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Acquisition of freezing tolerance in early autumn and seasonal changes in gall water content influence inoculative freezing of gall fly larvae, *Eurosta solidaginis* (Diptera, Tephritidae)

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Abstract

We examined seasonal changes in freeze tolerance and the susceptibility of larvae of the gall fly, *Eurosta solidaginis* to inoculative freezing within the goldenrod gall (*Solidago* sp.). In late September, when the water content of the galls was high (~55%), more than half of the larvae froze within their galls when held at -2.5 °C for 24 h, and nearly all larvae froze at -4 or -6 °C. At this time, most larvae survived freezing at ≥ -4 °C. By October plants had senesced, and their water content had decreased to 33%. Correspondingly, the number of larvae that froze by inoculation at -4 and -6 °C also decreased, however the proportion of larvae that survived freezing increased markedly. Gall water content reached its lowest value (10%) in November, when few larvae froze during exposure to subzero temperatures ≥ -6 °C. In winter, rain and melting snow transiently increased gall water content to values as high as 64% causing many larvae to freeze when exposed to temperatures as high as -4 °C. However, in the absence of precipitation, gall tissues dried and, as before, larvae were not likely to freeze by inoculation. Consequently, in nature larvae freeze earlier in the autumn and/or at higher temperatures than would be predicted based on the temperature of crystallization (T_c) of isolated larvae. However, even in early September when environmental temperatures are relatively high, larvae exhibited limited levels of freezing tolerance sufficient to protect them if they did freeze.

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1. Introduction

For many ectotherms, winter survival is closely tied to microclimatic conditions within their hibernacula (Danks, 1991). Since winter cold is often linked to low relative humidity, moist hibernacula may provide important protection from severe desiccation. Hibernacula may also buffer against exposure to extreme cold and, thereby, avoid cooling to the temperature of crystallization (T_c), at which ice forms spontaneously within body fluids. Consequently, laboratory investigations of isolated ectotherms, removed from their hibernacula,

may not necessarily reflect responses that occur in nature.

Contact with environmental ice may cause inoculative freezing of body fluids as external ice lattice propagates through a body orifice, or directly across the integument (Salt, 1963). For freezing intolerant species that rely on various physiological mechanisms to depress their T_c , inoculative freezing may limit an organism's supercooling capacity and, thereby, reduce winter survival. In contrast, freezing tolerant ectotherms commonly increase their chances of survival by producing ice nucleating proteins or other ice nucleators to insure that freezing begins at high subzero temperatures (Duman, 2001; Zachariassen, 1985). The induction of internal freezing by ice inoculation is the most effective mechanism known to induce freezing at warm temperatures because ice may form as soon as environmental temperatures reach the melting point of body fluids; whereas, even the most efficient ice nucleating proteins or ice nucleating

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active bacteria do not induce freezing until the organism has supercooled several degrees (Lee, 1991; Lee et al., 1991, 1995b).

The larvae of the goldenrod gall fly, *Eurosta solidaginis*, over winter within spherical stem galls on goldenrod (*Solidago* spp.). Larvae prepare for winter by accumulating substantial lipid stores before they enter diapause in mid-October (Irwin et al., 2001). Through the autumn they also acquire freezing tolerance to <-40 °C, in part due to the accumulation of glycerol, sorbitol and low-molecular-weight cryoprotectants (Baust and Lee, 1982). Accumulation of glycerol is triggered by drying of the gall tissues as the aboveground portions of the plants senesce and die in early autumn (Rojas et al., 1986). Concomitantly with the drying of their galls, overwintering larvae also acquire exceptional levels of desiccation resistance (Ramløv and Lee, 2000).

During the summer and early in the autumn larvae of *E. solidaginis* that have been removed from their gall are freeze intolerant and supercool to -13 and -17 °C before reaching their T_c (Morrissey and Baust, 1976). In mid-autumn, T_c values increase markedly to approximately -9 °C and remain unchanged until pupariation in the spring. This increase is associated with the appearance of calcium phosphate crystals within the Malpighian tubules that have sufficient ice nucleating activity to account for the T_c of the larvae (Mugnano et al., 1996). In addition to the endogenous ice nucleator, the larvae are also susceptible to inoculative freezing. Moistened larvae freeze in the range of -3 to -5 °C (Bale et al., 1989a; Layne et al., 1990; Shimada et al., 1993). Furthermore, Layne et al. (1990) demonstrated inoculative freezing of larvae is caused by ice that forms within gall tissues. Growing galls with a high water content readily induced the freezing of their larvae compared to dry, dead galls (Layne, 1993; Layne et al., 1990).

