ZOOLOGICAL SCIENCE 14: 849–853 (1997)

Winter Survival and Freeze Tolerance in a Northern Cockroach, *Periplaneta japonica* (Blattidae: Dictyoptera)

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ABSTRACT—Cold tolerance of overwintering nymphs of a cockroach, *Periplaneta japonica*, was examined in terms of the supercooling capacity and lower lethal temperature. The supercooling point of overwintering nymphs fell in a relatively narrow range of temperature from −6 to −9°C and no correlation was observed between the supercooling point and body size. In the temperature range from −5 to −8°C, a significant proportion of cockroaches could tolerate a 12 hr period of tissue freezing. The freeze tolerance capacity differed between nymphal instars, but the supercooling capacity was similar for all nymphs. In a freezing trial at −6 and −7°C, none of the first instar nymphs recovered after tissue freezing, whereas many mid (from 3rd to 5th) and final (8th) instar nymphs survived freezing. Glucose, *myo*-inositol, *scyllo*-inositol and trehalose were found in overwintering nymphs, but neither the array nor the content except for trehalose differed among the nymphal instars. Unexpectedly, the concentration of trehalose was negatively correlated to freeze tolerance. Winter survival of this cockroach may be based on both the freeze tolerance and microhabitat selection.

INTRODUCTION

Periplaneta japonica Karny occurs in cool-temperate regions and is common in northern Japan. It is semivoltine and overwinters twice as a nymph in northern areas (Tsuji and Mizuno, 1972; Shindo and Masaki, 1995), but partly univoltine in a southern population (Tanaka and Uemura, 1996). Nymphal development is under the control of photoperiod and temperature, and all nymphal instars except for the first one have an ability to enter diapause (Tsuji and Mizuno, 1972; Shindo and Masaki, 1995), although this species may overwinter in any nymphal instar including the first one (Tanaka and Uemura, 1996).

Overwintering nymphs of *P. japonica* are found in decayed wood material or under the bark of trees (Tabaru and Kobayashi, 1971; Tsuji and Tabaru, 1974; Shindo and Masaki, 1995; Tanaka and Uemura, 1996). Since such hibernacula are not always covered by insulative snow (K.Tanaka, unpublished observations), overwintering individuals may encounter a subzero temperature. Thus, it seems essential for this cockroach to have cold tolerance for successful overwintering.

Although *P. japonica* is the most northern species of all Japanese cockroaches, its cold hardiness has received relatively little attention. Tsuji and Mizuno (1973) assessed the cold hardiness of last instar nymphs and adults to test their possible overwintering success under outdoor conditions in central Japan. In their study, however, the lowest temperature used was +5.5°C and no investigation was carried out at subzero temperatures. The purpose of the present study is to examine the cold tolerance of this cockroach in more detail, particularly whether or not they can survive freezing. Here, we determined the supercooling point (SCP), the temperature at which spontaneous freezing occurs, and the lower lethal temperature of nymphs overwintering in Tsukuba, central Japan.

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MATERIALS AND METHODS

Insects

Our stock cultures of *P. japonica* used for the present study originated from adults and nymphs collected in an oak forest of the Toyosato Park, Ibaraki in June 1993. They were reared in plastic containers (20 × 35 × 26 cm) and were fed insect feed (Oriental Yeast Co. Tokyo, Japan) and slices of carrot by the method of Shindo and Masaki (1995). Newly hatched nymphs were placed on a veranda facing north on the 3rd floor of a building of NISES, Tsukuba, Japan on different dates from spring to autumn in 1995. Nymphs encountered winter at different instars, depending on the date of hatching: the later the time of hatching the earlier the nymphal instar in winter (Tanaka and Uemura, 1996). In the present study, cockroaches in January and February were regarded as overwintering individuals.

Supercooling point (SCP)

To determine the SCP, cockroaches were individually put into a test tube (10 mm diam. × 40 mm height) in which the specimen was

in contact with the tip of a thermocouple connected to a computer (PC-9801F). The test tube was covered twofold with a glass vial (30 mm diam. × 65 mm height) and a plastic vial (60 mm diam. × 100 mm height) to reduce the cooling rate to ca. 1°C/min when placed in a freezer (ca. −24°C). The SCP was determined by a release of the latent heat due to ice formation within the body of the test animal. The same method was used to determine the SCP of haemolymph. 1 µl of haemolymph was collected in a glass capillary (Drummound 5 µl) by cutting a leg of the cockroaches. The glass capillary was then attached to the thermocouple to record the SCP of haemolymph.

