CRYOBIOLOGY 27, 562–568 (1990)

Regulation of Supercooling and Nucleation in a Freeze Intolerant Beetle (*Tenebrio molitor*)[1]

SEAN L. JOHNSTON AND RICHARD E. LEE, JR.[2]

Department of Zoology, Miami University, 1601 Peck Boulevard, Hamilton, Ohio 45011

The mealworm beetle, *Tenebrio molitor* (Tenebrionidae, Coleoptera), is a freeze-susceptible species that does not survive cooling to below the supercooling point (i.e., tissue freezing) in any stage of development. Most insects of a given age and developmental stage exhibit a relatively narrow range of supercooling point (SCP) values. However, *Tenebrio* larvae reared at 24°C on dry wheat bran displayed a wide range of values extending from −1.5 to −24.3°C. Larvae acclimated at 5°C had slightly lower SCPs; however, the range of individual values remained highly variable. SCPs were significantly correlated ($r = 0.59$) with body weight: small larvae demonstrated a greater capacity for supercooling than larger individuals. In an attempt to identify the specific site of nucleation, the SCPs of hemolymph, fat body, and gut contents were determined and compared to whole body SCPs. Hemolymph and fat body samples generally supercooled to −15°C or lower for larvae whose whole body SCPs were −5°C or higher. Only on some occasions could whole body SCPs be explained on the basis of the efficacy of ice nucleators within the gut.

The supercooling point (SCP) is defined as the temperature at which body water spontaneously freezes. For freeze-intolerant insects it represents the absolute limit of low temperature tolerance, although some insects experience cold shock injury and death at temperatures above the SCP (2, 12, 13). Frequently, insects exhibit a seasonal pattern in their capacity to supercool: SCPs decrease through the autumn reaching their lowest values in winter followed by a reduction in their supercooling capacity in the spring (19). As a consequence, for many freeze-intolerant insects regulation of the SCP is a critical factor in the cold-hardening process to ensure survival in winter.

At relatively high subzero temperatures, in the range that would be experienced naturally by insects, the limit of supercooling is believed to be determined by the endogenous activity of heterogeneous ice nucleating agents (19), although inoculative freezing may also be important in some species (10). These nonaqueous agents serve as the initial nucleus upon which the ice lattice grows. The activity of the most efficient nucleating agent in the insect's body will determine the onset of ice nucleation and, thus, the SCP value. Although it is commonly assumed that efficient ice nucleators are found in gut contents (7), few studies have critically examined the nature of this process. A number of freeze-tolerant insects limit supercooling of their body fluids by producing ice nucleating proteins and lipoproteins in the hemolymph (8, 9). Nucleating agents also have been associated with the cellular matrix (5).

As do a number of insects, *Tenebrio molitor* larvae produce low levels of thermal hysteresis or antifreeze proteins in their hemolymph (8, 16). When these larvae are acclimated to a short photoperiod or low temperature thermal hysteresis of the hemolymph increases, while at the same

Received March 20, 1989; accepted May 31, 1989.

[1] We thank S. L. Chitwood, M. W. Mabry, and W. G. Leary for technical assistance. Drs. D. L. Claussen, J. P. Costanzo, and A. L. Rypstra provided helpful suggestions on this study. Supported in part by NSF Grants DCB-8517875 and 8811317.

[2] To whom correspondence and reprint requests should be addressed.

0011-2240/90 $3.00

time lower lethal temperatures and SCPs are lowered (16). Cold-acclimated larvae did not significantly elevate titers of the cryoprotectant glycerol (16).

In preliminary studies we found that, unlike other species, the SCPs for larvae of the mealworm beetle, *T. molitor*, were highly variable. This result suggested to us that *T. molitor* might serve as a useful model system for the study of nucleation and supercooling in insects. This report examines the nature of supercooling and nucleation in larvae of *T. molitor*. Specifically, the effect of the following parameters on supercooling capacity was assessed: relative humidity, acclimation temperature, body weight, and feeding. We also sought to identify the body compartment in which nucleation occurs.