Seasonal changes in the plant's tissues may have a dynamic impact on the microenvironment of galling insects that not only feed and develop but also remain within their hosts through the winter. The purpose of this study was two-fold. Since little is known concerning the initial acquisition of freeze tolerance in *E. solidaginis* larvae, we characterized the seasonal time-course of cold tolerance paying special attention to survival of freezing at high subzero temperatures early in the autumn. Secondly, we examined the relationship between the water content and the temperature of crystallization (T_c) of goldenrod galls and their larval inhabitants within the context of environmental temperatures and precipitation that they experience naturally during the autumn and winter.

2. Methods and materials

Stem galls from goldenrod containing *E. solidaginis* larvae were collected from the Miami University Ecol-

ogy Research Center, Oxford, Ohio, every 3–4 weeks from late September through December 1999, with a final collection made in February 2000. Dry collection refers to sampling dates in which there had not been rain or other precipitation during the previous 7 days. The water content of galls ($n = 30$) or larvae ($n = 30$) was determined by weighing them individually on a Mettler balance (AG 245), then drying them at ~ 65 °C to a constant mass.

Larval T_c was measured by placing individual larva ($n = 10$) inside a 2-ml polyethylene microcentrifuge tube. The tube's opening was plugged with plastic foam, which anchored the sensing junction of a thermocouple against the larva. Each tube was then placed into a glass test tube suspended in a refrigerated bath and, after equilibrating to 0 °C, was cooled at a rate of 1.0 °C min^{-1} . The T_c was identified by the appearance of an exotherm, caused by the freezing of water in the larvae; the larval body temperature was monitored at 5-s intervals using a multi-channel data logger (Omega RD3752). Gall T_c was measured ($n = 9-10$) by taping the sensing junction of a thermocouple to the gall, then placing galls into a large beaker suspended in a refrigerated bath. Galls were first equilibrated to 0 °C, and then cooled until an exotherm was observed.

Larval survival of freezing or exposure to subzero temperatures was evaluated by exposing isolated larva placed individually in 0.5-ml microcentrifuge tubes ($n = 8$) or galls containing larvae ($n = 30$) to -2.5 , -4 , -6 or -20 °C for 24 h; this duration of exposure was selected because it more closely approximated the short-term cold exposures that occur in the autumn. Since galls often do not contain larvae, we split galls, checked for the presence of larvae and then resealed the galls with larvae in their original position using a small amount of tape following methods used by Irwin and Lee (2000). As galls may not spontaneously freeze immediately when exposed to these temperatures, aerosol coolant was briefly applied to the gall exterior after they equilibrated to the target temperature to initiate ice nucleation within the plant tissue and standardize treatment conditions. After 24 h larvae and galls were removed from the bath, opened and immediately classified as either frozen or unfrozen based on whether the plant tissue was hardened by ice formation, and whether ice could be seen melting following removal from the refrigerated bath. Frozen larvae appeared yellowish (compared to unfrozen larvae) and ice could be seen and felt beneath the cuticle. Larvae were placed in petri dishes and held at 15 °C for 24 h when survival was assessed; larvae that moved spontaneously or in response to gentle prodding were judged to have survived.

The osmolality of larval hemolymph ($n = 8$) was determined using a Wescor 5500 vapor pressure osmometer. After puncturing the cuticle, 10 μl of clear hemolymph was drawn into a capillary tube. The time course

for the freezing of galls and their larvae ($n = 30$ for each time interval) was determined by cooling galls to $-4\text{ }^{\circ}\text{C}$ and removing treatment groups after 1, 2, 4, 5, 6 and 24 h. Water content was then obtained for each gall and larva as described above.

