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Cold tolerance strategy of the freeze-intolerant chrysomelid, *Aulacophora nigripennis* (Coleoptera: Chrysomelidae), in warm-temperate regions

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Abstract. We investigated the physiological adaptations for winter survival in a freeze-intolerant chrysomelid, *Aulacophora nigripennis*, in warm-temperate regions. The adults showed a decreased supercooling point (SCP), increased chill tolerance and high *myo*-inositol content during winter. Chill tolerance at 0°C appears to be a more suitable indicator of their cold hardiness than SCP because they die at 0°C without freezing and normally are not exposed to subzero temperatures below their SCP (< –4°C). The temporal pattern for changes in chill tolerance correlated closely with that for fluctuations in *myo*-inositol content, indicating that this compound has a role in chill tolerance. Topical application of a juvenile hormone analogue, pyriproxyfen, at the dose which can terminate their diapause decreased both chill tolerance and *myo*-inositol content, suggesting that cold tolerance of this beetle is linked to the diapause program. The thermal response in relation to *myo*-inositol metabolism changed seasonally. This may in part contribute to forming a seasonal profile of *myo*-inositol pool in a natural population. This study provides one model for the cold tolerance strategy of freeze-intolerant insects in relatively mild temperate regions.

INTRODUCTION

Much of the cold hardiness work has been done on insect species from extreme environments (alpine or polar regions) (reviewed in Sømme, 1982; Zachariassen, 1985; Lee, 1991; etc.) Among such insects, freeze-intolerant species depress their supercooling point (SCP) greatly during winter to avoid lethal tissue freezing. The SCP is considered as the lower limit of temperature that a freeze intolerant individual can survive, but many workers also pointed out the importance of time exposure at low temperatures above the SCPs (Payne, 1927; Salt, 1936; Lozina-Lozinskii, 1974; Merivee, 1978; Zachariassen, 1985; Bale, 1993; Sømme, 1996).

The role of low-molecular weight sugars and polyols as cryoprotectants is probably the most extensively studied aspect of insect cold hardiness. In freeze-intolerant species, these compounds not only provide colligative depression of SCP (reviewed in Zachariassen, 1985; Storey & Storey, 1991), but also act to stabilize membrane and protein structure at freezing or non-freezing low temperatures (Crowe et al., 1983; Quinn, 1985; Williams, 1990).

A univoltine chrysomelid, *Aulacophora nigripennis*, is distributed in sub-tropical and warm-temperate regions. The beetle emerges as an adult in mid or late summer, feeds mainly on leaves of *Trichosanthes cucumeroides*, and then leaves the host plant in early-October. The insect overwinters in crevices of stone walls or under bark after a period of swarming (Saito, 1985). In March or April, the adults emerge from the overwintering site and mate before dispersing. This beetle enters diapause in late summer and terminates diapause in February (Watanabe & Tanaka, 1997, 1998b).

At present, there are few studies dealing with cold adaptations of freeze-intolerant insects in warm-temperate regions. Our previous studies showed that *A. nigripennis* is a freeze-intolerant species and accumulates a relatively large amount of *myo*-inositol, a polyol found in relatively few insects during winter (Watanabe & Tanaka, 1997, 1998b). The present paper reviews recent studies to address the following questions in relation to the cold tolerance strategy of this beetle: What is a suitable indicator of cold hardiness of this beetle? What is the possible role of *myo*-inositol for winter survival? Is its cold tolerance strategy linked to the diapause program? How is *myo*-inositol metabolism regulated?

MATERIAL AND METHODS

Overwintered adults were collected from plants of *T. cucumeroides* in July 1996 and June 1997 in Tsukuba (central Japan). Adults that were swarming in early or mid-October 1996 or 1997 were put in plastic containers (diameter 12.9 cm, height 7.2 cm) according to Watanabe & Tanaka (1998b). This outdoor population was kept throughout winter in corrugated carton boxes (26 × 28 × 22.5 cm) in a shaded place on the south side (from 1996 to 1997) and the north side (from 1997 to 1998) veranda of our laboratory. Temperatures at the artificial overwintering sites (south and north) and those in open shade overhung by a concrete wall in a net house were recorded every 30 min by using a thermo recorder (RT-10, Tabai espec Co., Osaka) from August 1996 to April 1997, from October 1997 to April 1998 and from November 1997 to April 1998, respectively.

