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Source: Zoological Science, 14(2) : 233-237

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.233

ZOOLOGICAL SCIENCE 14: 233–237 (1997)

Photoperiodic Control of Adult Diapause, Cold Hardiness, and Inositol Accumulation in a Beetle, *Aulacophora nigripennis* (Coleoptera, Chrysomelidae)

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ABSTRACT—Adults of *Aulacophora nigripennis* terminated diapause when transferred from outdoor conditions to a long photoperiod before February but remained in diapause when transferred to a short photoperiod. Both mating and vitellogenesis occurred at either photoperiod in adults examined in February or later. The supercooling point (SCP) of adults was about -6°C in summer and -10 to -11°C in late autumn and winter. Summer adults rapidly decreased the SCP to about -16°C after deprivation of food for 4 days at 25°C, suggesting that the gut contents play an important role in lowering the SCP. Among the cryoprotectants known in insects, only *myo*-inositol was found accumulated in overwintering adults of *A. nigripennis*. Female adults accumulated more *myo*-inositol than males in February. An exposure of overwintering beetles to 25°C caused a rapid decrease in *myo*-inositol level even at a short photoperiod. This indicated that the reduction in inositol may be independent of the termination of diapause.

INTRODUCTION

Insects have evolved various adaptations to survive the adverse seasons. Many species of insects distributed in the temperate region enter diapause before winter, reduce the loss of their reserves by lowering their metabolism and enhance cold hardiness. They have been classified into two major categories in terms of cold hardiness strategy. One includes freeze-tolerant species, which can survive ice formation in their body tissues and fluids, and their supercooling point (SCP), the spontaneous freezing temperature, tends to show no conspicuous seasonal change (Lee, 1991). The other category includes freeze-intolerant insects in which the SCP declines greatly before the onset of winter. Their supercooling capacity is influenced by a number of factors: ice nucleating proteins, ice nucleating bacteria, antifreeze proteins, inoculative freezing, gut contents, low-molecular-weight antifreezes and so on (Lee, 1991).

One of the most striking characteristics of overwintering insects is their capacity to accumulate high concentrations of low-molecular-weight polyols and sugars such as glycerol, sorbitol, mannitol, ethylene glycol, ribitol, erythritol, inositol, fructose, trehalose and glucose (Somme, 1982; Lee, 1991). Among them, glycerol is the most common low-molecular-weight cryoprotectant in overwintering insects (Somme, 1982). On the other hand, there are only a few reports of sole accumulation of inositol (Hoshikawa, 1981, 1987; Hoshikawa *et al.*, 1988). However, we know relatively little about the relationships between inositol accumulation and cold hardiness, and the mechanisms controlling these phenomena.

Aulacophora nigripennis is a univoltine chrysomelid known as a pest of gourds and carnations. According to Saito (1985), it emerges as an adult in July to August, and feeds actively on leaves of gourds, particularly *Trichosantbes cucumerides*. Adults leave the host plants in October, swarm for a while, and then move to their overwintering sites such as crevices of stone walls. In April, they swarm again around the overwintering sites, mate actively and then disperse. Though, their life cycle is well-studied, there is no report about their cold hardiness and the mechanism controlling diapause.

The main purpose of our study is to understand the overwintering strategy of *A. nigripennis*. We investigate seasonal changes in the photoperiodic response controlling imaginal diapause and in SCP during winter. We also present evidence of *myo*-inositol accumulation in their body during winter.

MATERIALS AND METHODS

A. nigripennis adults were collected in Tsukuba, Ibaraki in August to October and in Ootaki, Chiba in April in 1995 and 1996. In August, feeding adults were found on a host plant, *Trichosantbes cucumerides*. About 20 swarming adults collected in October were placed in a plastic container (dia.12.9 cm, height 7.2 cm) under outdoor conditions during

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winter and given only water. The lid of the container was perforated and covered with nylon mesh for ventilation. These beetles were regarded as overwintering adults and collected regularly from October to the following March. Overwintered adults aggregating on walls or under roof eaves of buildings were collected in April.