To measure field temperatures experienced by the galls, probes attached to StowAway and Hobo-Pro data loggers were inserted into small holes punched into galls ($n = 11$). Data loggers were programmed to record gall temperature every 15 min from 18 October 1999 through 8 March 2000. The software programs Logbook 2.01 and BoxCar Pro PPC 3.5d2 were used to download data loggers and obtain printouts of gall field temperatures. As some loggers failed during the field season, complete field temperatures were not obtained for all galls. T_c of these instrumented galls was determined by identifying exothermic events in the temperature record. The lowest temperature to which the gall cooled prior to each exotherm was reported as the T_c . The duration of the exotherm was determined as the time from the beginning of the exotherm until the gall temperature returned to the T_c value.

Instat v.2.00 was used to analyze gall and larval T_c values and water content with a Kruskal–Wallace test and to analyze regressions comparing the number of larva freezing at different temperatures and water contents. Two-factor ANOVAs were used to determine effects of temperature and month on larval survival and freezing within galls were performed using Statview v.5.0.

3. Results

3.1. Seasonal changes in gall water content and its effect on inoculative freezing of larvae

In September, goldenrod plants were in flower, and the stem and leaves were green. At this time the water content of galls was $54.7 \pm 1.5\%$ (mean \pm SEM). For dry collections (i.e., no precipitation during the previous 7 days) from September through November the water content of galls decreased markedly as the above ground portions of the plant senesced, gradually turned brown and dried (Fig. 1A). In the November 16 collection, the water content of the galls reached its lowest level of $10.5 \pm 0.6\%$. In contrast, larval water content remained constant ($\sim 60\%$) throughout the entire sampling period (Fig. 1A, Kruskal–Wallis, $KW = 5.19$, $P = 0.27$).

The T_c of galls decreased from $-4.7 \pm 0.2\text{ }^{\circ}\text{C}$ in September to $-7.6 \pm 0.2\text{ }^{\circ}\text{C}$ in October (Fig. 1B). For dry collections in November and February no T_c values were reported because no exotherms were observed even though galls were cooled to $-20\text{ }^{\circ}\text{C}$; presumably this result was due to the very low water content of the galls. The T_c of isolated dry larvae was significantly lower dur-

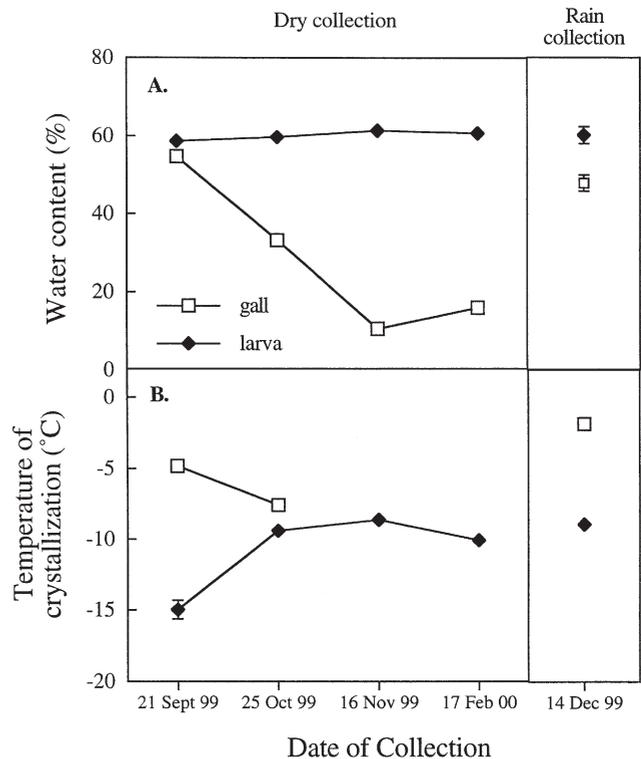


Fig. 1. Seasonal changes in water content and the temperature of crystallization (T_c) for larvae of *Eurosta solidaginis* and their galls. Dry collection indicates no precipitation within the previous 7 days. Rain collection indicates several days of rain immediately prior to gall collection. (A) Mean water content (\pm SEM) of galls and larvae. (B). Temperature of crystallization (T_c) of galls and larvae. During November and February gall T_c was undetectable due to low water content of the plant tissues.

ing September compared to T_c values from all other sampling periods (Fig. 1B, ANOVA, $F_{4,35} = 43.52$, $P < 0.0001$).