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The SCPs of adults between August 1996 and April 1997 were determined using the methods 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.

To determine chill tolerance, each of 12 to 28 pairs of adults taken from outdoors between August 1996 and June 1997 was put into a petri dish (diameter 9 cm, height 2 cm) and then buried in crushed ice in a styrene foam container. 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 ice every three days for most samples or every day for August and September samples, and warmed up gradually to the temperature of 15 or 20°C (ca. 0.26°C/min.) in a styrene foam container at room temperature. Survival was judged by their walking activity and only those capable of walking normally were regarded as survivors. After removing dead individuals, dishes containing the survivors were again cooled as mentioned above. This procedure was repeated until all the individuals died. Thus, in the course of the experiment, the experimental beetles were exposed to cyclic warming, 3 to 4 h, every 1 or 3 days. Chill tolerance was expressed as how long they survived at this temperature.

To examine seasonal changes of sugar and polyol contents, adults taken from outdoors to the laboratory between July 1996 and June 1997 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 gas chromatography as described by Watanabe & Tanaka (1997).

To examine the effect of diapause termination on cold hardiness, 1 μl of acetone solution with 5 μg of pyriproxyfen (PPF), a juvenile hormone analogue (JHA), was topically applied on the ventral side of the abdomen of the adults taken from outdoors in 27 January. This treatment induces diapause termination in this beetle (Watanabe & Tanaka, 1998a). The adults were held for 7, 15 or 30 days under the same outdoor conditions after PPF application and then used to determine chill tolerance at 0°C and their polyol contents.

In the experiments to examine seasonal changes in thermal response of polyol metabolism, adults were transferred to the laboratory on 9 October 1997, 27 November, 27 December, 27 January 1998, 27 February or 27 March, and incubated for 15 or 30 days at 5, 10, 15, 20 or 25°C under a 12 L : 12 D (short day) photoperiod or at 0°C under continuous dark conditions. Polyol contents of these individuals were determined by gas chromatography (mentioned above) or high performance liquid chromatography (HPLC) using the following methods: adults were homogenized with 4 ml of 80 % ethanol and 100 μg of erythritol and the homogenate was centrifuged at 3,000 g for 15 min. The supernatant was dried under a stream of nitrogen gas at 60°C. The dried residue was dissolved in 50 μl of distilled water and the solution analyzed after membrane filtration (0.45 μm). The analysis was carried out using HP HPLC system (HP 1050 series, Hewlet Packard) equipped with a guard column (Carbo-c refill cartridge, 30 × 4.6 mm i.d.) and a main column (aminex HPX-87C, Ca^{2+} form, 300 × 8.7 mm i.d.) (Bio-rad Co., California). The main column was submerged in a water bath at 80°C. Evacuated, distilled water was used as solvent at a flow rate of 1.0 ml/min.

RESULTS

The SCP of adults changed seasonally. Adults collected in August had a relatively high SCP with small variance

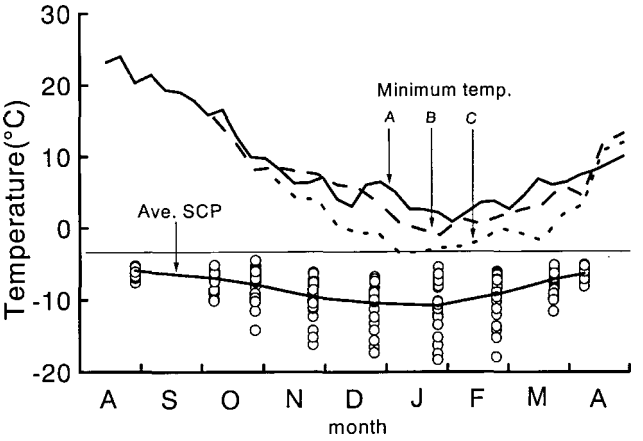

Fig. 1. Seasonal changes of supercooling point (SCP) of *Aulacophora nigripennis* adults (N = 11–15) collected from the field or from outdoors compared with weekly (or every 10 days) minimum temperatures at the three sites. The open circles are individual SCPs. Site A (——) and B (– – –) are the covered shade places on the south (from 1996 to 1997) and north side (from 1997 to 1998) of the veranda of the laboratory, respectively. Site C (- - -) is the open shaded location in a net house (from 1997 to 1998). Data of SCPs from Watanabe & Tanaka (1998b); data of temperatures at site A from Watanabe & Tanaka (1999).

in both sexes (Fig. 1). Their SCPs gradually decreased toward mid-winter and increased again from late February. The variance of SCP for overwintering adults was rather large but most individuals had a relatively high SCP ranging from –6 to –8°C.