A pair of adults sampled on various dates were placed with squash leaves in a 300 ml plastic cup at 12-hr light/12-hr dark (short day) or 16-hr light/8-hr dark (long day) and 25°C. Adults in diapause are usually characterized by reduced ovarian development in females (Danks, 1987) and suppressed mating activity in males (Solbreck, 1972). We used these characteristics to judge if insects were in diapause or not. They were checked at least twice a day until the first mating was seen. Mating usually lasted longer than 12 hr. Females were dissected 20 or 50 days after a transfer to the laboratory to determine if vitellogenesis in the ovary occurred or not.

Total lipid content was measured as follows. Insects were dried in an oven at 80°C for 12 hr, and then kept in 1.5 ml of ether for 3 days to extract lipids [a method of Tanaka and Okuda (1996) with minor changes]. The ether solution was changed once during that period. Wet weight, dry weight and lean dry weight were measured for each individual.

The supercooling point (SCP) was measured by the method of Morimoto (1991) with minor changes. The tip of a copper-constantan thermocouple was attached to the dosal abdomen of an adult with rubber bond. The cooling rate was about 1°C/min. SCP determinations were made for feeding adults collected in the field in August and the following May, and non-feeding ones collected in October and kept under outdoor conditions between October to the following April. Adults in May were given squash leaves for about 1 month.

Low-molecular-weight carbohydrates and polyols were detected by gas chromatography as described by Shimada *et al.* (1984). Insects were homogenized individually with 4 ml of 80% ethanol and 100 μg of erythritol added as an internal standard. Then the homogenate was centrifuged at 3000 × g for 10 min and the supernatant was evaporated completely in a stream of N_2 gas at 59.5°C. To the residue, 0.05 ml trimethylsilylating reagent (TMSI-C, GL Sciences Inc., Tokyo) was added and the solution heated at 65°C for 40 min. The resulting derivatives were injected to a gas chromatograph (Shimazu, Kyoto, GC-4CMPF) with a grass column (3 mm × 3 m) containing 1.5% OV-1. The column was heated from 130 to 270°C at 5°C/min and kept at the last temperature for 10 min. Compounds were identified from retention time of a standard mixture of known carbohydrates.

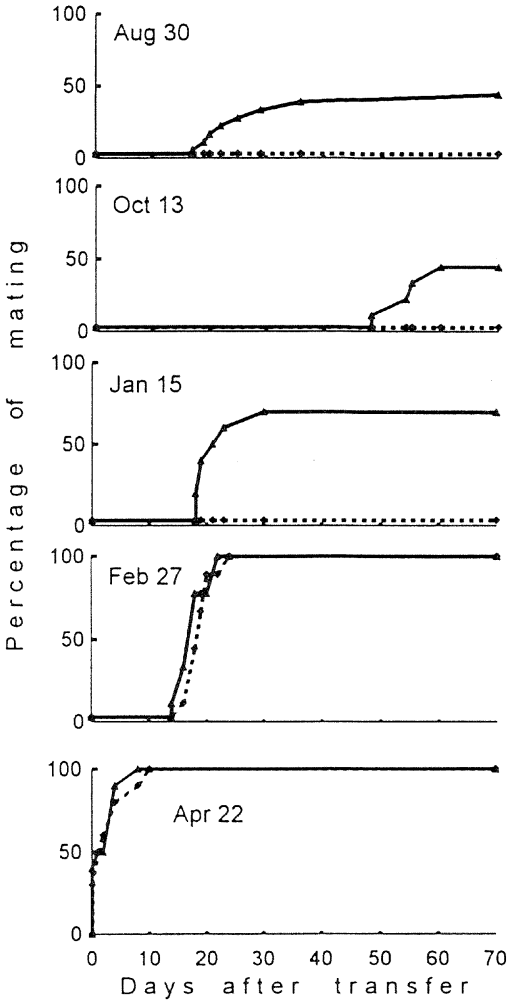

Fig. 1. Pre-mating period of field-collected *A. nigripennis* male adults at 12-hr light/12-hr dark (◆ - - ◆) or 16-hr light/8-hr dark (▲ — ▲) photoperiod and 25°C (N=9-20).