Concomitant with seasonal drying of the galls the proportion of larvae freezing after 24-h exposure to -2.5 , -4 and $-6\text{ }^{\circ}\text{C}$ also decreased (ANOVA, $F_{4,8} = 12.407$, $P = 0.0017$). At these temperatures inoculative freezing of larvae was high during September and decreased to 0% by the November sampling period. The percent of larvae freezing at -6 and $-4\text{ }^{\circ}\text{C}$ was positively correlated with the mean water content of the galls during the sample period (linear regression: $-6\text{ }^{\circ}\text{C}$, $F_{1,3} = 26.43$, $P = 0.036$; $-4\text{ }^{\circ}\text{C}$, $F_{1,3} = 25.11$, $P = 0.038$). Throughout the study all larvae froze when held at $-20\text{ }^{\circ}\text{C}$, a result that is consistent with T_c values between -15.0 and $-8.6\text{ }^{\circ}\text{C}$ for isolated larvae (Figs. 1 and 2).

Through the autumn the T_c of galls was directly correlated with the water content of the gall between 15 and 85% (Fig. 3, $P < 0.0001$, $r^2 = 0.20$, $n = 72$). For galls with a water content $< 15\%$ freezing exotherms were not detected. In addition, when gall water content fell below approximately 20%, less than 1% of the larvae froze when exposed to $-4.0\text{ }^{\circ}\text{C}$ for 24 h (Fig. 3); this result

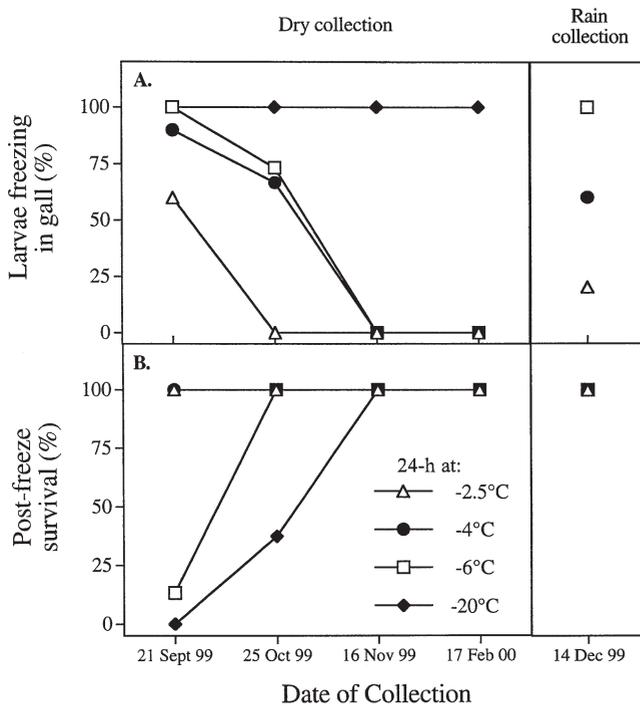


Fig. 2. Seasonal changes in the proportion of *Eurosta solidaginis* larvae freezing (2A) and surviving (2B) in galls cooled to -2.5 , -4 , -6 or -20 °C for 24 h. Dry collection indicates no precipitation within the previous 7-day period. Rain collection indicates precipitation within the previous 7-day period.

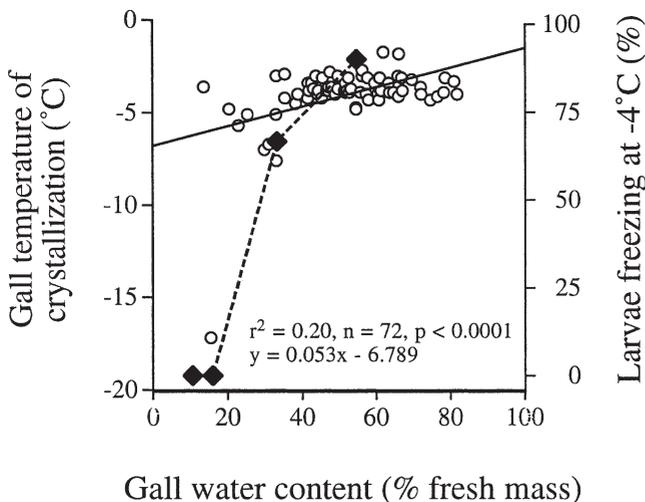


Fig. 3. Water content of the gall in relation to its temperature of crystallization (T_c) and to the proportion of *Eurosta solidaginis* larvae that froze within its tissues. Open circles indicate the T_c of the gall. Closed diamonds indicate the proportion of larvae freezing at -4 °C.

suggests that insufficient ice was present within the gall tissue to induce freezing in the larvae.