Freeze tolerance

To assess the lower lethal temperature, test animals were cooled at 1°C/min to a certain temperature within the range from −5 to −12°C. When body temperature of the test animals reached the desired level, they were removed from the freezer. Both frozen and non-frozen individuals were transferred to 25°C and supplied with food for 30 days. Survival was assessed by observing their walking activity or moulting during this period; only those which showed normal walking or moulting to the next nymphal instar or the adult stage were regarded as survivors. To assess the long-term freeze tolerance, the test animals were cooled at 1°C/min to a certain temperature ranging from −5 to −8°C and kept at that temperature for 12 hr. After the freezing trials, the test specimens were transferred to 25°C and supplied with food for 30 days. Their survival was assessed as described above. In addition, subsequent reproductive capability of some females that had been frozen at either −6 or −7°C as final instar nymphs was monitored. During the 12 hr freezing trials, the body temperature of test animals was individually monitored to determine when freezing occurred. To compare the freeze tolerance among nymphal instars, first (1st), mid (from 3rd to 5th) and final (8th) instar nymphs were applied to 12 hr freezing trials.

Sugar and polyol contents

Cockroaches were homogenized individually with 4 ml of 80% ethanol in a glass homogenizer and then 1 mg of erythritol was added as an internal standard according to the method of Shimada *et al.* (1984). The homogenate was centrifuged at 3, 000 × g for 15 min and the supernatant evaporated in a stream of N_2 gas at 59.5°C until dry. To the residue, 0.05 ml of trimethylsilylating reagent (TMSI-C, GL Science Inc., Tokyo) was added and the solution heated at 65°C for 40 min. The resulting derivative was injected into a gas chromatograph (GC-4CMPF, Shimadzu, Kyoto) with a glass column (3 mm × 3 m) containing 1.5% OV-1. The column was heated from 130 to 270°C at 5°C/min and then kept at the final temperature for 10 min. Compounds were identified based on the retention time of standard mixtures of known carbohydrates.

RESULTS

Thermal conditions

Ambient temperatures recorded at a research plot of NISES during the study period are given in Table 1. The lowest temperature recorded in the winter of 1995–96 was −8.5°C on 22 February. Daily maximum temperature was always above 0°C, thus indicating that subzero temperatures never lasted 24 hr.

Supercooling points

Figure 1 plots the SCPs of overwintering nymphs against their body weight. In spite of the large variation in body weight (range: 3.7–589.8 mg), the SCPs fell in a relatively narrow range of temperature from −6 to −9°C and no significant correlation was observed between SCPs and body weight (r^2 = 0.011, P>0.05). Thus, the SCP of this cockroach was not influenced by the nymphal stage. A comparison between SCPs of the whole animals and haemolymph indicated that the SCP was about 16 deg lower for the haemolymph (−23.1 ± 1.8°C [mean ± s.d.], n = 7) than for the whole cockroach (−7.4 ± 0.5°C, n = 6) (t-test, t = 19.1, P<0.01).

Table 1. A summary of temperature records at a research plot of NISES, Tsukuba in the winter of 1995–96

Month	Minimum temperature (°C)	Maximum temperautre (°C)	Mean temperature (°C)	No. of frost days*
September	11.5	31.2	20.9	0
October	4.5	26.0	16.8	0
November	−2.5	22.3	7.0	6
December	−7.6	15.9	4.2	28
January	−6.9	11.2	3.4	25
February	−8.5	21.8	2.8	23

* The day on which minimum temperature fell below 0°C.

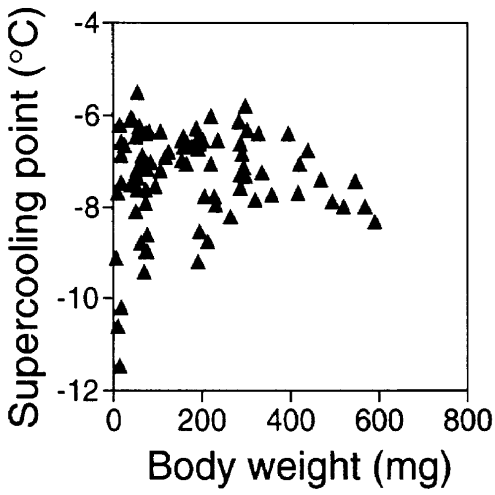

Fig. 1. Effect of body size (wet weight) on individual supercooling points of *P. japonica* nymphs in January and February.