RESULTS

Photoperiodic control of diapause

Adults were collected in the field at various times of the year and exposed to short day or long day conditions and 25°C to examine their seasonal changes of the photoperiodic response controlling diapause. Mating behavior (Fig. 1) and ovarian development in females (Fig. 2) were observed only at a long day photoperiod until January. About a half of adults collected in August mated within 35 days, while the other half apparently entered diapause because they did not mate for 70 days (Fig. 1) and the lipid content increased from 14.9% (N=25) in August to 38.2% (N=20) on the 70th day of transfer. Reproductively active males and females collected in July contained 13.7% of lipid (N=16). Adults collected in October and January started mating after the 48th day and 19th day of transfer, respectively. Adults collected in February onward mated even under short day conditions. This may indicate that male adults had terminated diapause by February. The females collected in January showed a long day type of photoperiodic response, but those collected in April underwent vitellogenesis either at a long or a short photoperiod. They mated actively in April, but vitellogenesis did not occur when they were given only water for 1 month (N=5).

Supercooling point

To examine the cold tolerance of overwintering adults, the SCP was determined for adults collected between August and the following May. None of them survived freezing. Feeding adults collected in August or May had relatively high SCPs with a small variance (mean ± SD, -6.52 ± 1.06°C in August, -6.06 ± 0.79°C in May) (Fig. 3). The mean SCP showed a conspicuous seasonal fluctuation. It dropped by about 4°C from August to November and remained at the low level (ca. -11°C) through January. In February onward, it rose gradually and finally returned to the previous high level in April. Adults checked in winter (November to the following March) had low SCPs compared with those collected in August (Mann-Whitney U test, $p < 0.001$), and their SCPs had a large variance among individuals (for example, -10.64 ± 3.71°C in November, -10.89

± 3.74°C in January) (mean ± SD).

We measured the SCPs of actively feeding and starved adults to test if the reduced SCP of adults observed during winter was due to exclusion of their gut contents. The SCPs of actively feeding adults collected in August decreased quickly after deprivation of food for 1 or 4 days at a long day photoperiod and 25°C (1day deprivation: -12.41 ± 1.56°C; 4 days: -15.67 ± 1.39°C) (mean ± SD), and the variance was relatively small.

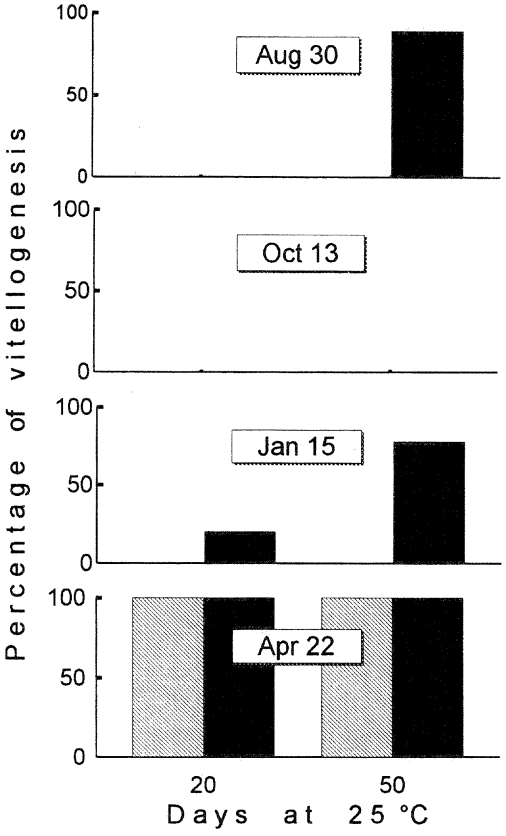

Fig. 2. Vitellogenesis occurring in the ovary after field-collected *A. nigripennis* females were reared for 20 or 50 days at 12-hr light/12-hr dark (slash column) or 16-hr light/8-hr dark (black column) photoperiod and 25°C (N=10-12).

