The Journal of Experimental Biology 207, 4407-4414
Published by The Company of Biologists 2004
doi:10.1242/jeb.01320

Partial link between the seasonal acquisition of cold-tolerance and desiccation resistance in the goldenrod gall fly *Eurosta solidaginis* (Diptera: Tephritidae)

Jason B. Williams*, Naomi C. Ruehl and Richard E. Lee, Jr
Department of Zoology, Miami University, Oxford, Ohio 45056, USA
*Author for correspondence (e-mail: willia37@muohio.edu)

Accepted 30 September 2004

Summary

Possible links between seasonal increases in cold-tolerance and desiccation resistance were examined in field-collected larvae of the goldenrod gall fly, *Eurosta solidaginis*. From 20 September to 30 October 2001, larvae exhibited a gradual increase in cold-tolerance culminating in 100% survival of freezing at –20°C for 24 h. The increase in cold-tolerance was probably due to a concomitant increase in cryoprotectants as measured by hemolymph osmolality (488–695 mOsmol kg^{-1}). In contrast to the gradual increase in cold-tolerance, larvae exhibited two distinct phases of reduced rates of water loss. The first phase was an abrupt sixfold decrease to 0.57 μg mm^{-2} h^{-1} between 3 and 16 October. The first phase of reduced rates of water loss was not correlated with changes in cold-tolerance; nor was it correlated with hemolymph osmolality and body water content, which remained constant throughout the study. The reduction in rates of water loss during the first phase were probably the result of decreased respiratory water loss as the larvae entered diapause, and possibly reduced cuticular water loss as larvae increased the amount of their cuticular hydrocarbons. Interestingly, the first phase of reduced water loss was associated with, and may have been cued by, a reduction in the water potential of the gall tissues surrounding the larvae. The second phase was a more subtle fourfold reduction in rates of water loss occurring between 16 October and 11 December. In contrast to the first phase, the second phase of increased desiccation resistance correlated closely with increases in hemolymph osmolality (568–870 mOsmol kg^{-1}). The correlation between seasonal increases in hemolymph osmolality and reduction in rates of water loss may represent a link between desiccation resistance and cold-tolerance in this species.

Key words: cold-tolerance, desiccation resistance, goldenrod gall fly, cryoprotectants.

Introduction

Many insects that overwinter in temperate and polar regions must tolerate not only extreme cold but desiccation stress. Recently, several reviews suggested that certain behavioral and physiological adaptations promoting cold-tolerance may also influence, or were originally adaptations for, desiccation resistance (Ring and Danks, 1994; Block, 1996; Danks, 2000). For instance, freeze-tolerant insects use glycerol and other low-molecular-mass polyols and sugars, termed cryoprotectants, to decrease the amount of body water that freezes at a given temperature, thereby preventing excessive cellular dehydration (Baust and Lee, 1981; Storey and Storey, 1992; Zachariassen, 1991). Increased cryoprotectant concentrations may also lower water loss rates by colligatively reducing the vapor pressure deficit between the insect's hemolymph and environmental water vapor (Ring and Danks, 1994; Bayley and Holmstrup, 1999; Sjursen et al., 2001). However, most studies of overwintering insects have focused on how adaptations promote low temperature survival with little attention to the possible effects these adaptations may have on water conservation.

Larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera, Tephritidae), have been used extensively as an insect model for studying freeze tolerance. The gall fly ranges throughout much of the United States and southern Canada where they induce stem galls on goldenrod plants (*Solidago* spp.; Uhler, 1951). Larvae feed on the mature, moist gall tissue throughout the summer. In early autumn, larvae cease feeding (as the goldenrod plant senesces) and overwinter as freeze-tolerant third instar larvae within their dried galls. Gall tissue offers little protection against winter extremes (Layne, 1993). Overwintering larvae experience ambient air temperatures and extremely desiccating conditions above the snow pack, although hydric parameters of the gall may change depending on precipitation (Layne, 1993).