Freeze tolerance

The proportion of cockroaches which froze increased as the temperature decreased (Fig. 2). In this experiment, a frozen individual was first obtained at −6°C and all individuals were frozen at −9°C. After an exposure to a temperature below −9°C, however, a significant portion of individuals resumed activity and development at 25°C. This means that this cockroach has some degree of freeze tolerance. The calculated median lower lethal temperature, the temperature at which 50% of test individuals died, was −8.3°C, while the mean SCP was −6.7°C.

To elucidate the freeze tolerant ability in more detail, final instar nymphs were exposed to various sub-zero temperatures for 12 hr and then their survival was assessed at 25°C. During the freezing trial, all test individuals were frozen at least within the first 3 hr. The results are summarized in Fig. 3. Even after the relatively long-term tissue freezing, a significant portion of individuals resumed activity and development

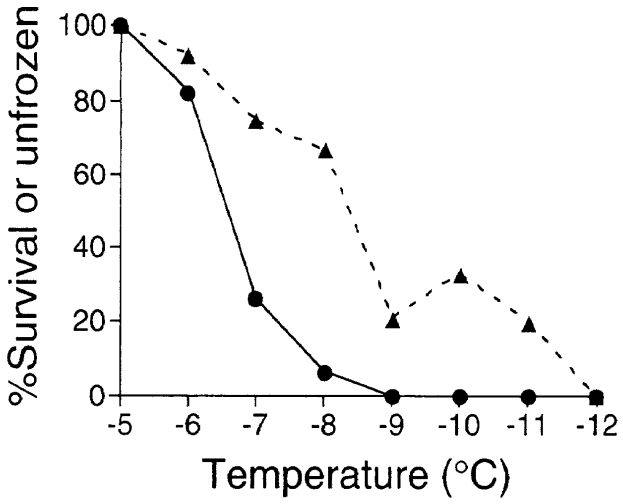

Fig. 2. Proportions of final instar nymphs of *P. japonica* that remain unfrozen by a short-term exposure to sub-zero temperatures (continuous line and circles) and subsequent survival (dashed line and triangles) assessed after transfer to 25°C. All unfrozen animals survived in this experiment. N = 9–18 per temperature.

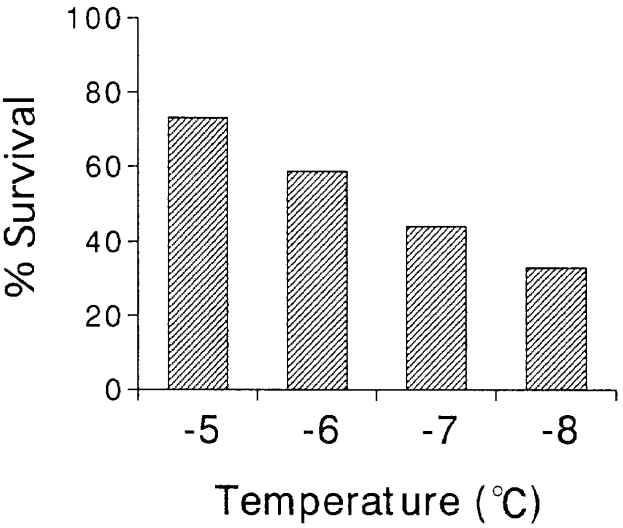

Fig. 3. Effect of 12 hr tissue freezing at various sub-zero temperatures on survival of final instar nymphs of *P. japonica*. After freezing, nymphs were transferred to 25°C and checked for survival. N = 11–22 per temperature.

at 25°C. The survival of frozen individuals was dependent on the temperature experienced. It exceeded 73% at −5°C, but fell to 33% at −8°C.

To assess the influence of tissue freezing on future reproductive capability, newly emerged females that had been frozen for 12 hr at −6 or −7°C as final instar nymphs were mated with a male and subsequent hatching success of eggs produced was measured at LD, 16:8 and 25°C. All females monitored (N=9) produced viable eggs, thus suggesting that tissue freezing at the nymphal stage did not influence the reproductive capability.