MATERIALS AND METHODS

Cultures of *T. molitor* were obtained from Carolina Biological Supply Co. and fed wheat bran. Whole body SCPs were determined using a refrigerated bath with a cooling rate of approximately 1°C/min and a 12-channel thermocouple chart recorder (13). The SCP was recorded as the lowest temperature reached prior to the release of the latent heat of crystallization. The SCP of the excised gut was determined by placing the entire gut into a 100-μl glass capillary tube and attaching a thermocouple to the outside of the tube. The SCPs of hemolymph, fat body, and gut contents were determined using approximately 5 μl samples in capillary tubes as described previously (14, 26).

In order to test the effect of relative humidity on the SCP, larvae were held in desiccators over dry calcium sulfate (0% RH) or a saturated solution of sodium chloride (75% RH). Environmental chambers were used to maintain acclimation temperatures to within 1°C. Since *T. molitor* is cannibalistic when deprived of food, larvae were held separately during starvation experiments.

The coefficients of variation and correlation coefficients were calculated as described by Sokal and Rohlf (22).

RESULTS

No individual survived the freezing of its body fluids. The SCPs of fourth instar larvae reared at 25°C were highly variable ranging from −1.5 to −24.3°C with a mean ± SEM of −12.4 ± 0.6°C (Fig. 1). The coefficient of variation (CV), 52.0, was substantially higher than the variability observed in SCPs from a variety of other insects (Table 1). Cold acclimation to 5°C for 2 weeks resulted in a decrease in the SCP to −15.2 ± 0.9°C (Fig. 1). However, this decrease was not statistically significant compared to the 25°C acclimation group and the SCP values were still highly variable (CV = 34.7).

The SCP was inversely related to the weight of the larvae acclimated to 25°C (Fig. 2). On average small, first instar larvae supercool to a greater extent than large, fourth instar ones. However, there remains a large amount of variability among SCP values regardless of weight. A few large individuals (>200 mg) sometimes supercooled to less than −20°C, while small larvae (<60 mg) did not exhibit SCPs above −5°C and rarely above −10°C. In a sepa-

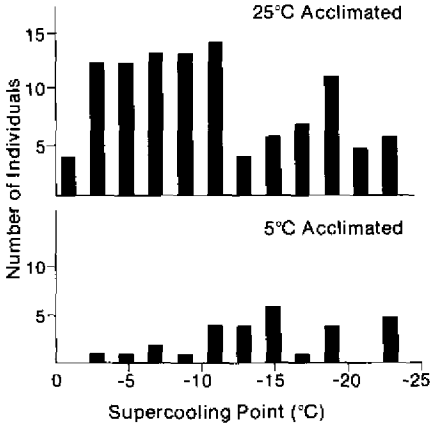

FIG. 1. Histograms of supercooling points for fed fourth-instar larvae of *Tenebrio molitor* acclimated to 25°C (top) and 5°C (bottom).

TABLE 1
Representative Data on the Variability of Supercooling Points (SCP, °C) for several insects[a]

Insect	SCP mean (×) (°C)	Standard deviation(s) (°C)	Range (°C)	Coefficient of variation (%)	Sample size (n)	Reference
Mealworm larvae (*Tenebrio molitor*)	−12.4	6.37	−1.5 to −24.3	52.0	109	This study
Goldenrod gall fly (*Eurosta solidaginis*)	−9.4	2.19	−5.0 to −11.9	23.2	16	(3)
Lady beetle (*Hippodamia convergens*)	−17.2	1.74	−14.0 to −21.5	10.1	15	(11)
Leafhopper (*Dalbulus maidis*)	−21.3	1.53	−17.1 to −21.3	7.2	14	(R. E. Lee and L. R. Nault, unpublished data)
Flesh fly pupae (*Sarcophaga crassipalpis*)	−24.2	0.81	−22.5 to −25.2	3.4	10	(13)

[a] The coefficient of variation of variation (CV) is equal to the standard deviation expressed as a percentage of the mean (22).

rate experiment the relationship between larval weight and the SCP for *T. molitor* acclimated to 5°C was examined. In this experiment no statistically significant relationship between weight and SCP was observed ($r = 0.068$, $n = 33$).

