JOURNAL OF EXPERIMENTAL ZOOLOGY 292:345–350 (2002)
DOI 10.1002/jez.10082

Energy and Water Conservation in Frozen vs. Supercooled Larvae of the Goldenrod Gall Fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae)

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ABSTRACT Insects that tolerate severe cold during winter may either supercool or tolerate ice forming within the tissues of the body. To compare the relative advantages of freezing and supercooling, we measured rates of CO_2 production and water loss in frozen and supercooled goldenrod gall fly larvae (*Eurosta solidaginis*). As an important first step, we measured the time required for ice content and metabolic rate to stabilize upon freezing. Ice content stabilized after only three hours of freezing at –5°C, whereas CO_2 production required 12 hours to stabilize. Subsequent experiments found that freezing greatly reduced both water loss and metabolic rate. Comparisons of supercooled and frozen larvae at –5°C indicated that CO_2 production fell 47% with freezing and water loss decreased 35%. As temperature decreased to –10 and –15°C, CO_2 production fell exponentially and was no longer detectable at –20°C with our measurement system. Our results demonstrate that freezing significantly reduces energy consumption during the winter and may therefore improve winter survival and spring fecundity. The advantages of freezing over supercooling would drive selection toward insect freeze tolerance and also toward higher supercooling points to increase the duration of freezing each winter. *J. Exp. Zool. 292:345–350, 2002.*

To survive temperatures below the freezing point of their tissues, insects use one of two mechanisms: supercooling, or freeze tolerance. Supercooling requires the absence of potent nucleators within the body fluids, and physiological or behavioral mechanisms to prevent external ice from crossing the body wall and initiating ice formation in the body. In contrast, freeze-tolerant insects survive freezing of the body fluids. This ice is substantially extracellular, although fat body cells of *Eurosta solidaginis* tolerate intracellular ice formation (Lee et al., '93). Freeze tolerance is supported by the production of very high concentrations of polyols and/or sugars that act as cryoprotectants to protect the tissues while frozen and to reduce cellular dehydration (Storey and Storey, '88; Baust and Nishino, '91).

Generally, freeze tolerance is considered to have evolved because of its advantages in the survival of extreme cold (Lee, '91), but freezing may also be advantageous by reducing energy consumption during the winter (Duman et al., '91; Lundheim and Zachariassen, '93). Because they possess a greater tolerance of low temperature, freeze-tolerant insects can overwinter in colder, more exposed sites, and thus will have very low metabolic rates and consequently use less energy than insects overwintering in warm, protected sites (Danks, '91). Also, there is evidence that frozen insects have lower metabolic rates than supercooled insects at the same temperature. Scholander et al. ('53) demonstrated that metabolism drops precipitously upon freezing and suggested that the metabolic rate of supercooled insects would fall with temperature as a continuation of the above-zero portion of the curve. Salt ('58) confirmed this to be the case with his measurements of supercooled flour moth larvae. Only two studies have directly compared the metabolic rates of frozen and supercooled insects within a species: Lundheim and Zachariassen ('93), and the unpublished work of Kanwisher cited in Asahina ('69). Lower metabolic rates in frozen insects may have

Grant sponsor: Sigma Xi, Grant-in-Aid; Grant sponsor: NSF; Grant number: IBN-0090204.
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Received 7 June 2001; Accepted 21 September 2001

been a strong selective force favoring freeze tolerance—those insects that freeze may conserve more energy through the winter, thus leaving more energy for egg production and mating activity in spring (e.g., Irwin and Lee, 2000). For species (including *E. solidaginis*) that do not feed between spring emergence and reproduction, energy savings associated with freezing may be particularly important. We measured metabolic rates in frozen and supercooled goldenrod gall fly larvae (*E. solidaginis*) to further test this hypothesis.

Lundheim and Zachariassen ('93) suggested another advantage of freezing versus supercooling—reduced water loss. There are two reasons why frozen insects may lose less water to the environment than those that are supercooled: (1) the vapor pressure of a frozen insect more closely matches that of the surrounding icy environment than supercooled insects which have a higher vapor pressure; and (2) frozen insects have lower metabolic rates than supercooled insects at the same temperature, thus decreasing respiratory water loss (water lost through the spiracles during gas exchange).