To survive the low temperatures and desiccating conditions of winter, larvae of *E. solidaginis* increase their cold-tolerance during the autumn and have extremely low rates of water loss. In early autumn, few larvae can survive –6°C for 24 h, but as the season progresses larvae readily survive freezing at –20°C (Lee and Hankinson, 2003). The seasonal increase in cold-

tolerance is correlated with the accumulation of the cryoprotectants glycerol and sorbitol, whose synthesis is triggered, respectively, by drying of the gall tissue and low temperature (Baust and Lee, 1982; Rojas et al., 1986; Storey and Storey, 1992). Recently, mid-winter *E. solidaginis* larvae were shown to have extremely low rates of water loss, rates comparable to heavily sclerotized desert beetles (Ramløv and Lee, 2000). However, it is unknown if resistance to water loss changes in this species from early autumn, when gall tissue is fully hydrated, to mid-winter, when their galls can be extremely dry. Also, if seasonal changes in the rate of water loss exist, are these reductions in rate of water loss linked to physiological process of increasing cold-tolerance?

Previous studies examined several parameters of cold-tolerance and their possible link to desiccation resistance in cold-hardy insects collected in mid-winter (c.f. Williams et al., 2002). By contrast, the purpose of the present study was to characterize seasonal changes in cold-tolerance and resistance to water loss in *E. solidaginis* larvae to determine if the acquisition of desiccation resistance is linked to increases in cold-tolerance. To investigate this question we measured survival after exposure to subzero temperatures, hemolymph osmolality as a measure of cryoprotectant production, resistance to water loss, and body water content of field collected larvae from early autumn to mid-winter. To identify possible environmental cues for seasonal increases in cold-tolerance and enhanced desiccation resistance we monitored ambient temperature, gall water content and gall water activity. In conjunction with the field study, we also examined the effect of mild desiccation stress on rates of water loss and cold-tolerance, prior to and after plant senescence and gall drying in the autumn.

Materials and methods

Insect collection

Galls containing third instar larvae of *E. solidaginis* Fitch were collected every other week from 20 September to 14 November 2001 and then again on 11 December 2001 and 15 January 2002 from the Miami University Ecology Research Center in Oxford, Ohio. All tests were initiated within 24 h of gall collection. To standardize for body size, only larvae weighing between 45–55 mg were used in this study.

Environmental and gall measurements

Beginning 1 September 2001, air temperature was monitored by the Miami University weather station located at the Miami University Ecology Research Center approximately 0.2–0.4 km from the collection sites. Because the dried gall tissue offers little insulative value, larval body temperature should closely track ambient air temperatures, particularly on cloudy days or at night (Layne, 1993).

Water activity of the galls was assessed by measuring the total water content of each gall and the water vapor potential of the gall tissue immediately surrounding the larvae. Gall water content was determined by weighing 10 galls that had contained larvae to ±0.1 mg using a Mettler Toledo AG245 balance (Mettler-Toledo Inc., Hightstown, NJ, USA), before and after drying in an oven at 65°C until they reached a constant mass. Water vapor potential of the gall tissue was determined by the psychrometric vapor pressure depression technique described by Hølmstrup and Westh (1994). Immediately after opening an occupied gall, 10–20 mg of gall tissue directly surrounding the larval chamber was transferred to a Wescor C-52 sample chamber (Wescor, Logan, UT, USA) and allowed to equilibrate for 30 min. Water potential was then determined with a Wescor HR 33T Dewpoint Microvoltmeter operated in the dewpoint mode. Measurements were taken on 10 randomly selected galls for the first three testing dates. However, only five of the 10 randomly selected galls on 30 October were moist enough to obtain a reading. No vapor pressures were measured after 30 October because gall tissues were too dry to measure.

Measurement of cold-tolerance

Larval cold-tolerance was assessed by measuring survival rates after exposure to various subzero temperatures. Ten larvae were placed in temperature-controlled baths and cooled at 1°C min^{-1} to either –2, –4, –8, or –12°C. A fifth group was placed in an insulated container that provided a cooling rate of approximately 1°C min^{-1} until it reached equilibrium in a –20°C freezer. After 24 h exposure to a treatment temperature, larvae were warmed to room temperature (~23°C) at 1°C min^{-1}. Larvae were then held for 24 h at room temperature and considered alive if they moved after being gently touched with a blunt probe. The –12°C experimental group was added on 3 October 2001 to increase sensitivity for detecting changes in cold-hardiness. Cold-tolerance tests were not done after 30 October when all larvae survived –20°C for 24 h and were considered to be highly cold-tolerant.