In order to evaluate the consistency of SCP values for an individual, three replicate determinations were conducted for individual larvae (Fig. 3). These data were selected from a larger set of results in order to illustrate the general pattern of response that we observed. Replicate SCP values were usually within 2°C of each other, the widest range observed was 5°C in one individual (Fig. 3). Since *T. molitor* larvae do not survive the first SCP determination these data suggest that regulation of the SCP is dependent on physical factors which continue to operate unchanged in dead larvae.

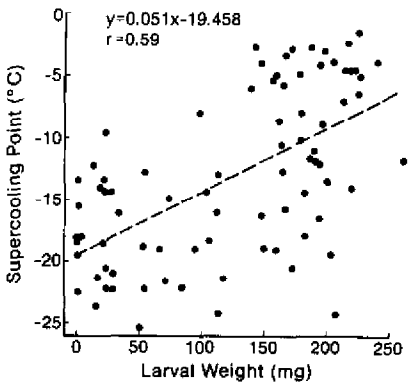

FIG. 2. Effect of larval weight on the supercooling point of *Tenebrio molitor* acclimated to 25°C.

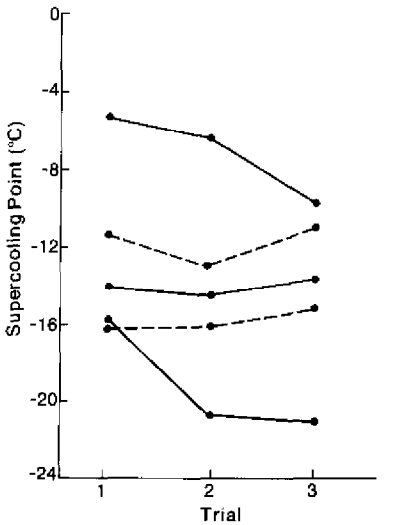

FIG. 3. Supercooling point values for five individual larvae of *Tenebrio molitor* determined in successive trials. Solid and dashed lines are used for clarity.

Eight groups of larvae (n = 30–37) were acclimated to combinations of temperature (5 or 24°C) and humidity (0 or 75% RH) and were continuously fed wheat bran or were starved for 9–13 days. Regardless of treatment, the variability of SCPs within a sample remained high (CV from 13.4 to 31.0). SCPs of larvae starved for 9 to 13 days were similar to those fed wheat bran, regardless of acclimation temperature (5 or 24°C) or the relative humidity (0 or 75%). At 24°C acclimation to a relative humidity of 75% significantly lowered SCPs ($P < 0.01$, Student's t test) compared to individuals held at 0% RH for both the fed and starved treatment groups. A similar effect of humidity was not observed in larvae held at 5°C. However, we repeated this acclimation experiment, using the same temperatures and humidities, and found no significant differences in the SCPs among the four treatment groups (i.e., mean SCP values ranged from −12.7 to −14.5°C).

The possible effect of different foods on the SCP was tested by feeding larvae wheat bran, potato, or apple. The type of food had no statistically significant effect on the mean (−9.3 to −11.6°C) or range of the SCPs.

Two additional experiments were conducted in an attempt to identify the specific site of nucleation within the body. In the first, the relationship between the whole body SCP and the SCP of the entire gut with its contents for fourth instar larvae was examined. After determining the whole body SCP the entire gut was dissected out, placed in a capillary tube and the SCP of the gut determined. The SCPs for both the gut and whole body from single larvae are shown in Fig. 4. The dashed line represents the theoretical relationship in which the whole body SCP is equal to that of the gut. Since 82% of the data points lie to the right of the line it suggests that the gut and its contents frequently supercool to a greater extent than the whole body.