The minimum temperatures at the south artificial overwintering site did not decrease below 0°C at any time during winter, whereas the temperatures at the north site sometimes declined below 0°C (Fig. 1). The temperatures in the net house were lower, but did not decline below –3.5°C.

Seasonal changes of chill tolerance are shown in Fig. 2A. Up to September, the beetles died quickly after exposure to 0°C. They gradually became cold-hardy in autumn and a high level of chill tolerance was maintained between November and early February. Their chill tolerance decreased rapidly from early February.

Adults accumulated a relatively large amount of *myo*-inositol (male > 8 μg per mg fw, female > 10 μg per mg fw) (Fig. 2B) during winter with trace amounts of *scyllo*-inositol (< 0.3 μg per mg fw) and glucose (< 0.7 μg per mg fw) (data not shown); in early October, the amount of *myo*-inositol was small, increasing rapidly thereafter and reached a maximum between November and early February, and was lost rapidly until mid-March.

Adults taken from outdoors in January survived for 152.7 days on an average at 0°C (Fig. 3). The diapause-terminated adults by PPF treatment when held outdoors for a further 30 days decreased their chill tolerance (mean 62.8 days) more rapidly than the control ones (140.6 days) (Mann-Whitney U test, $p < 0.01$).

Adults taken from outdoors in January contained a relatively large amount of *myo*-inositol (Fig. 4). The diapause-terminated adults by PPF treatment decreased their content more rapidly than the control ones between

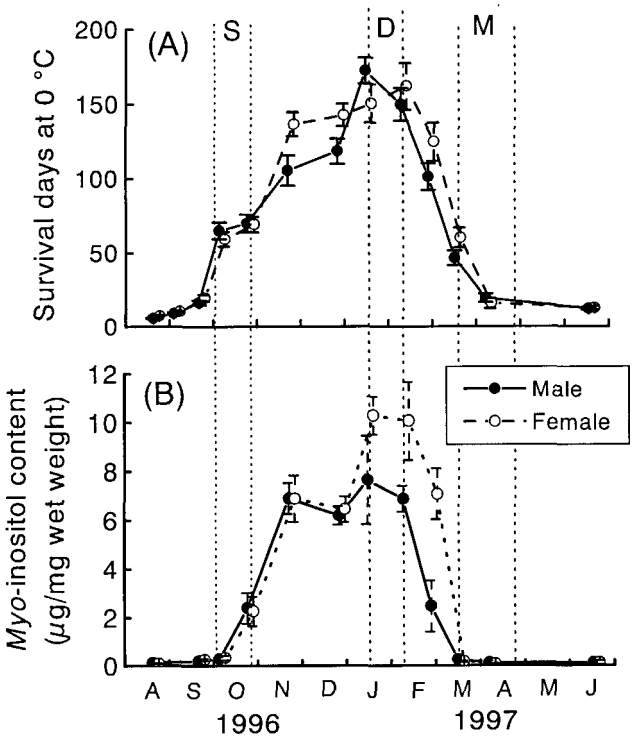

Fig. 2. Seasonal changes of chill tolerance at 0°C (A) and *myo*-inositol content (B) in *Aulacophora nigripennis* adults collected in the field or taken from outdoors between August 1996 and June 1997. Chill tolerance is shown as mean ± SE survival days at 0°C. Each point of *myo*-inositol content is the mean ± SE. Males, solid line with closed symbols; females, broken line with open symbols. Periods of swarming (S), diapause termination (D) and mating (M) are divided by vertical lines. For chill tolerance, N = 12–19 except for adults collected in August (N = 28 pairs); *myo*-inositol, N = 4–5. Data from Watanabe & Tanaka (1998b).