3.2. Effect of precipitation on gall water content and T_c values

Precipitation increased the water content and T_c of the gall, and restored the gall's capacity to induce inoculat-

ive freezing in the larvae at high subzero temperatures (Fig. 1). In December, galls were collected following several days of rain causing the galls to rehydrate to $48.0 \pm 0.1\%$, a value which was comparable to green galls collected in September (Fig. 1A, $KW = 97.353$, $P < 0.0001$). The mean T_c of these rehydrated galls was -1.8 ± 0.4 °C, a value which was higher than T_c s for galls from all dry collections (Fig. 1B, $KW = 23.572$, $P = 0.0001$). Furthermore, the capacity of the galls to induce inoculative freezing of larvae was restored; within the rehydrated galls all larvae froze when exposed to -6 or -20 °C for 24 h, while 60% of the larvae froze when held at -4 °C (Fig. 2A). However, rehydration of gall tissues had no effect on the T_c value of isolated larvae (Fig. 1B).

3.3. Seasonal acquisition of freeze tolerance in *E. solidaginis* larvae

Interestingly larvae collected in the earliest sample in September were already tolerant of freezing for 24-h at -2.5 and -4 °C, although few survived freezing at lower temperatures (Fig. 2). During the next month larvae increased their freezing tolerance markedly with all larvae surviving freezing for 24-h at -6 °C and $>35\%$ survival at -20 °C. By December 14 all larvae survived the lowest test temperature of -20 °C. This pattern of increasing cold tolerance matched steady increases in hemolymph osmolality (indicative of the accumulation of sorbitol and other low molecular weight cryoprotectants) through the autumn, which reached 911 mosmol/kg in December (Table 1).

3.4. Temporal relationship between the freezing of the gall and inoculative freezing of its larvae

To examine the temporal relationship between the freezing of the gall and the subsequent inoculative freezing of the larvae within its tissues, we placed galls at -4 °C and removed groups of 15–20 larvae after 1, 2, 4, 5, 6 or 24 h of exposure (Fig. 4). The galls used in this study were collected in mid-October with a water content of approximately 45%. Since at this time the T_c of larvae removed from their gall was near -9 °C, we selected the test temperature of -4 °C to insure that the only way that larvae would freeze was by inoculative contact with ice in the gall tissues. This assumption was confirmed by the fact that at no time were frozen larvae found within unfrozen galls.

During the first hour of exposure 60% of the galls froze, while $<15\%$ of the larvae within their tissues did so. Within 6 h, all galls had frozen. However, larvae lagged behind with only 75% freezing after 6 h. Even after 24 h of exposure 10% of the larvae remained unfrozen.

Table 1

Body mass ($n = 30$) and hemolymph osmolality ($n = 8$) of field-collected larvae of *Eurosta solidaginis* during the autumn and winter

	Date of Collection (1999–2000) ^{a,b}				
	21 Sept	25 Oct	16 Nov	14 Dec	17 Feb
Larval mass (mg)	49.0 ± 2.0	49.1 ± 2.2	39.2 ± 2.5	40.8 ± 2.0	43.5 ± 2.4
Hemolymph osmolality(mosmol/kg)	636 ± 21	730 ± 16	825 ± 21	911 ± 17	895 ± 24

^a All samples were collected during dry periods (i.e., no precipitation during the previous seven days) with the exception of the December sample that was collected following several days of rain.

^b Values are mean ± SEM.

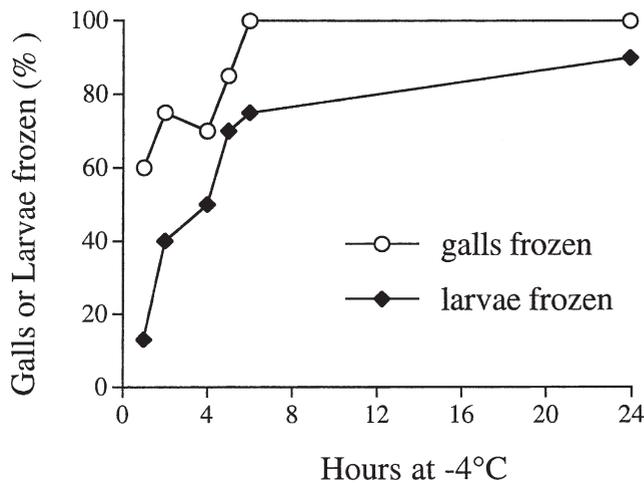


Fig. 4. Temporal relationship between the freezing of the gall and inoculative freezing of its larvae during 24 h of exposure to -4.0 °C. Each point is based on $n = 15$ –20 larvae.