The goldenrod gall fly, *E. solidaginis,* is freeze tolerant, supercools to –10°C depending on season and latitude (Baust and Lee, '81), and is also susceptible to inoculative freezing (Layne et al., '90). Thus, we could measure the metabolic rate of supercooled larvae, briefly cool the larvae below their supercooling point to initiate freezing, then measure the metabolic rate again in frozen larvae. This work was facilitated by recent advances in technology which allowed us to measure the very low rates of metabolism and water loss of *E. solidaginis* larvae at subzero temperatures. We used this experimental model to: (1) describe the relationship between metabolic rate (as measured by CO_2 production) and temperature in both supercooled and frozen larvae; and (2) directly explore the relationship between metabolic rate and water loss in both frozen and supercooled *E. solidaginis* larvae.

MATERIALS AND METHODS

Larvae were collected at Miami University's Ecology Research Center near Oxford, OH (39° 31′ 57″ N, 84° 43′ 23″ W) by removing galls from the stem of goldenrod plants. Collections were made in midwinter to ensure the larvae were in diapause. The larvae were stored in the galls at –22°C until used in the respirometry experiments.

We measured metabolic rate using a TR-3 respirometry system (Sable Systems Inc., Henderson, NV). This system includes carbon dioxide/water analyzers, signal conditioners to reduce electrical noise, thermocouple inputs for simultaneous temperature measurement, an air pump and flow controller, and an interface to allow real-time analysis with Sable System's data acquisition/analysis software. The analyzers were calibrated against commercially available standard gases and the humidity of the room air, and the system was configured for flow-through respirometry. Larvae were held individually or in groups (see below) in small glass respirometry chambers that were held within a jacketed beaker. Ethanol, cooled by a refrigerated bath, flowed within the wall of the beaker. Preliminary experiments demonstrated that this apparatus allowed precise control of larval body temperature and that body temperature closely tracked the temperature measured on the outer surface of the respirometry chambers.

Experiment 1: Changes in metabolic rate as ice accumulates

To compare the metabolic rates of frozen and supercooled larvae, we first needed to measure the time required for the metabolic rate to stabilize after the initiation of freezing. Individual larvae were placed in respirometry chambers at 4°C with a small piece of moist filter paper in contact with the larval cuticle. Upon transfer to –5°C, the water in the paper quickly froze, thus inducing freezing within the larva. The metabolic rates of the larvae were measured at 6, 12, 24, 48, and 96 hr after the initiation of freezing. The means were compared with repeated-measures ANOVA followed by Bonferroni multiple comparisons (Statview 5.0, SAS Institute Inc., Cary, NC).

A second experiment measured the ice content of groups of five larvae 1, 3, 6, 12, 24, and 96 hr after the initiation of freezing (four groups of five larvae were measured at each time interval). The larvae were transferred from the cold bath using pre-chilled forceps into 10 ml of room-temperature water in a calorimeter. The change in temperature of the water was recorded (± 0.01°C) by a thermistor connected to a MacLab data acquisition system. The larvae were subsequently blotted to remove surface water, weighed (± 0.01 mg), dried to constant mass at 60°C, then reweighed to allow calculation of water content and dry tissue mass. Ice content was calculated as in Lee and Lewis ('85). The ice contents were compared using one-way ANOVA followed by Bonferroni multiple comparisons (Statview 5.0).

Experiment 2: Relationship between metabolic rate and temperature

To determine the relationship between temperature and metabolic rate, we measured the CO_2 production in groups of supercooled *E. solidaginis* larvae (n = 5 pools of 8 larvae) at –5°C. After a 24 hr exposure to –20°C to induce freezing, we moved the larvae back to –5°C for 24 hr, then measured metabolic rate again. We subsequently measured metabolic rate at –10, –15, and –20°C with a 24 hr acclimation period at each temperature before a measurement was made. Repeated-measures ANOVA followed by Bonferroni multiple comparisons (Statview 5.0) were used to compare the samples.