15 and 30 days after the treatment (Mann-Whitney U test,$p < 0.01$). While PPF treatment did not affect the levels of *scyllo*-inositol and glucose so much (data not shown) and both were always small (< 0.3 μg per mg fw) before and after the treatment.

The thermal response of this beetle in relation to *myo*-inositol metabolism changed seasonally (Fig. 5). In October when the adults had only a small amount of *myo*-inositol, the subsequent exposure to any temperature ranging from 0 to 25°C significantly stimulated its accumulation (Mann-Whitney U test, $p < 0.01$). The rate of the accumulation was relatively higher at temperatures between 5 and 20°C, especially at 10°C, whereas it was lowest at 0°C. Adults taken from outdoors in late November further accumulated *myo*-inositol at temperatures between 0 and 20°C for the first 15 days, but thereafter exposure to relatively high temperatures (more than 15°C) caused significant depletion of its content (Mann-Whitney U test, $p < 0.01$). In adults taken from outdoors between late December and late February, the exposure to higher temperatures caused depletion of *myo*-inositol more rapidly, and the rate of depletion became faster as the season progressed. The temperatures of 0 or 5°C did not decrease *myo*-inositol content so far even in midwinter. In late March when the adults already had lost

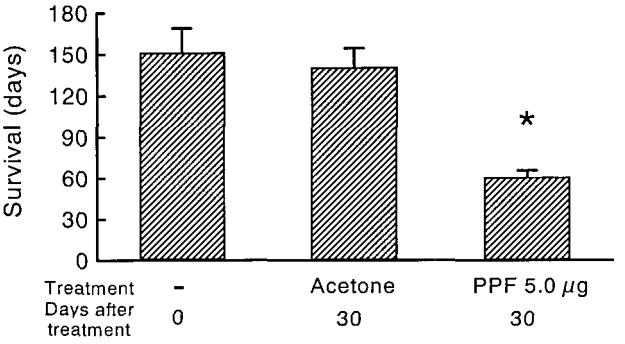

Fig. 3. Effect of pyriproxyfen on chill tolerance at 0°C in overwintering adult females of *Aulacophora nigripennis*. Adults topically applied with 5 μg of pyriproxyfen were held outdoors for a further 30 days, and then transferred to 0°C. The column at day 0 shows the data of the non-treated individuals taken on 27 January. Asterisk shows significant difference ($P < 0.01$) between the PPF-treated and control individuals at the same date. Each column shows mean ± SE (N = 12).

myo-inositol, they accumulated low amounts again at 5°C.

DISCUSSION

What is a suitable indicator of cold hardiness of this beetle?

For freeze-intolerant insects, SCP is considered as the lower limit of temperature that an individual can possibly survive, but they often die at the temperatures above the SCPs (Salt, 1936; Hansen & Kuusik, 1970; Knight et al., 1986; Bale et al., 1988; Clough et al., 1990; Chen et al., 1991). In such species, chilling injury may be a more im-

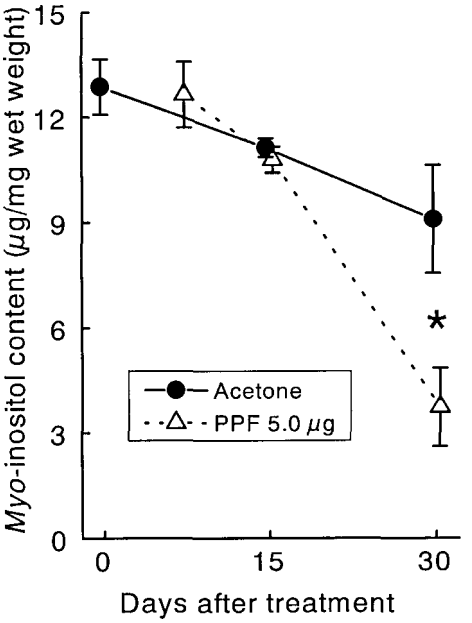

Fig. 4. Effect of pyriproxyfen on *myo*-inositol content in overwintering adult females of *Aulacophora nigripennis*. Adults topically applied with 5 μg of pyriproxyfen were held outdoors for a further 7, 15 or 30 days, and then used for measurements of polyol contents. The point at day 0 shows the data of the non-treated beetles taken on 27 January. Asterisk shows significant difference ($P < 0.01$) between the PPF-treated and control individuals at the same date. Each point shows mean ± SE (N = 5).