3.5. Environmental temperatures and T_c of galls in the field

To better understand the effect of early frosts experienced by the galls and larvae naturally, we inserted temperature probes, attached to temperature loggers, into small holes in galls on standing stems of goldenrod ($n = 7$). We analyzed temperature records for four of the coldest days in October 1999 (Table 2). Exotherms associated with the freezing of the galls were clearly evident from these recordings. Mean T_c for the galls varied between -2.3 and -5.2 °C while individual T_c values ranged between -1.3 and -10.5 °C. The mean cooling rate of the galls prior to freezing varied between -0.02 and -0.15 °C/min. The duration of the freezing exotherm as ice formed in the gall varied for individual galls from as little as 1 h 18 min to 4 h 20 min with a mean duration of 2–3 h. Generally, upon the completion of the freezing exotherm, the temperature of the gall continued to decrease reaching temperatures as much as 4.7 °C below the T_c of the gall. However, in some instances gall temperature never dropped below the T_c .

To estimate the number of larvae that would be expected to freeze under the conditions described in

Table 2 we used our laboratory-based data on T_c s and inoculative freezing of galls and larvae that were collected from the same field on October 25, 1999 (see Figs 1 and 2). If the estimate was based on the T_c of isolated larvae <5% of the larvae would be expected to freeze on these 4 days (Table 2). However, if we assume that larvae were induced to freeze by inoculation when the gall froze then we would predict that 45–50% more of the larvae would freeze on 3 of the 4 days. Lastly, if the number of larvae that froze was based on the minimum temperature of the gall our estimate increased to 69–79%. The mildest frost occurred on the fourth day, October 24, on which no larvae were expected to freeze based on the T_c of either the gall or larvae, however based on the minimum gall temperature it is likely that 35% of the larvae would freeze (Table 2).

4. Discussion

Recent reports have highlighted the important role that microclimatic conditions, and interactions between cold-hardy ectotherms and their hibernacula have on winter survival. Collembola and other soil invertebrates undergo a protective dehydration that lowers the equilibrium freezing point of their body fluids until it equals the water vapor pressure of surrounding ice-laden soil (Holmstrup and Zachariassen, 1996; Holmstrup et al., 2002). The innate capacity of painted turtle hatchlings to supercool and survive extreme cold may be compromised by the presence of ice nucleating agents in the soil (Costanzo et al., 2000). Warm hibernacula can reduce winter survival and reproductive output by elevating the metabolic rate and excessively depleting energy reserves (Irwin and Lee, 2000, 2003). The present study documented the dynamic role that moisture conditions within the gall have on overwintering gall fly larvae.

4.1. Seasonal changes in gall water content and its effect on inoculative freezing of larvae

Gall water content decreased markedly from early- to mid-autumn. The decreases observed in our study were

Table 2

Temperature and temperature of crystallization (T_c) recorded from galls in the field during four of the coldest days in October 1999^a

	Date 19 Oct	20 Oct	24 Oct	26 Oct
Gall T_c (°C)	-4.2 ± 0.8 (-8.7, -2.8)	-5.2 ± 1.0 (-10.5, -2.2)	-2.3 ± 0.8 (-6.7, -1.3)	-3.8 ± 0.8 (-8.2, -1.9)
Duration of freezing exotherm in gall (h:min)	3:19 ± 0:13 (2:35, 4:21)	2:09 ± 0:16 (1:18, 3:24)	2:38 ± 0:04 (2:17, 2:47)	3:19 ± 0:11 (2:20, 4:00)
Rate of cooling (°C/min) prior to freezing of the gall	-0.10 ± 0.01 (-0.17, -0.07)	-0.15 ± 0.02 (-0.22, -0.10)	-0.02 ± 0.001 (-0.02, -0.03)	-0.10 ± 0.02 (-0.04, -0.17)
Minimum temperature (°C) after the gall froze	-5.8 ± 0.8 (-10.4, -4.4)	-6.5 ± 0.9 (-11.2, -4.5)	-2.8 ± 0.7 (-6.9, -1.4)	-5.7 ± 0.8 (-10.1, -4.4)
<i>Estimated % of larvae freezing^b</i>				
Based on T_c of isolated larvae	5	10	0	0
Based on T_c of the gall	50	62	0	47
Based on the minimum temperature to which gall cooled after freezing	70	79	35	69