Hemolymph osmolality provided a measure of the seasonal accumulation of cryoprotectants. Hemolymph osmolality (N=10) was determined by drawing 7–10 μl of hemolymph into a capillary tube through a small incision in the larva's cuticle. The hemolymph was then analyzed in a Wescor Vapro 550 Hemolymph Osmometer.

Measurement of desiccation resistance

Resistance to desiccation was examined using measures of water loss rate in units of μg mm^{-2} h^{-1}, and body water content as a ratio of wet mass to dry mass. To determine water loss rates, 10 individuals per test date were weighed to ±0.01 mg to obtain a fresh mass. Larvae were then re-weighed after being desiccated at 5°C over Drierite (W. A. Hammond Drierite Co., Ohio, USA), providing a 4% RH, until they lost 5–10% of their fresh mass. Body water content was determined by placing the desiccated larvae in an oven at 65°C until a constant dry mass was obtained.

Cuticular surface area was estimated from initial wet mass using an equation derived from the best fit line for larvae of known mass and surface area. Surface area was calculated for 10 individuals of varying mass by puncturing the cuticle,

expelling the internal contents by gently flattening the cuticle on millimeter-squared paper, and estimating the surface area (Williams et al., 2002; Ramløv and Lee, 2000). The derived equation was $y=0.912x+4.204$, $r^2=0.804$, where y=surface area in mm^2 and x=mass in mg.

Effect of moderate desiccation stress on cold-tolerance and desiccation resistance

Larvae collected on 5 October and 2 November were used to determine the effects of mild water stress on cold-tolerance and desiccation resistance. Larvae were either held over a saturated solution of sodium sulfate producing a RH of 95% or over a saturated solution of sodium chloride producing a RH of 76% at 15°C. After 10 days of exposure to these conditions, larval cold-tolerance, water loss rate and body water content were measured using the techniques described previously. In contrast to the previous techniques, 15 larvae per treatment were used in these experiments as opposed to 10, and cold-tolerance was determined using only the –8 and –12°C treatment conditions. Larvae collected on 3 October and 30 October as described in the previous sections, were compared with the 95% and 76% RH experimental groups and referred to as field groups.

Statistical analyses

Seasonal data were analyzed using a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. To identify differences between gall and larval water activity for a given date, unpaired *t*-tests were used. When determining the effect of moderate desiccation on cold-tolerance and desiccation resistance, a one-way ANOVA followed by Student–Newman–Keuls test were used to indicate significant differences between treatment groups for a given date. To identify differences in survival between larvae subjected to moderate desiccation stress a chi-squared analysis was used. A significance level of P=0.05 was used for all tests. Linear regression analyses were used to estimate surface area of the larvae, as well as the relationships between events of cold hardening and acquisition of desiccation resistance.

Results

Seasonal acquisition of cold-tolerance and desiccation resistance

Daily minimum air temperatures gradually decreased from early September to late December (Fig. 1). Temperatures decreased to below 5°C for the first time on 6 October and to below 0°C on 7 October. However, minimum daily temperatures were not consistently below 0°C until mid-December.

In contrast to the gradual decrease in air temperature, gall water content decreased dramatically during two weeks in October as the goldenrod plants senesced. Galls were well hydrated from 20 September to 16 October, ranging from 1.9 to 2.1 mg water per mg dry mass (Fig. 2A). However, between 16 October and 30 October, gall water content decreased significantly (P<0.05) to 0.4 mg water per mg dry mass. Gall water content reached a minimum value of 0.2 mg water per mg dry mass on 15 January.