Experiment 3: Metabolic rate and water loss in frozen and supercooled larvae

To measure the metabolic rate and water loss of *E. solidaginis* larvae, groups of fifteen individuals (n = 4) were placed in the respirometry chambers at –5°C. After 36 hours of acclimation at –5°C, the rates of CO_2 production and water loss were determined. The larvae were then cooled to –20°C for 12 hours to stimulate ice formation within the larval tissues. The larvae were warmed to –5°C and again acclimated for 36 hours before measuring CO_2 production and water loss. Means were compared using a one-tailed, paired-sample *t*-test (Statview 5.0).

RESULTS

Experiment 1: Changes in metabolic rate as ice accumulates

Metabolic rate took much longer to stabilize than did ice content. After the initiation of freezing at –5°C, at least 12 hours were required before the metabolic rate stabilized (Fig. 1A). Twelve hours after freezing began, metabolic rate continued to fall slowly but was not significantly lower than the 12 hr sample. It is interesting to note that, although metabolic rate decreased for at least 12 hours, the ice content had reached its peak only three hours after freezing (Fig. 1B). Beyond three hours, ice content stabilized, never falling statistically lower than the 3 hr sample.

Experiment 2: Relationship between metabolic rate and temperature

Metabolic rate fell rapidly with temperature (Figs. 2,3). In fact, at –20°C, the metabolic CO_2 production could no longer be distinguished from the background noise of the system (< ~0.05 µl

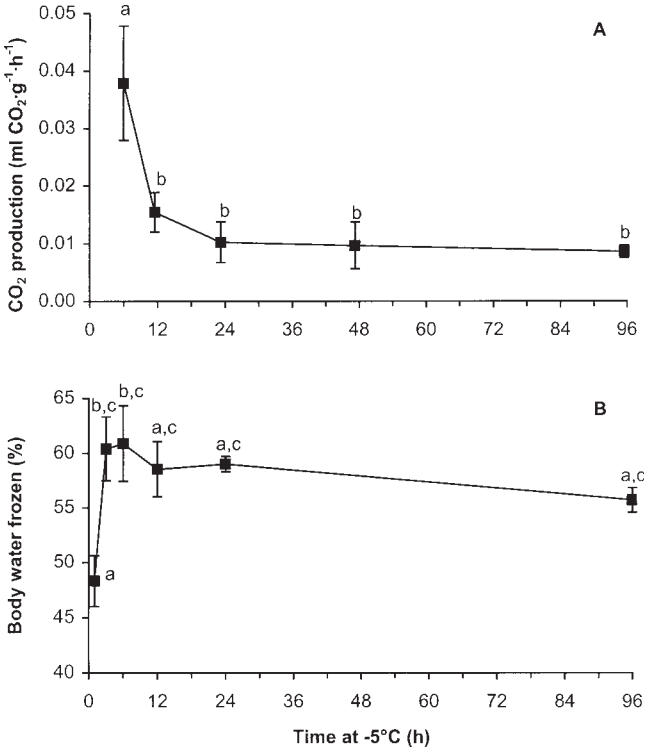

Fig. 1. Changes in metabolic rate (**A**) and ice content (**B**) of goldenrod gall fly larvae (*Eurosta solidaginis*) with time held at –5°C. Larvae were inoculated (stimulated to freeze) by the presence of wet filter paper. Data are presented as mean ± SEM. Sample size is five larvae for each time interval in A, whereas each point in B represents four groups of five pooled larvae. Means not sharing a letter were significantly different (Bonferroni multiple comparisons, alpha = 0.05).