Polyol and sugar contents

The gas chromatography assays of whole body extracts indicated at least three kinds of compounds which were identified as *myo*-inositol, *scyllo*-inositol and glucose. Tables 1 and 2 show changes in polyol and sugar contents in field-collected adults and in those exposed to a low temperature in the laboratory. A large quantity of *myo*-inositol was found in adults measured in February, and its level decreased until April (Table 1). Those individuals collected in October and exposed to a short day photoperiod and 15°C for 3 months had also a large amount of *myo*-inositol (Table 2). The amount of *myo*-inositol decreased significantly when such individuals were exposed to 25°C for 2 weeks either at a long or short photoperiod. Neither mating behavior nor vitellogenesis occurred even for more than 1.5 month under a short day photoperiod and 25°C. This suggests that they were still in diapause. Females tended to have a larger amount of *myo*-inositol than males.

DISCUSSION

Winter diapause is often induced by a short day photoperiod and is accompanied by lipid accumulation (Danks, 1987) and *A. nigripennis* appears to follow such a rule. The intensity of diapause is known to change as diapause development proceeds, normally decreasing in individuals collected later in the diapause period (Tauber and Tauber, 1976). *A. nigripennis*, however, shows some extent different seasonal trends. Number of days to start mating or vitellogenesis at a long day photoperiod, a criterion of diapause intensity, first increased from August to October and then decreased by spring (Figs. 1 and 2). This may be in part due to the fact that a half of males and most of females collected in August have not entered diapause yet. In many species, diapause development is completed in the field before the winter solstice, so that the subsequently lengthening days have no effect on diapause (Danks, 1987). The present data suggest that adults of *A. nigripennis* do not complete diapause development before February. During the period from February to April, mating occurs any time if they were transferred to

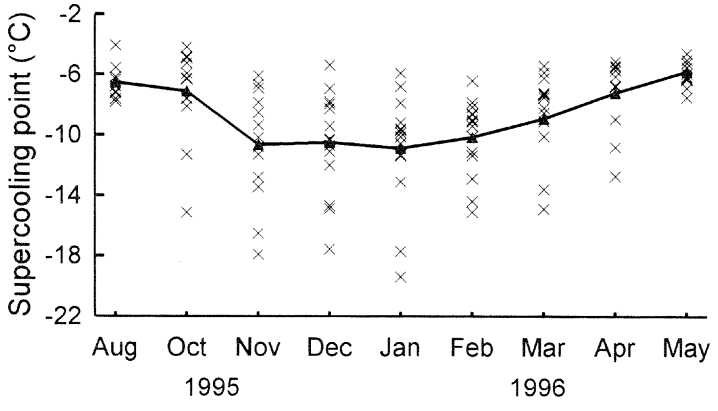

Fig. 3. Seasonal changes of supercooling points in *A. nigripennis* (× - individual value, ▲ - mean value).

Table 1. Polyol and sugar contents of field-collected *A.nigripennis* adults[a]

Collected date	*Myo*-inositol		*Scyllo*-inositol		Glucose	
	♂	♀	♂	♀	♂	♀
Feb 7	1.75 ± 0.88	5.60 ± 0.63	0.05 ± 0.05	0.06 ± 0.02	0.27 ± 0.17	0.22 ± 0.17
Apr 3	0.16 ± 0.06	0.19 ± 0.11	0.16 ± 0.05	0.75 ± 1.25	0.18 ± 0.07	0.17 ± 0.11

[a] Mean ± SD (μg/mg wet weight), N= 3 or 4.