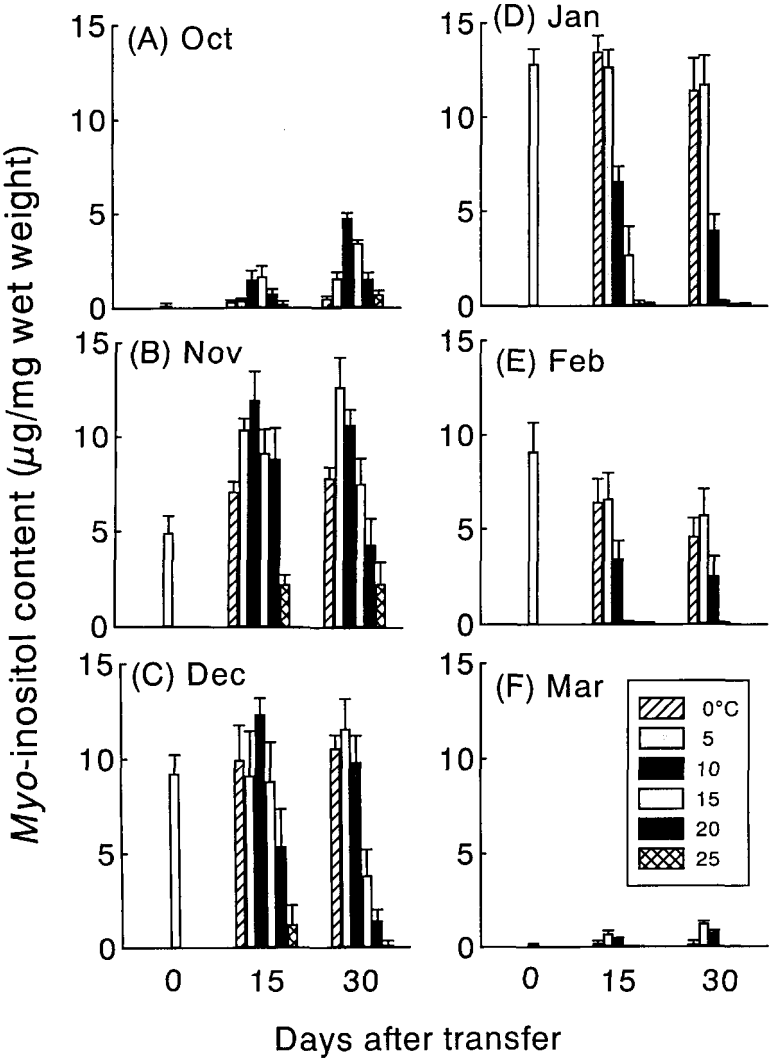

Fig. 5. Seasonal changes of the thermal responses in relation to *myo*-inositol metabolism of adult females of *Aulacophora nigripennis*. Adults collected on 9 October (A), taken from outdoors on 27 November (B), 27 December (C), 27 January (D), 27 February (E) or 27 March (F) were incubated for 15 or 30 days at 0°C without light or at 5, 10, 15, 20 or 25°C and 12L : 12D (SD) photoperiod. The column at Day 0 represents values before incubation. Each column shows mean ± SE (N = 4–5).

portant cause of low temperature mortality than the freezing injury. This may hold true for *A. nigripennis*. Although winter temperatures at the study sites sometimes fell below 0°C, this beetle had SCPs lower than –4°C even in their summer active phase. Moreover, *A. nigripennis* adults normally overwinter under bark or in crevices of stone walls, which provide protection against low air temperature. Under the circumstances, the overwintering adults would rarely be exposed to freezing temperatures.

Even so, overwintering beetles would face chilling temperatures around 0°C in their natural habitats. Such temperatures proved lethal to the summer active beetles; most of them died within a week at 0°C (Fig. 2). Therefore, enhancement of chill tolerance during the approach to winter is essential for winter survival. This means that chill tolerance is probably a suitable indicator of cold tolerance in this beetle.