A comparison of freezing tolerance among the first, mid (3rd to 5th) and final (8th) instar nymphs is shown in Fig. 4. In

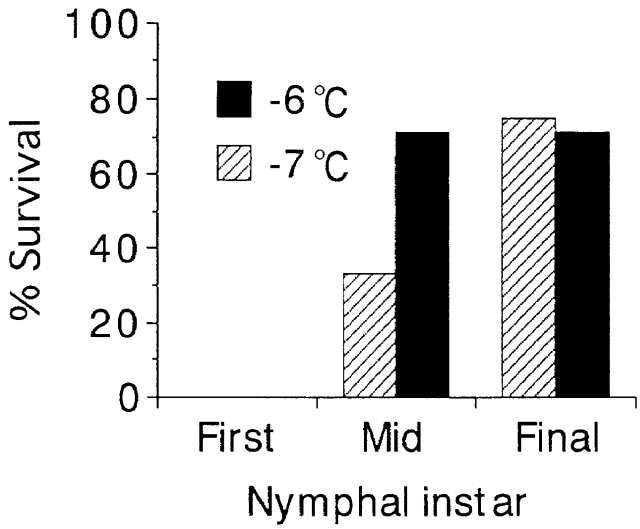

Fig. 4. Effect of 12 hr tissue freezing on survival of first, mid (from 3rd to 5th) and final (8th) instar nymphs of *P. japonica* at −6 or −7°C. N = 6–14 per instar.

this experiment, all individuals were kept at −6 or −7°C for 12 hr before they were transferred to 25°C. After an exposure to either −6 or −7°C, none of the first instar nymphs recovered after relatively long-term tissue freezing, whereas a significant portion of the mid and last instar nymphs did recover. The values for last instar nymphs in this experiment were greater than those in Fig. 3. However, the differences between the two experiments based on rather small sample sizes were statistically insignificant (Fisher's exact test, $P>0.05$ in either case).

Sugar and polyol contents

Gas chromatography assays indicated that at least four compounds were present in the whole body extracts of *P. japonica*. From the retention time, they were identified as glucose, *myo*-inositol, *scyllo*-inositol and trehalose (Table 2). Among the nymphal instars examined, no apparent difference was found in the array and content of carbohydrates except for trehalose which tended to decrease as nymphs grew older ($F=20.158$, $P<0.01$).

DISCUSSION

To tolerate subzero temperatures, at least two options have been exploited by terrestrial arthropods (see recent re-

Table 2. Mean ± S.D. concentrations of polyhydric alcohols and sugars in whole body extracts of first, mid (from 3rd to 5th) and final (8th) instar nymphs of *P. japonica* in late January. Values are expressed as μg/mg wet weight

		Polyhydric alcohols and sugars			
Instar	n	Glucose	*scyllo*-Inositol	*myo*-Inositol	Trehalose
First	3	0.2 ± 0.0	0.7 ± 0.1	1.1 ± 0.2	32.0 ± 2.0
Mid	4	0.2 ± 0.0	1.0 ± 0.2	1.5 ± 0.4	28.5 ± 2.2
Final	3	0.1 ± 0.1	0.9 ± 0.3	1.2 ± 0.4	19.4 ± 2.4

views by Block, 1990; Duman *et al.*, 1991). One is freeze tolerance in which the animal can tolerate extracellular freezing, and the other is supercooling because animals are susceptible to freezing.

The present results indicate that overwintering nymphs of *P. japonica* have some degree of freeze tolerance and a significant portion of individuals survived freezing lasting at least 12 hr (Fig. 3). This result may be ecologically relevant because sub-zero temperatures occur only during certain hours of the day even in mid-winter (Table 1). We used successful moulting to the next instar or normal walking as a criterion of survival in test animals. This method should be sufficient to draw the above conclusion, but one may argue that reproductive capability should also be checked for these animals to define the significance of the freeze tolerance observed in the present study. We monitored subsequent reproductive capability of some females that had been frozen as final instar nymphs. As expected, such individuals produced viable eggs after mating with a male. To our knowledge, this is a third example of freeze tolerant cockroach following the previous two species; *Parcoblatta pennsylvanica* (Duman, 1979) and *Cryptocercus punctulatus* (Hamilton *et al.*, 1985), although the reproductive capability post-thaw was not assessed in these two species.

In January and February, overwintering nymphs of *P. japonica* retained a relatively high SCP (Fig. 1) compared to other overwintering insects (see Somme, 1982; Zachariassen, 1980, 1985). This fact may indicate that they have some active ice-nucleating agent in their body. It has been well known that some freeze-tolerant species produce ice nucleating agents or proteins in the haemolymph, and these agents are thought to control ice formation and the rate of its spread over the body, thereby avoid lethal intracelluler freezing (see reviews by Zachariassen, 1985; Duman *et al.*, 1995). In *P. japonica,* however, no ice nucleating activity was observed in the haemolymph which had a SCP of as low as –23°C (Table 2). Thus, ice nucleators may exist in other tissues. In other freeze-tolerant insects, ice nucleators are found in the alimentary canal (Shimada, 1989), muscle tissue (Tsumuki and Konno, 1991), fat body cells and Malpighian tubules (Mugano *et al.*, 1996).