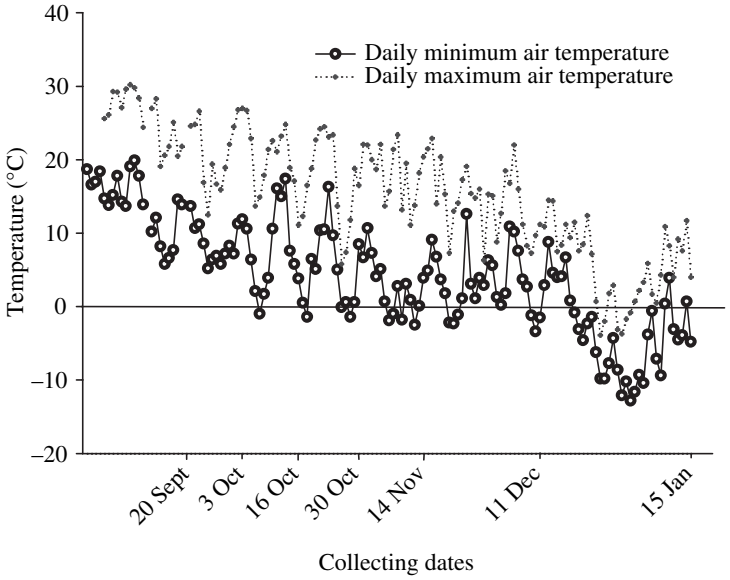

Fig. 1. Daily minimum and maximum air temperatures taken from 1 September 2001 to 31 December 2001 at the weather station located at the Miami University Ecology Research Center, Oxford Ohio, USA.

Even though gall water content decreased markedly during the study period, larval body water content remained statistically unchanged (Fig. 2B). No trends were evident in values for body water content, which ranged between 1.44 and 1.71 mg water per mg dry mass.

As air temperatures decreased through the autumn and winter, larval cold-tolerance gradually increased (Fig. 3). Larvae collected in September already had a modest level of cold-tolerance, as all larvae survived a 24 h exposure to –2°C and 90% survived –4°C; however, no larvae survived –20°C. Larvae were judged to be extremely cold-tolerant on 30 October, as all individuals survived –20°C for 24 h. Notably, throughout the study larvae tolerated temperatures that were 10–20°C lower than were measured in the field.

The gradual increase in cold-tolerance of *E. solidaginis* larvae was mirrored by steady increases in hemolymph osmolality (Fig. 2C). Values for hemolymph osmolality increased significantly (P<0.05) at each successive testing date after 16 October and ranged from an initial value of 488 mOsmol kg^{-1} to the final measure of 967 mOsmol kg^{-1}.

In contrast to the gradual increase in larval cold-tolerance, there were two distinct periods in which water loss rates decreased during the autumn. The first phase of reduced rates of water loss was a substantial sixfold decrease that occurred between 3 October (3.5 μg mm^{-2} h^{-1}) and 16 October (0.6 μg mm^{-2} h^{-1}; Fig. 2D). This initial reduction in the rate of water loss was followed by a second phase in which rates of water loss decreased more slowly over an 8 week period

(Fig. 2D). Even though the second phase of reduced rates of water loss was not as dramatic as the one that occurred in early October, the 3.9-fold decrease was significantly different when the 16 October, 30 October, 14 November and 11 December data were analyzed using an ANOVA followed by Student–Newman–Keuls test (Fig. 2D). Interestingly, the decrease in rates of water loss during this period correlated strongly with increases in hemolymph osmolality levels, r^2= 0.94 (Fig. 4). It is important to note that the data for the larvae collected on 15 January were excluded from this analysis because these individuals were probably no longer in the refractory phase of diapause (Irwin et al., 2001).