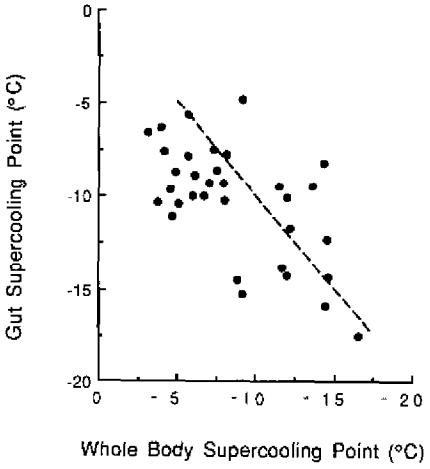

FIG. 4. Relationship between the whole body supercooling point (SCP) and the SCP of its gut including contents in larvae of *Tenebrio molitor*. Dashed line represents the theoretical relationship in which the whole body SCP is equal to the SCP of the gut.

In a second experiment we compared the whole body SCP to that of three separate body compartments: gut and its contents, hemolymph, and fat body. The data reported in Fig. 5 are only those for which all four SCP values were obtained from a single individual. Furthermore, only larvae with a relatively high SCP of −10°C or higher were selected since their limited capacity for supercooling suggested the pres-

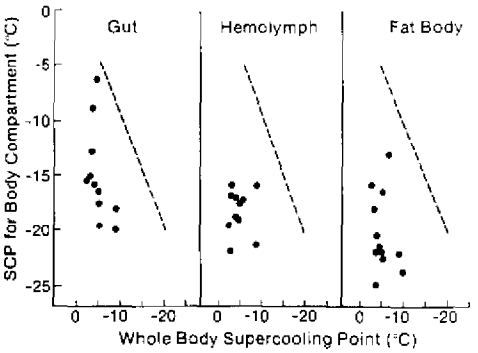

FIG. 5. Relationship between the whole body supercooling point (SCP) and the SCP of that individuals gut, hemolymph, and fat body for larvae of *Tenebrio molitor*. Dashed line represents the theoretical relationship in which the whole body SCP is equal to the SCP of the body compartments.

ence of relatively efficient ice nucleating agents within the body. All hemolymph and fat body samples had relatively low SCPs in the range of −13 to −25°C that were always less than that of the whole body SCP (Fig. 5). In 2 of 11 individuals, the SCPs of the gut contents were high enough to suggest that nucleating agents in the gut could account for the relatively high SCP of the whole body.

DISCUSSION

Exposure to low temperature commonly serves as the primary trigger for winter cold-hardening in insects (3, 19). In freeze-susceptible species this process usually includes a depression in the whole body SCP. A similar response was observed in the present study as acclimation to 5°C resulted in a decrease in SCPs (Fig. 1). However, unlike cold-hardening in other species in which the coefficient of variation generally decreases with cold acclimation, *T. molitor* larvae continued to exhibit a wide range of SCP values. Patterson and Duman (16) also reported highly variable SCPs for warm- and cold-acclimated larvae of *T. molitor*.

Insect populations composed of individuals containing either very high or very low numbers of ice nucleators are expected to have a narrow range of freezing temperature distributions (21). As illustrated by the selected examples in Table 1, many studies of insect cold-hardiness have reported relatively narrow SCP ranges. Alternatively, populations composed of individuals with differing concentrations of nucleators would be expected to have broad freezing temperature distributions (21). Therefore, the high degree of variability in SCPs that we observed throughout this study suggests a wide range in the number or the efficacy of nucleators in *Tenebrio* larvae.

It was also surprising to find supercooling points as high as −1.5°C in some larvae. Some bacteria are extremely potent nucleating agents capable of catalyzing ice nucleation in this range (15). Recently, our laboratory demonstrated that ice nucleating bacteria have the potential to increase markedly the SCP of insects (24). It is possible that ice nucleating bacteria are present in the gut of *Tenebrio* and responsible for these extremely high SCPs.

