the survival time at 5 and 0°C showed similar seasonal trends, the beetles always survived much longer at 0°C than at 5°C. This may indicate that the mortality at higher temperature is caused by depletion of energy reserves or water rather than by chilling injury. Because the chilling trial was done under relatively humid environments, the survival time at 5 and 0°C seems to represent the degree of starvation tolerance rather than that of cold tolerance. Thus, the set up of low temperatures the insects were exposed to should be chosen carefully when we evaluate chilling tolerance.

Low-molecular weight sugars and polyols including glycerol, trehalose and inositol have been reported as

cryoprotective agents in many species of insects (reviewed in Sømme, 1982; Lee, 1991; Storey & Storey, 1991). These compounds not only provide colligative depression of SCP (reviewed in Zachariassen, 1985; Storey & Storey, 1991) but also may function to stabilize membrane and protein structure at low temperature or low humidity (reviewed in Crowe et al., 1983; Carpenter & Crowe, 1988). Adult *H. axyridis* mainly accumulated *myo*-inositol during the winter. A similar phenomenon is also known in other beetles belonging to the Chrysomelidae and Coccinellidae (Hoshikawa, 1981, 1987; Hoshikawa et al., 1988; Košťál et al., 1996; Watanabe & Tanaka, 1998). In this study, the seasonal change of *myo*-inositol content correlated significantly with the pattern for change of SCP, lower lethal temperature and chilling tolerance at -5°C. *Myo*-inositol may have some role in the control of cold tolerance in this beetle, although it is still unclear how *myo*-inositol acts.

Almost all of the *H. axyridis* adults succeeded in overwintering at the artificial hibernation site in Tsukuba. Judging from the survival time data at -5 to 5°C, the optimum temperature for overwintering would be somewhere between 0 and -5°C. This study also showed that this beetle enhanced their supercooling capacity and cold tolerance at moderate (-5°C) and severe (-10 or -15°C) low temperatures in the approach of winter. Such phenomena were also reported in other freeze-intolerant arthropods such as the apple blossom weevil, *Anthonomus pomorum* (Košťál & Šimek, 1996) and the house spider, *Achaearanea tepidariorum* (Tanaka, 1993). However, in the case of *H. axyridis* at this study site, the minimum ambient temperatures did not decline below -5°C throughout winter (Fig. 1B). Even autumn adults that had not established high cold tolerance yet were able to survive short-term (1 day) and long-term (around 100 day) exposure to -5°C. This would indicate that before the winter season, this beetle has enough cold tolerance to endure winter low temperatures normally encountered in this study site. The following enhancement of cold tolerance may be important for winter survival during exceptionally severe winters in this study area. Furthermore, such regulation would be essential in populations distributed in the northern or higher latitude areas where this beetle could be exposed to more severe low temperatures.

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