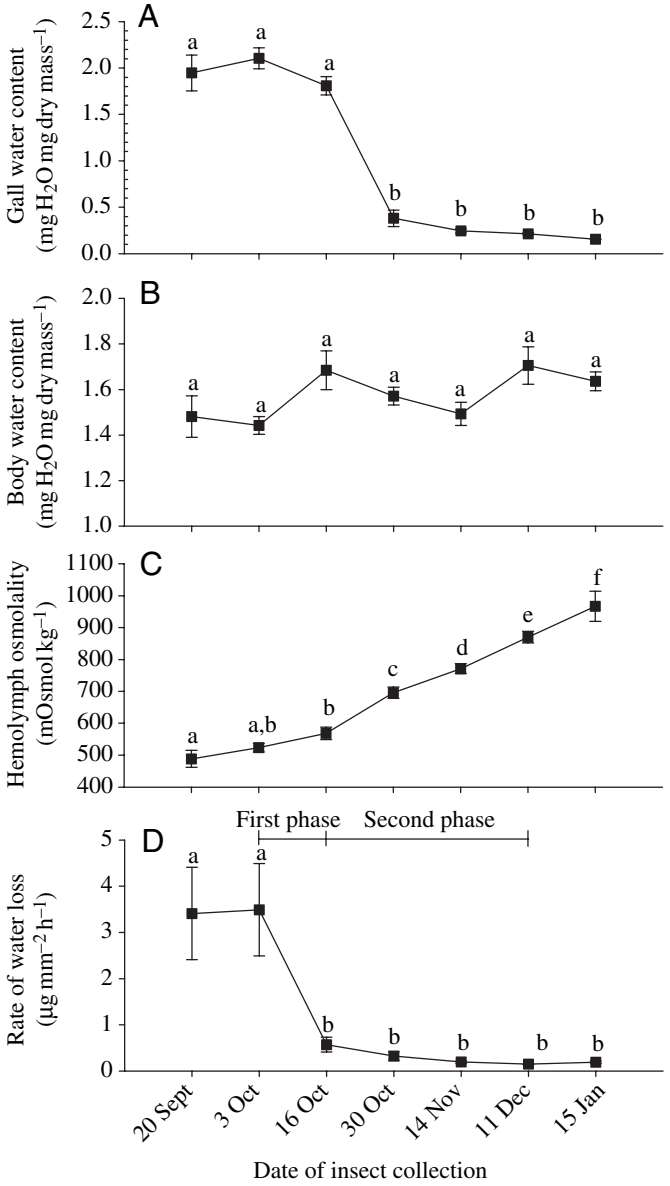

Fig. 2. Gall water contents (A), body water contents (B), hemolymph osmolalities (C) and rates of water loss (D) for *E. solidaginis* larvae collected from 20 September 2001 to 15 January 2002. Data points not sharing a letter are significantly different (P<0.05). Values are mean ± S.E.M., N=10.

The water potential of the gall tissue decreased from 20 September to 16 October, ranging between –9.1 and –12.7 bars (Fig. 5). By 30 October, gall tissue was considerably drier, only 5 of the 10 randomly selected galls were moist enough to obtain a measure of water potential. Water potential of the gall tissue was significantly higher (P<0.05) than the water potential of larval hemolymph on 20 September and 3 October, suggesting larvae were not subjected to desiccation stress at this time. However, the water potential of the gall tissue and the insect's hemolymph did not differ on 16 October, indicating the gall was transitioning between a non-desiccating and desiccating environment for the larvae.

Cold-tolerance and desiccation resistance after moderate desiccation stress

To determine whether desiccation stress could induce changes in rates of water loss and cold-tolerance, larvae were collected on two different dates and were subjected to desiccating conditions in the laboratory. One group of larvae were collected on 5 October, when the goldenrod plant tissue was green and moist, and a second group on 2 November, after the plant had senesced and dried.

Larvae collected on 5 October were subjected to either 95% RH or 76% RH at 15°C for 10 days prior to assessing their cold-tolerance and desiccation resistance. Even though there was an apparent trend toward increased survival at –8°C and –12°C for larvae in both the 76% and 95% RH treatment groups, these differences were not significant when compared with field samples taken on 3 October (Fig. 6). Body water content for all larval groups for the 5 October treatments were the same, averaging 1.49 mg water mass mg^{-1} dry mass (Fig. 7A). In contrast, moderate desiccation stress enhanced desiccation resistance as rates of water loss were significantly lower (P<0.05) for larvae in the 95% RH and the 76% RH groups, 2.24 and 0.83 μg mm^{-2} h^{-1} respectively, than the field group (Fig. 7B). These data suggests that mild desiccation stress induced an enhanced desiccation resistance in the larvae.

A second group of larvae collected on 2 November were tested for desiccation resistance after 10 days exposure to 95 or 76% RH at 15°C. As with the 5 October collection, the November-collected control group had the same body water content as the 95% and 76% RH experimental groups, ~1.68 mg water mass mg dry $mass^{-1}$ (Fig. 7A). In contrast to the 5 October collection, water loss rates were very low and there were no differences in rates of water loss between larvae in the control and experimental groups (Fig. 7B). These results suggest that larvae were highly resistant to desiccation prior to being collected and subjected to these conditions on 2 November. Cold-tolerance was not examined for this collection date as the larvae were previously deemed to be extremely cold-tolerant on 30 October.