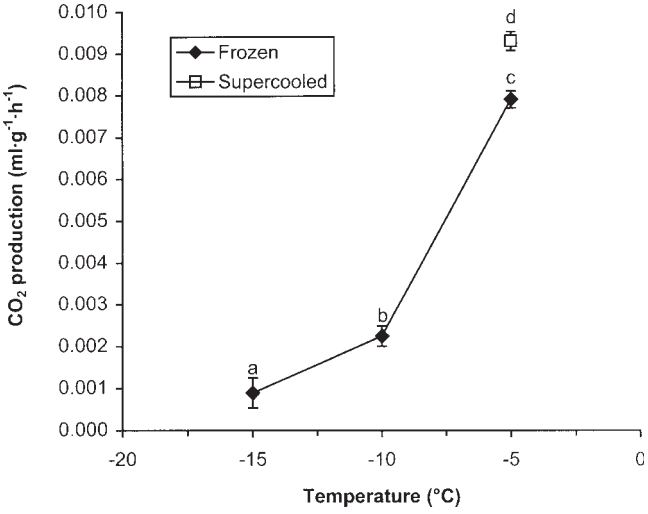

Fig. 2. The effect of temperature on mean (± SEM) metabolic rate (CO_2 production) in frozen and supercooled third-instar goldenrod gall fly larvae (*Eurosta solidaginis*). Each point is based on five pools of eight larvae each. Means not sharing a letter were significantly different (Bonferroni multiple comparison, repeated measures ANOVA, alpha = 0.05).

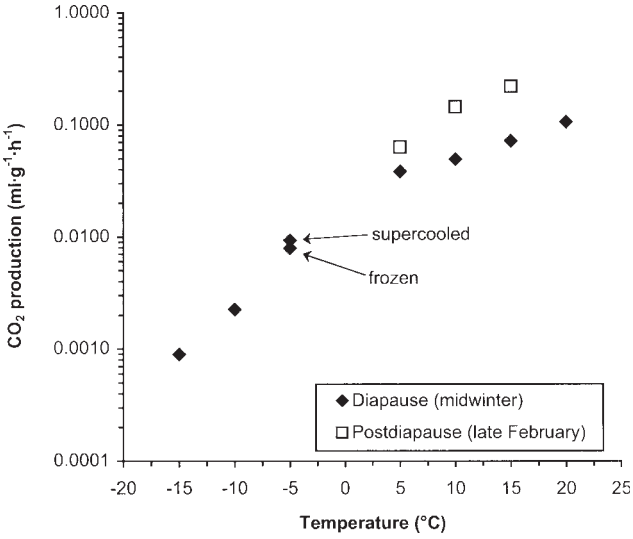

Fig. 3. The effect of temperature on mean (± SEM) metabolic rate (CO_2 production) of third-instar goldenrod gall fly larvae (*Eurosta solidaginis*). Subzero data from this study; data for temperatures above 0°C from Irwin et al. (2001). Bars indicating standard error of the mean are smaller than the symbols.

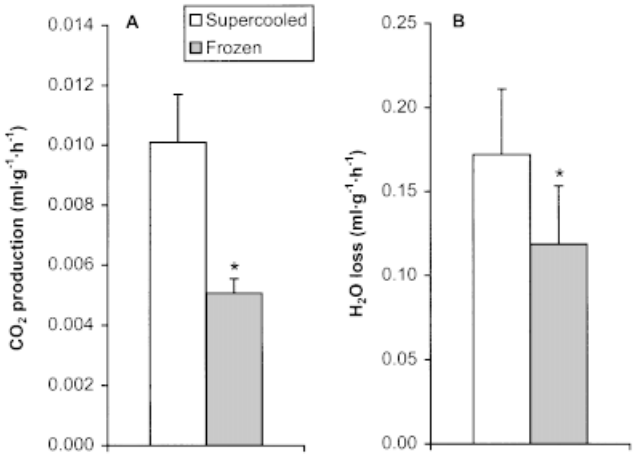

Fig. 4. Metabolic rate (CO_2 production) (**A**) and water loss (**B**) of frozen and supercooled goldenrod gall fly larvae (*Eurosta solidaginis*) at –5°C. Data are presented as mean ± SEM. Frozen larvae had significantly lower rates of metabolism and water loss (paired sample *t*-test, $P < 0.05$). Each mean is based on four pools of 15 larvae each.