Lower lethal temperature is often used as an indicator of chill tolerance for freeze-intolerant insects, but in most cases, is determined by the experiments with relatively short-term low temperature exposure (for example 24 h). Under field conditions, however, overwintering insects may be exposed to low temperature for a relatively long period of time. In an ecological context, therefore, evaluation of chill tolerance must be carried out with long-term exposure similar to those that the species normally encounters in the field. In the present study, we evaluated the chill tolerance of this beetle from the survival time at 0°C and found that only individuals collected in winter can survive for relatively long periods of time at that temperature (more than 120 days).

What is the possible role of *myo*-inositol for cold hardiness?

Accumulation of low molecular weight carbohydrates has been correlated with an increase in insect supercooling ability and chill tolerance (Lee, 1991). One of the possible functions of such compounds in insect cold hardiness is the colligative effect on supercooling ability. It is believed that large polyol contents provide depression of the SCP (Zachariassen, 1985; Storey & Storey, 1991).

Adults of *A. nigripennis* accumulate *myo*-inositol at ca. 1% of body weight during winter and its accumulation is correlated with depression of the SCP (R^2 = 0.572 in males and females from October to March). This concentration, however, is much lower than that (> 10%) at which polyol can depress the freezing point in an aqueous system (MacKenzie, 1977). Furthermore, even during mid-winter when all adults accumulate a large *myo*-inositol content, individuals with a relatively high SCP of about –7°C are dominant in the overwintering population. This suggests that SCP depression is not directly caused by the colligative effect of *myo*-inositol. The SCP depression in early-winter may be explained partly by exclusion of gut contents which may contain ice nucleating agents because this beetle stops feeding after swarming (Saito, 1985). Thus, *myo*-inositol would not affect the supercooling ability of this beetle.

Polyols including inositol may also function to stabilize membrane and protein structure at freezing or non-freezing low temperature (Crowe et al., 1983; Quinn, 1985; Williams, 1990), or serve as a salt or desiccation stress protectant (Bohnert et al., 1995; Sheveleva et al., 1997). In a natural population of *A. nigripennis*, the acquisition of chill tolerance correlated closely with *myo*-inositol levels during winter (R^2 = 0.925 in males and females from October to March); adults have a large amount of *myo*-inositol between November and early February, only when they also have high chill tolerance. Moreover, topical application of JHA to overwintering beetles decreased both chill tolerance and *myo*-inositol content (Figs 3, 4). These observations strongly suggest that *myo*-inositol has some role in the chill tolerance of this beetle. However, it is still unknown how *myo*-inositol acts to enhance chill tolerance in this beetle.

Is the cold tolerance strategy linked to the diapause program?

Both cold hardiness and the related polyol accumulation are closely linked to the diapause syndrome in *Bombyx mori* (Chino, 1958), *Hyalophora cecropia* (Wyatt & Meyer, 1959) and *Sarcophaga crassipalpis* (Lee et al., 1987). In this study, topical application of JHA at a level which can terminate diapause stimulates both loss of chill tolerance and reduction of *myo*-inositol content in overwintering adults (Figs 3, 4). Also, in pre-overwintering adults, JHA treatment suppressed both enhancement of chill tolerance and *myo*-inositol accumulation (unpublished data). These suggest that the two variables related to cold tolerance are at least under the control of the same endocrine system of JH, although it is not clear if JH regulates cold hardiness directly or indirectly through diapause termination in this beetle. In contrast to this study, JH or JHA treatment stimulates polyol production in species with larval or pupal diapause (Tsumuki & Kanehisa, 1981; Lee et al., 1988; Pullin & Bale, 1989). The difference seems to be on account of the stage specific action of JH in relation to diapause; JH would act to maintain diapause in species with larval or pupal diapause, whereas it would act to break diapause in species with adult diapause such as *A. nigripennis*. Ecdysone injection into *Chilo suppressalis* larvae (Tsumuki & Kanehisa, 1981) or *Pieris brassicae* pupae (Pullin & Bale, 1989) or HCl treatment to *B. mori* eggs (Yaginuma & Yamashita, 1977) also terminates diapause and decreases polyol contents. Thus, diapause termination seems to stimulate polyol catabolism regardless of diapause stage or the kind of hormone applied. In a natural population of *A. nigripennis*, both chill tolerance and *myo*-inositol content seem to decline rapidly after the time of diapause termination (Fig. 2). Therefore, the decreases of chill tolerance and *myo*-inositol content by JHA treatment would be an indirect effect of the accompanying diapause termination than the direct action of JH. Thus, the cold tolerance of this beetle appears to be linked to the diapause program similar to adults of *Pyrrhocoris apterus* (Hodková & Hodek, 1994).