In *P. japonica*, the median lower lethal temperature of overwintering nymphs after a brief exposure to sub-zero temperatures was –8.3°C. Because this value is close to the minimum ambient temperature in Tsukuba (–8.5°C) (Table 1), it is possible that overwintering nymphs suffer some freezing injury. To avoid such a risk and overwinter successfully, microhabitat selection in seeking for a protected hibernaculum may be an important strategy. This cockroach is known to overwinter in decayed wood material or under the bark of trees (Tabaru and Kobayashi, 1971; Tsuji and Tabaru, 1974). Although we do not have precise data for the microhabitat temperature of this species, such hibernacula would provide some protection against freezing temperatures. Winter survival of *P. japonica* is thus likely to be ensured by both behavioural microhabitat selection and freeze tolerance capacity.

It is noteworthy that none of the first instar nymphs recovered after freezing trials at –6 and –7°C, while some mid and final instar nymphs survived freezing (Fig. 4). This may suggest that first instar nymphs are susceptible to freezing. At present, however, we cannot rule out completely the possibility that they are potentially tolerant to freezing because various other factors often influence the expression of freeze tolerance. In the carabid beetle, *Upis ceramboides*, for example, the cooling rate is critical for freezing survival at –50°C for 1 hr, and a slight change in cooling rate from 0.3 to 0.5°C/min resulted in a change in the survival rate from 100 to 0% (Miller, 1978). Recent studies also demonstrated that several arthropod species survive freezing only when inoculative freezing occurs (e.g. Tanno, 1977; Shimada and Riihimaa, 1988; Gehrken *et al.*, 1991; Tursman *et al.*, 1994). In the present study, the test animals were cooled relatively rapidly (ca. 1°C/min) and inoculative freezing was not applied. Our results have also demonstrated that the array and content of sugars and polyols, potential cryoprotectants, did not differ greatly among the nymphal instars (Table 2). In fact, the concentration of trehalose in the most susceptible stage, i.e., the first instar, was the highest. Therefore, the quantity or quality of cryoprotectants does not explain the observed high susceptibility of first instar nymphs to sub-zero temperatures.

The above finding that first instar nymphs are less cold-hardy than others, together with the fact that the former lack the capacity to enter diapause (Shindo and Masaki, 1995), might suggest some differences in overwintering success between the first and the other nymphal instars in *P. japonica*. However, first instar nymphs hatching late in the growing season can overwinter in central Japan (Tanaka and Uemura, 1996). This also appears to be true for northern populations because a sample collected in mid-October in Hirosaki (40.3°N) contained first instar nymphs, which could overwinter successfully when reared under outdoor conditions in central Japan (36.1°N) (Tanaka and Uemura, 1996). It is likely that first instar nymphs avoid the risk of freezing by remaining deep inside their winter hibernacula. During the growing season, nymphs normally remain hidden during the day, but appear on the bark of trees at night. However, first instar nymphs, which show a relatively strong tendency to aggregate, are seldom found at exposed sites at night. This behavioural difference may have left the first nymphal instar devoid of freeze tolerance. To understand the thermal adaptation of this insect fully, more information about behaviour of the nymphs and the thermal conditions of their microhabitats is necessary.

ACKNOWLEDGMENTS

This research was supported by a KA-SHIN-CHOU project of the Science and Technology Agency of Japan. Experiments were undertaken while KT was a research fellow at the Department of Insect Physiology and Behavior, National Institute of Sericultural and Entomological Science, Tsukuba, Japan. Thanks are also due to Nobuo Morimoto, National Institute of Agro-environmental Science, for letting us use the apparatus for SCP determinations and Noriko Kenmochi, Sumi Ogawa and Hiroko Ikeda for technical assistance.

Finally, we wish to thank Kimio Shimada, Hokkaido University, for giving us invaluable suggestions throughout the present study and kindly reviewing the manuscript, and two anonymous reviewers for helping to improve the presentation of our data and to sharpen our arguments.

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(Received April 24, 1997 / Accepted June 27, 1997)