^a Values shown are mean ± SEM, $n = 7$; minimum and maximum values are in parentheses.

^b Estimated percentage of larvae freezing is based on laboratory studies of inoculative freezing and T_c determinations of isolated larvae collected from the same field on October 25, 1999 (see Figs 1 and 2).

similar to ones for *E. solidaginis* populations in New York and Texas (Baust et al., 1979; Layne, 1993; Layne et al., 1990). The water content of galls was 63–66% for green galls in early autumn collections decreasing to a minimum of 17% for dried galls in mid-winter (Baust et al., 1979). During September and October the decrease in gall water content was associated with a decrease in gall T_c from -4.7 to -7.6 °C (Figs 1 and 3). However, once the gall tissues had reached a critical level of dryness (~18–20%), we were unable to detect a T_c for the galls, and larvae were no longer induced to freeze by ice within the gall tissues (Figs 1 and 2). The small amount of water remaining in the galls either did not freeze because it was “bound” and therefore unfreezeable (Zachariassen, 1991), or if it did freeze the heat released was not detectable using our sensors and resulted in insufficient ice to inoculate larvae within the central chamber of the gall.

Drying of the gall did not affect the relative water content of the larvae (Fig. 1). Previous studies also found that larvae maintain their water content near 60–64% during the autumn and winter (Layne and Kuharsky, 2001; Lee et al., 1995a; Ramløv and Lee, 2000; Rojas et al., 1986). However, larvae do respond to the drying of their hibernaculum by producing glycerol (Rojas et al., 1986) and by markedly increasing their resistance to desiccation (Ramløv and Lee, 2000; Williams, et al., manuscript in preparation).

4.2. Effect of precipitation on gall water content and T_c values

The potential for inoculative freezing of the larvae was not limited to the autumn. Dry galls readily became rehydrated by contact with rain or melting ice and snow, which also restored their capacity to induce larval freezing (Fig. 2). After several days of rain, galls collected in December had a water content of 48% compared to only 10% for dry galls in November (Fig. 1). Throughout the winter, goldenrod galls in Pennsylvania and New York frequently rehydrate to >40% with some galls exceeding 60% (Layne, 1993; Layne and Medwith, 1997). Consequently, cycles of larval freezing and thawing depend not only on the larval T_c , but also on the hydration status of gall tissues.

Larvae remain susceptible to inoculative freezing until they pupariate in the spring (Shimada et al., 1993). At this time, the T_c of isolated larvae decreases to <-17 °C due to the disappearance of ice-nucleating calcium phosphate crystals in the Malpighian tubules (Mugnano et al., 1996). Although larvae lose their freeze tolerance during metamorphosis, the newly formed puparium is resistant to inoculative freezing (Shimada et al., 1993). Similarly, diapausing pupae of *Sarcophaga crassipalpis*, which may contact ice when they overwinter in the soil, are more resistant to inoculative freezing than non-diapausing ones (Kelty and Lee, 2000). Increased resist-

ance to inoculative freezing is associated with seasonal changes in the integument and in hemolymph and epidermal antifreeze proteins (Olsen et al., 1998; Duman, 2001).

4.3. Seasonal acquisition of freeze tolerance in *E. solidaginis* larvae

Most freezing tolerant insects only survive freezing in the winter and must undergo a period of cold-hardening in the autumn. Previous studies of the seasonal acquisition of freezing tolerance in *E. solidaginis* larvae generally used fairly extreme criteria requiring survival at temperatures from -25 to -40 °C (Bale et al., 1989b; Bennett and Lee, 1997; Lee and Lewis, 1985; Lee et al., 1993). In contrast, our approach was to use relatively mild criteria for freezing tolerance to allow us to identify early stages in seasonal cold-hardening.