Table 2. Effects of transfer from 12-hr light/12-hr dark photoperiod and 15°C (12L12D, 15°C) to diapause-maintaining (12L12D, 25°C) or breaking (16L8D, 25°C) conditions on polyol and sugar contents of *A.nigripennis* diapause adults[a]

	Myo-inositol		*Scyllo*-inositol		Glucose	
	♂	♀	♂	♀	♂	♀
12L12D, 15°C[b]	3.95 ± 2.79	6.28 ± 2.84	0.04 ± 0.04	0.06 ± 0.05	0.10 ± 0.07	0.18 ± 0.04
Transfer from 12L12D, 15°C to[c]						
16L8D, 25°C	0.12 ± 0.04	0.18 ± 0.18	0.01 ± 0.01	0.04 ± 0.05	0.18 ± 0.16	0.34 ± 0.19
12L12D, 25°C	0.07 ± 0.02	0.20 ± 0.10	0.04 ± 0.04	0.06 ± 0.05	0.10 ± 0.07	0.18 ± 0.04

[a] Mean ± SD (μg/mg wet weight), N=3~8.
[b] Adults were collected in October and stored at 12L12D and 15°C for 3 months without food.
[c] They were given squash leaves for 2 weeks before measurements.

warm conditions, indicating that male adults may complete diapause development by February.

In freeze-intolerant species, SCPs have been used as an index of cold hardiness. Many insect species decrease the risk of freezing by enhancing their supercooling ability during winter (Lee, 1991). *A. nigripennis* decreased its SCP by ca. 4°C on average between November and February, though the variance within the population was relatively large as reported in several other insects (Block, 1982; Cannon and Block, 1988; Morimoto, 1991). Deprivation of food for 4 days at 25°C induced a 9°C decrease in SCP, and the subsequent feeding raised the SCP to the original level (data not shown). This observation suggests that the gut contents influence the SCP greatly as reported for many other insects such as *Agrotis orthogonia*, *Megachile rotundata* and *Pieris brassicae* (Salt, 1953; Krunic and Radovic, 1974; Parish and Bale, 1990 etc.). In the field, adults may not empty their gut completely and thus show large variance in SCPs in winter. However, it should be noted that adults collected in March or April had relatively high SCPs even though they had not been fed. Therefore, the physiological basis for the control of SCP remains unsolved. Some insects such as aphids and the house fly are known to die at a temperature much above their SCP (Bale *et al.*, 1988; Coulson and Bale, 1990). Therefore, it is important to examine the relationship between the SCP and lower lethal temperature in *A. nigripennis*.

The synthesis of cryoprotectants typically begins in early autumn in many species of insects. The maximal levels of such compounds are maintained over midwinter and disappear as spring comes (Storey and Storey, 1991). The synthesis of polyols and sugars is induced by low temperature (Hayakawa and Chino, 1981; Baust, 1982; Storey and Storey, 1983 etc.) and often related to the diapause state (Chino, 1957; Lee *et al.*, 1987; Pullin and Bale, 1989; Tanaka, 1995). *Myo*-inositol is the only carbohydrate accumulated in *A. nigripennis* adults. A similar situation is known in a few other overwintering beetles including two Chrysomelids (*Paridea angulicollis* and *Gastrophysa atrocyanea*) and five Coccinellids (*Henosepilachna pustulosa*, *Eocaria muiri*, *Propylea japonica*, *Halyzia sedecimguttata* and *Epilachna vigintioctomaculata*) (Hoshikawa, 1981, 1987; Hoshikawa *et al.*, 1988). The inositol content in *A. nigripennis* tended to be higher in females than in males. A transfer of field-collected adults to 25°C caused disappearance of *myo*-inositol from their body quickly, though they were still in diapause. Thus, the reduction in inositol is independent of the termination of diapause.

The present study has provided only one aspect of cold hardiness for *A. nigripennis*. More information is necessary for better understanding of the overwintering strategy of this beetle. Such information includes temporal variation and sexual differences in inositol accumulation and their relations to the dynamics of diapause, SCPs and lower thermal limit for survival.

ACKNOWLEDGMENTS

We thank Hiroko Ikeda, Noriko Kenmochi and Sumi Ogawa for rearing insects, Nobuo Morimoto for letting us use a device for SCP measurements and Toru Arakawa for collecting insects. Many thanks are due to Seiji Tanaka for critical reading of the manuscript and helpful advice and Toyomi Kotaki for many useful suggestions.

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(Received November 14, 1996 / Accepted January 7, 1997)