Discussion

During the autumn and winter, *E. solidaginis* larvae exhibited two phases of reduced rates of water loss. A rapid

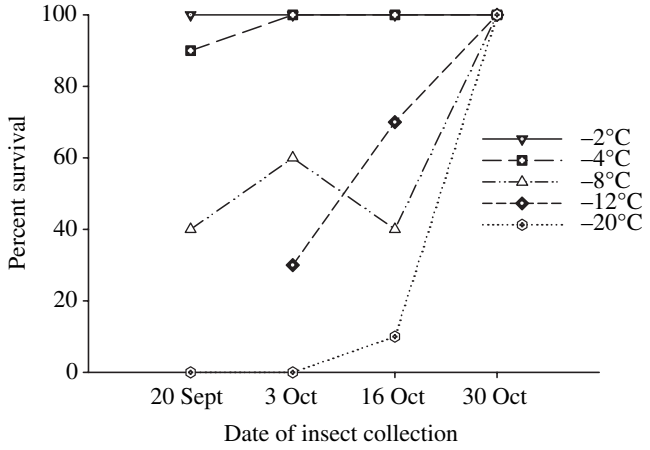

Fig. 3. Seasonal changes in cold-tolerance of *E. solidaginis* larvae (*N*=10), as indicated by survival after 24 h exposure to –2, –4, –8, –12, or –20°C from 20 September to 30 October 2001.

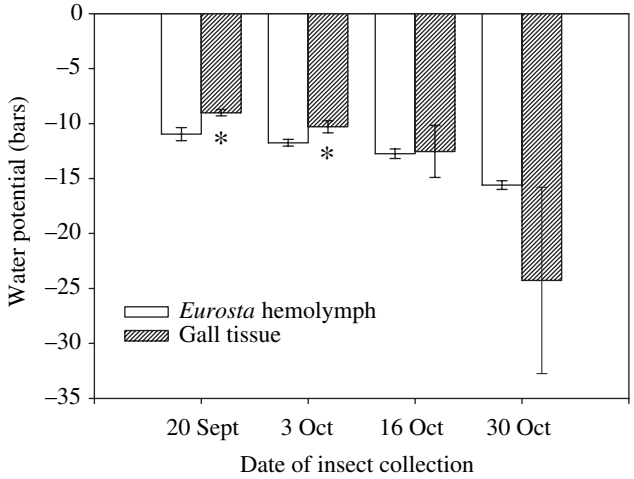

Fig. 5. Water potentials for goldenrod gall tissue and *E. solidaginis* hemolymph from 20 September to 30 October 2001. Water potential of the larval hemolymph was mathematically derived from osmolality values in Fig. 2C. An asterisk indicates a significant difference between gall and larval values for the same date of collection (P<0.05). Values are mean ± S.E.M., N=10 for all values except gall tissue measurements on 30 October where N=5.

sixfold reduction in the rate of water loss occurred in a 2 week period beginning on 3 October, which did not appear to be linked to changes in cold-tolerance. A second, more subtle 3.9-fold decrease took place over a 6 week period beginning on 16 October, which may be linked to increasing hemolymph osmolality and cold-tolerance.

The first phase of increased resistance to water loss is probably due to decreased respiratory transpiration as larvae entered diapause. Water loss through transpiration is positively linked to the activity level of a given insect. High levels of metabolic activity due to flight (Nicolson and Louw, 1982) or elevated temperatures (Ahearn, 1970), increase respiratory water loss. Irwin et al. (2001) showed that *E. solidaginis* larvae from southwest Ohio reduce their metabolic rate by more than 75% between 1 and 15 October, when they enter diapause. Diapause is defined as a genetically determined state of low metabolic activity, suppressed development and heightened resistance to environmental extremes that lasts longer than the adverse conditions (Danks, 1987; Tauber et al., 1986). A reduction of metabolic rate and consequent decrease in respiratory transpiration, as larvae entered diapause, most likely contributed importantly to the rapid decrease in rates of water loss between 3 and 16 October.