How is *myo*-inositol metabolism regulated?

In many species of insects, polyol metabolism is regulated not only hormonally, but also environmentally (reviewed in Storey & Storey, 1991). It is generally known that polyol synthesis is accelerated by low temperatures (Hayakawa & Chino, 1981; Storey & Storey, 1983; Nordin et al., 1984) and the trigger temperature with maximal rate of synthesis is typically in the 0 to 5°C range (Storey & Storey, 1988). In *A. nigripennis*, the thermal responses of the overwintering beetles in *myo*-inositol metabolism changed seasonally. In October and November, the adults accumulate *myo*-inositol at a wide range of temperatures between 0 and 25°C, but only low temperature (< 10°C) was effective for its accumulation in December. In February, the *myo*-inositol pool was decreased irrespective of temperatures exposed. *Myo*-inositol metabolism of this beetle thus gradually shifts from synthesis to degradation as the season progresses. This may be related to the progress of diapause development and diapause termination.

In a natural population, the *myo*-inositol content of *A. nigripennis* began to increase in October, reached a high level between November and January and decreased rapidly in February (Fig. 2B). This seasonal profile would be produced in part by the seasonal changes in thermal response mentioned above (Fig. 5; Watanabe & Tanaka, 1999). October adults accumulated *myo*-inositol even at relatively high temperatures of 15 to 25°C (Fig. 5A). At this time, *myo*-inositol metabolism may be in a synthesis phase and the adults generate the accumulation of *myo*-inositol efficiently, despite ambient temperature fluctuations. Because high temperature generally causes degradation of polyol and sugar contents (Hayakawa & Chino, 1981; Pio & Baust, 1988; Churchill & Storey, 1989), the observed thermal response in this beetle would save energy reserves by inhibiting energetically expensive cyclic synthesis and degradation of polyols, which are caused by temperature fluctuations (Churchill & Storey, 1989). In addition, because *myo*-inositol correlates with chill tolerance in this species (Fig. 2A, B), this response, in which this beetle accumulates *myo*-inositol at 10 or 15°C more than at 5°C (Fig. 5A), would be adaptive so as to ensure *myo*-inositol accumulation before winter. Around mid-December, the thermal response in relation

to *myo*-inositol metabolism in this beetle changed drastically: only low temperatures of 0 to 10°C were effective in maintaining or increasing *myo*-inositol content (Fig. 5B). This means that the *myo*-inositol pool might be catabolized when the insects were exposed to higher temperatures. At this study site, the ambient temperatures rarely reached to 10°C after mid-December (data not shown), so that the *myo*-inositol content in the natural population in fact remained high during December and January (Fig. 2B). On the other hand, February adults degraded *myo*-inositol irrespective of the ambient temperature exposure (Fig. 5E). At this time, the enzymatic machinery geared to *myo*-inositol catabolism would be activated. Under the circumstances, *myo*-inositol content in the natural population decreased rapidly in February despite the relatively low ambient temperatures and was lost until March (Fig. 2B). The missing *myo*-inositol is not recovered to glycogen but may be used as a metabolic fuel in relation to post-diapause development (Watanabe & Tanaka, 1998b).

CONCLUSION

In this study, we have demonstrated at least in part the physiological adaptations for winter survival of *A. nigripennis* in relatively warm-temperate regions; the adults can survive winter not by increased supercooling ability but by enhancement of chill tolerance. *Myo*-inositol accumulation seems to be related rather to enhancement of chill tolerance than to SCP decrease. Control of chill tolerance and *myo*-inositol are likely to be linked to the diapause program. *Myo*-inositol metabolism is also regulated by temperature conditions. The thermal response in relation to *myo*-inositol metabolism changed seasonally. This may contribute to forming the seasonal profile of the *myo*-inositol pool efficiently in a natural population. This study supports one of the models of cold tolerance strategy in freeze-intolerant insects in relatively warm-temperate regions.

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