In our first sample, collected on September 21, the galls were green and environmental temperatures were relatively high (~ 20 °C). Nonetheless, larvae were already tolerant of freezing at -2.5 and -4 °C, suggesting that seasonal cold-hardening had begun already (Fig. 2). At this time hemolymph osmolality was 636 mosmol/kg; this value is approximately 100–150 mosmol/kg higher than Layne (1991) reported for hemolymph of larvae collected in August, and suggests that cryoprotectant accumulation had begun already in our September-collected larvae. Our data documenting modest levels of freeze tolerance in early autumn are consistent with Layne's report (1991) for a more northerly, New York population of *E. solidaginis* that survived freezing at -6 °C in September.

For freeze tolerant species, insuring that ice forms at temperatures slightly below 0 °C is critical for survival (Duman, 2001; Lee et al., 1996). The centipede *Lithobius forficatus* survives freezing to -6 °C, only if ice nucleation is induced by contact with ice at -1 °C; if the centipede supercools slightly and freezing begins spontaneously at -3 °C it does not survive (Tursman et al., 1994). An even more dramatic example is the fly larva *Chymomyza costata* in which inoculative freezing is necessary to attain maximum levels of freeze tolerance (Shimada and Riihimaa, 1988). Supercooled larvae that froze at -20 °C were unable to develop normally. In contrast, larvae that inoculatively froze at -2 °C could survive cooling to -70 °C. Induction of freezing at high subzero temperatures slows the rate of ice growth and allows the organism to make physiological adjustments that moderate the deleterious mechanical and osmotic stresses associated with freezing (Lee et al., 1996; Zachariassen, 1985). In early autumn when *E. solidaginis* larvae have not yet acquired their full complement of cryoprotectants, the protection afforded by inoculative freezing might be especially important in promoting its capacity to survive freezing.

4.4. Significance of hydration level of gall on larval freezing tolerance

Relatively low rates of cooling and freezing are important for freeze tolerance in a number of insects including *E. solidaginis* larvae (Bale et al., 1989b; Miller, 1978; Shimada and Riihimaa, 1988). Layne (1993) reported that hydrated galls cooled more slowly than desiccated galls. In addition to low rates of cooling, the high water content of galls in September would result in larger and more protracted exotherms as the galls froze; for the larvae this would slow cooling, even temporarily stopping cooling or causing mild warming. This exothermic release would also reduce the rate of ice formation within larval tissues.

During mild frosts, the heat released as the galls freeze may prevent larvae from freezing. In October, exotherms from freezing galls lasted for 2–3 h (Table 2). Since the September-collected galls had considerably more water than in October-collected galls (Fig. 1), the heat released as they froze would be greater and may prevent larvae from freezing inoculatively during mild frosts of short duration. This idea is supported by the data of Fig. 4; after 4 h of exposure to -4 °C, 75% of the galls had frozen, while only 50% of the larvae within these galls froze. When, and if, larvae freeze by inoculation is also influenced by the nature of the insect's cuticle, the temperature of exposure, and the duration of exposure (Salt, 1963).

Even if larvae freeze, survival is related to the amount of body water that freezes; mortality is often associated with the freezing of $\sim 65\%$ body water (Costanzo et al., 1995; Storey and Storey, 1988). Lee and Lewis (1985) found that less than 40% of larval water froze after 4 h at -23 °C. In the present study (Fig. 1, Table 2), an early season frost would cause galls to freeze at ~ -5 °C and release an exotherm that would raise the gall temperature by 1–3 °C for several hours; under these conditions even if the larvae began to freeze only a small, innocuous amount of internal ice would form (for a detailed discussion of factors affecting ice content in ectotherms see Claussen and Costanzo, 1990).

Plant galls provide a unique, and in some cases a dynamic, hibernaculum for overwintering insects. In the case of *E. solidaginis*, contact with gall tissues may induce larvae to freeze inoculatively at higher temperatures and much earlier in the season than predicted by the T_c of isolated larvae and prevailing environmental temperatures. Furthermore, throughout the winter contact with rain or melting ice can cause gall tissues to rehydrate rapidly and restore their capacity to induce inoculative freezing in larvae. These data further support the importance of understanding links between water relations, including desiccation resistance, and cold tolerance (Block, 1996; Danks, 2000; Ring and Danks, 1994).

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