$CO_2 \cdot h^{-1}$) and thus was removed from further analyses. Each 5°C drop in temperature caused a large and statistically significant reduction in CO_2 production (Fig. 2). If the metabolic rate is plotted on a log-axis, we see that metabolic rates fell consistently with temperature, at least within the subzero range. However, the metabolic rates of frozen larvae were lower than metabolic rates of unfrozen larvae (Fig. 3).

Experiment 3: Metabolic rate and water loss in frozen and supercooled larvae

Freezing reduced both water loss and CO_2 production. Carbon dioxide production fell 47% with freezing ($t = 3.44$, $P = 0.021$) and water loss fell 35% ($t = 5.45$, $P = 0.007$) (Fig. 4). Metabolic rates in this portion of the experiment were quite similar to those measured in Experiment 2 (compare Fig. 4A with Figs. 1A, 2).

DISCUSSION

Our study addressed the question of why some species avoid freezing, while others have mechanisms by which they can survive extensive internal ice formation. By understanding the influence of freezing and supercooling on metabolic rates and water loss of insects, we can form hypotheses regarding selective forces driving the evolution of improved freeze tolerance.

Gall fly larvae rapidly accumulated ice during freezing at –5°C until ~60% of their body water was frozen. This is only slightly lower than the value of 64% measured by Lee and Lewis ('85). However, in our experiment, ice accumulated much more rapidly. This may have been due to our arrangement of the larvae on moist filter paper placed on the bottom of a beaker submerged in the cold bath. This system likely removed the latent heat of fusion more efficiently than the apparatus used by Lee and Lewis ('85), thus accelerating the rate of ice formation.

Upon the initiation of freezing, ice content stabilized more quickly than did metabolic rate. After an equilibrium level of ice content was reached, physiological changes resulting in reductions of the metabolic rate were still occurring. One possibility is that oxygen present in the tissues was gradually consumed; thus, CO_2 production gradually diminished, but eventually stabilized at a low overall level of oxidative metabolism. In fact, the measurable CO_2 production at low, subzero temperatures (at least as low as –15°C) suggests that the tissues remain metabolically active and even carry on some small amount of aerobic metabolism. There are other reports that suggest the existence of metabolic activity in frozen insects. For example, sorbitol concentration continues to increase in *E. solidaginis* larvae frozen at temperatures as low as –15°C (Baust and Lee, '82), and diapause development also occurs in frozen *E. solidaginis* larvae (Irwin et al., 2001).

Our measurements of the metabolic rate of su-

percooled larvae were similar to previous reports for other insects. The metabolic rates of supercooled *E. solidaginis* larvae at –5°C were approximately 10 μl $CO_2 \cdot g^{-1} \cdot h^{-1}$— very close to the value of ~12 μl $O_2 \cdot g^{-1} \cdot h^{-1}$ observed in flour moth larvae also measured at –5°C (Salt, '58), despite the very different analytical methods used between our study and his. However, Kanwisher's unpublished observations (as reported in Asahina, '69) of cecropia pupae at –5°C were considerably lower (~1 μl $O_2 \cdot g^{-1} \cdot h^{-1}$), possibly due to the allometric scaling of metabolic rate in this relatively large insect.

The measurements of metabolic rate in the frozen versus supercooled state were also comparable to earlier reports. Previously only Lundheim and Zachariassen ('93) and the unpublished work of Kanwisher (Asahina, '69) reported measurements of metabolic rate in supercooled and frozen insects. Lundheim and Zachariassen ('93) observed a 69% reduction of metabolic rate of *Upis ceramboides* upon freezing at –5°C, slightly larger than the 47% decrease in our study (Fig. 3). Kanwisher's data and ours both show that, when plotted on a log axis, metabolic rate falls consistently with temperature, even in frozen animals (Fig. 3; Asahina, '69). As an insect's body temperature decreases, more ice forms (Lee and Lewis, '85), which further limits cellular metabolism and thus reduces the metabolic rate.