Perhaps the principal finding of this study was the demonstration of the effect of body size on the SCP of conspecific larvae. Somme (23) summarized SCPs from a variety of overwintering freeze intolerant insects. Inspection of these data indicate that small insects supercool to a greater extent than larger ones. Insect eggs and small arthropods such as collembola and mites commonly have SCPs of −20°C or lower, while large insects frequently have SCPs of −15°C or higher. Thus, the effect of body size on the SCP that we observed is consistent with the general pattern observed among a wide variety of terrestrial arthropods.

Physical studies of water have shown that the capacity for supercooling is inversely related to the volume of the sample (1). The water content of larvae in Fig. 2 ranged from approximately 0.5 to 200 μl, with a corresponding range of SCPs between −24.3 and −1.5°C. These data correspond roughly to the values for the heterogeneous nucleation of water in physical systems: 1 μl to 1 ml volumes of water have generally been reported to nucleate between −30 to −10°C (1).

The major difference between the SCPs of *T. molitor* and those reported in physical studies of water was that some larvae exhibited only a very limited capacity to supercool, with SCPs above −5°C. This observation suggests the presence of relatively efficient ice nucleating agents in the body. Obviously, these agents should be considered independently from the ice nucleating proteins produced by freeze-tolerant insects which are believed to function by insuring extracellular ice nucleation

at relatively high subzero temperatures. Since *T. molitor* is a freeze-susceptible species, the agents affecting ice nucleation within its tissues probably serve some other primary function, while only "inadvertently" serving as nucleating agents (13).

One limitation of our approach to identifying the specific location of nucleators within mealworm larvae is the inherent difference in the amount of tissue and, as a consequence, the water volume in the sample used for the determination of SCPs in the whole body versus individual body compartments. For example, some larvae used for whole body supercooling points (Fig. 5) weighed 200 mg, while the corresponding supercooling point of the hemolymph was based on a 5 μl sample. Considering the physical effect of volume on the supercooling point, we would expect the SCP of the individual body compartments to be lower than that of the whole body unless potent nucleators were present. This result is essentially what we observed (Figs. 4 and 5). Thus, our experimental approach would tend to make it more difficult to identify the specific site of nucleation. Nevertheless, we found that 18% of the time the supercooling point of the gut was higher than that of the whole body (Fig. 4). In addition, in two of eleven larvae we were able to identify the gut as the likely site of nucleation (Fig. 5).

Previous studies on the effect of starvation on the SCP have produced varying results (23). Salt (18, 20) suggested that gut contents commonly contain efficient ice nucleating agents that regulate the SCP. In the Antarctic mite, *Alaskozetes antarcticus,* feeding decreases the capacity to supercool (25).

In contrast, other studies, including this one, have not found a correlation between feeding/starvation and nucleation (2). Most insects empty their gut within 1–2 days after food is removed. Whereas, *T. molitor* does not empty its gut, but retains fecal material in the hindgut when starved. This may partly explain why feeding and starvation did not significantly alter SCPs in this species. Another factor that might affect ice nucleation in the gut is the activity of the rectal complex and its role in water vapor absorption (17). In *T. molitor* the rectal complex in the hindgut is able to remove water vapor from unsaturated atmospheric air. This mechanism results in extreme dehydration of the fecal pellets in the hindgut such that they are only 10–15% water (17). This extensive dehydration may decrease the activity of potential nucleating agents in the gut by reducing the availability of freezable water in the gut. Along similar lines, Cannon (6) hypothesized that internal changes in water content may alter the activity of nucleators in an Antarctic mite.

In their review Baust and Rojas (4) point out problems inherent in understanding the regulation of ice nucleation in insects. Our study further emphasizes the complexity of this problem and the difficulty of identifying specific mechanisms regulating supercooling and nucleation in insects.

REFERENCES

1. Angell, C. A. Supercooled water. *In* "Water: A Comprehensive Treatise" (F. Franks, Ed.), Vol. 7, p. 1–81, Plenum, NY, 1982.
2. Bale, J. S. Insect cold hardiness: Freezing and supercooling an ecophysiological perspective. *J. Insect Physiol.* 33, 899–908 (1987).
3. Baust, J. G., and Lee, R. E. Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginis*. *J. Insect Physiol.* 27, 485–490 (1981).
4. Baust, J. G., and Rojas, R. R. Review—Insect cold hardiness: Facts and fancy. *J. Insect Physiol.* 31, 755–759 (1985).
5. Baust, J. G., and Zachariassen, K. E. Seasonally active cell matrix associated ice nucleators in an insect. *Cryo-Lett.* 4, 65–71 (1983).
6. Cannon, R. J. C. Effects of contrasting relative humidities on the cold tolerance of an Antarctic mite. *J. Insect Physiol.* 32, 523–534 (1986).
7. Cannon, R. J. C., and Block, W. Cold tolerance of microarthropods. *Biol. Rev.* 63, 23–77 (1988).
8. Duman, J., and Horwath, K. The role of hemolymph proteins in the cold tolerance of insects. *Annu. Rev. Physiol.* 45, 261–270 (1983).
9. Duman, J. G., Neven, L. G., Beals, J. M., Olson,

K. R., and Castellino, F. J. Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators, in the larvae of the cranefly *Tipula trivittata*. *J. Insect Physiol.* **31**, 1–8 (1985).

10. Humble, L. M., and Ring, R. A. Inoculative freezing of a larval parasitoid within its host. *Cryo-Lett.* **6**, 59–66 (1985).
11. Lee, R. E. Physiological adaptations of Coccinellidae to supranivean and subnivean hibernacula. *J. Insect Physiol.* **26**, 135–138 (1980).
12. Lee, R. E., Chen, C-P., and Denlinger, D. L. A rapid cold-hardening process in insects. *Science* **238**, 1415–1417 (1987).
13. Lee, R. E., and Denlinger, D. L. Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. *Physiol. Entomol.* **10**, 309–315 (1985).
14. Lee, R. E., Zachariassen, K. E., and Baust, J. G. Effect of cryoprotectants on the activity of hemolymph nucleating agents in physical solutions. *Cryobiology* **18**, 511–514 (1981).
15. Lindow, S. E. The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* **21**, 363–384 (1983).
16. Patterson, J. L., and Duman, J. G. The role of the thermal hysteresis factor in *Tenebrio molitor* larvae. *J. Exp. Biol.* **74**, 37–45 (1978).
17. Ramsay, J. A. The rectal complex of the mealworm *Tenebrio molitor*, L. (Coleoptera, Tenebrionidae). *Philos. Trans. R. Soc. London, B* **248**, 279–314 (1964).
18. Salt, R. W. The influence of food on cold hardiness of insects. *Can. Entomol.* **85**, 261–269 (1953).
19. Salt, R. W. Principles of insect cold-hardiness. *Annu. Rev. Entomol.* **6**, 55–74 (1961).
20. Salt, R. W. Location and quantitative aspects of ice nucleators in insects. *Canad. J. Zool.* **46**, 329–333 (1968).
21. Salt, R. W. Analysis of insect freezing temperature distributions. *Canad. J. Zool.* **48**, 205–208 (1970).
22. Sokal, R. R., and Rohlf, F. J. "Introduction to Biostatistics," Freeman, San Francisco (1973).
23. Somme, L. Supercooling and winter survival in terrestrial arthropods. *Comp. Biochem. Physiol. A* **73**, 519–543 (1982).
24. Strong-Gunderson, J. M., Lee, R. E., Lee, M. R., and Riga, T. J. Ingestion of ice-nucleating active bacteria increases the supercooling point of the lady beetle *Hippodamia convergens*. *J. Insect Physiol.* **36**, 153–157 (1990).
25. Young, S. R., and Block, W. Experimental studies on the cold tolerance of *Alaskozetes antarcticus*. *J. Insect Physiol.* **26**, 189–200 (1980).
26. Zachariassen, K. E., Baust, J. G., and Lee, R. E. A method for quantitative determination of ice nucleating agents in insect hemolymph. *Cryobiology* **19**, 180–184 (1982).