Although metabolic rates were quite low in the subzero-temperature range, slight differences in microenvironmental temperature may be important for energy conservation. For example, *E. solidaginis* larvae overwintering below the snowpack (as on a fallen goldenrod, *Solidago* spp., stem) consume significantly more energy during cold winter days than others overwintering above the snowpack, ultimately resulting in reduced adult fecundity (Irwin and Lee, unpublished data).

The duration for which larvae remain frozen would also influence energy consumption. Based on air temperature data collected for the winter of 1998–1999 (Irwin and Lee, unpublished data), we can calculate the difference in energy metabolism through the winter if larvae were to remain supercooled or frozen. If we assume a supercooling point of –10°C (Irwin and Lee, unpublished data) and a melting point of –2°C (Lee and Lewis, '85), a larva would have been frozen for approximately 391 hours (16.3 days) between November of 1998 and April of 1999. (Note that although a larva would freeze at –10°C, it would not thaw until warmed to –2°C.) If the percent reduction in metabolic rate with freezing at –5°C (Fig. 4) holds true for the other subzero temperatures, then a larva would be reducing its energy consumption by 47% over this 391 hr period. Moreover, in nature, the moist gall tissues inoculate the larvae at temperatures at least as high as –2.5°C (Hankison and Lee, unpublished data) so that larvae are frozen for longer periods, and thus conserve even more energy than is estimated when assuming that freezing occurs at the supercooling point. Under laboratory conditions, Layne and Kuharsky (2001) did not find a difference in the body mass or lipid content of supercooled and frozen larvae held for eight weeks at –5°C, but the high variability in body mass may have prevented them from detecting these differences. Layne and Kuharsky (2001) did observe that supercooled larvae were less likely to reach adulthood that those held frozen. Studies of effects more subtle than body size, such as reproductive output (Irwin and Lee, 2000; unpublished data), may allow us to detect the benefits of energy conservation by freezing.

There is considerable evidence that elevation of the supercooling point enhances the ability to survive freezing by preventing rapid ice formation (Zachariassen and Hammel, '76), but we suggest that energy savings may be another reason for insects to increase their supercooling point. Potential energy savings associated with freezing may explain why *E. solidaginis* larvae overwintering in Minnesota increase their supercooling points during the winter when the probability of freezing is highest (Baust and Lee, '81) and why *E. solidaginis* larvae, despite their great resistance to water loss, are highly susceptible to inoculative freezing (Layne et al., '90). These two factors (elevated supercooling point and susceptibility to inoculation) would increase the amount of time larvae are frozen, and thus enhance energy conservation during the winter. Energy conservation during the larval stage has a direct effect on potential reproductive output of female *E. solidaginis* upon reaching adulthood (Irwin and Lee, 2000); thus, one would expect strong selection for an elevation of the supercooling point in this species.

Our data also support Lundheim and Zachariassen's ('93) finding that freezing reduces water loss in insects. Their study reported that the rate of water loss of *Pytho depressus*, a Scandinavian bark beetle, fell 29% with freezing at –5°C, a similar result to the 35% reduction in *E. solidaginis* larvae under similar conditions (Fig. 4). Layne and

Kuharsky (2001) also detected a reduced rate of water loss in frozen *E. solidaginis*, but only after six weeks of exposure—not after shorter exposures or at the subsequent eight weeks of exposure. Given that *E. solidaginis* larvae have very low rates of water loss even above freezing (on par with that of desert beetles; Ramløv and Lee, 2000), and that galls are frequently soaked by precipitation (Layne, '93), water loss of *E. solidaginis* in nature may be at negligible levels.

We conclude from this study that freezing may be advantageous by improving the conservation of energy through the winter. Further studies should examine the duration and conditions of freezing in nature and its influence on subtle measures of organismal fitness such as emergence rates, ability to reproduce, fecundity, and viability of offspring. Given the low rates of water loss in unfrozen *E. solidaginis*, it is unlikely that reductions in water loss induced by freezing play an important role in water conservation.

ACKNOWLEDGMENTS

We thank Shala Hankison for assistance with collecting the ice content data and members of the Cryolab for help collecting galls.

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