In addition to reduced transpiration, increased levels of cuticular lipids may have contributed to the first phase of reduced rates of water loss. Water loss for dormant insects primarily occurs as water diffuses across their cuticle and during respiratory transpiration (Edney, 1977; Hadley, 1994). Cuticular water loss is primarily regulated by the amount and

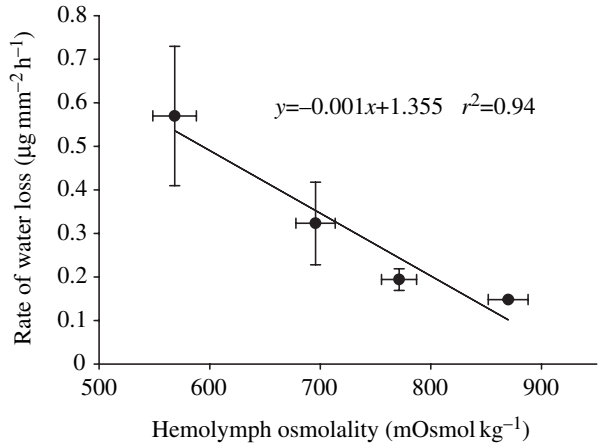

Fig. 4. Rates of water loss versus hemolymph osmolality in *E. solidaginis* larvae collected from 16 October 2001 to 11 December 2001. To ensure all larvae were in the refractory phase of diapause for this comparison, data collected on 15 January 2001 were not used.

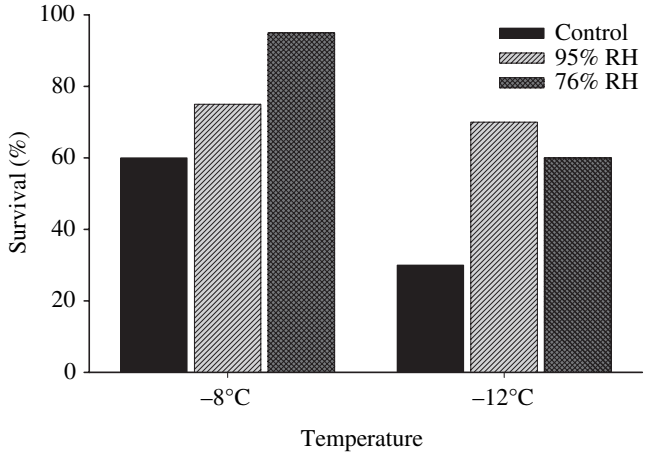

Fig. 6. The effects of moderate desiccation stress (95 or 76% RH) at 15°C for 10 days on cold tolerance of larvae (*N*=20) collected on 5 October 2001. Field group data was taken on larvae collected and analyzed on 3 October 2001.

type of epicuticular lipids on the integumental surface (see references in Hadley, 1994; Gibbs, 1998). Dormant stages of insects, which are at risk of dehydration, such as larvae of the flesh fly *Sarcophaga crassipalpis* (Yoder et al., 1992), the tobacco hornworm, *Manduca sexta* (Bell et al., 1975; Coudron and Nelson, 1981) and the moth *Mamestra configurata* (Hegdekar, 1979), increase the amount of their epicuticular lipids to diminish water loss. Epicuticular hydrocarbons increase 40-fold in *E. solidaginis* from late summer to mid-winter (D. R. Nelson and R. E. Lee, 2004) and may have contributed to the rapid phase one decrease rates of water loss (Fig. 2D). However, it is unknown if epicuticular lipids increased over the 13-day period that constituted phase one in the present study.

Gall water content has been used as the primary indicator of desiccation stress in galling insects (Irwin et al., 2001; Layne and Medwith, 1997; Lee and Hankinson, 2003). However, this technique is unable to detect slight changes in water potential of the gall tissue immediately surrounding the insect that could profoundly impact its physiology and water balance. The springtail *Folsomia candida* increases its drought tolerance after being exposed to a water potential deficit between its environment and hemolymph of only 17 bars (Sjursen et al., 2001). Small changes in water potential between the body fluids of *E. solidaginis* larvae and its gall tissue may also influence its resistance to water loss. For instance, on 20 September and 3 October the water potential of larval hemolymph was significantly lower than the water potential of the surrounding gall tissue (Fig. 5), indicating the larvae were in a potentially hydrating environment. By contrast, between 16 and 30 October the water potential of the gall tissue decreased markedly, indicating a shift to a dehydrating environment. This small change in water potential deficit between the gall tissue and larval hemolymph correlates closely with the phase one reduction in rates of water loss (Fig. 2D) and may be a cue that triggers larvae to increase their resistance to desiccation and to enter into diapause.

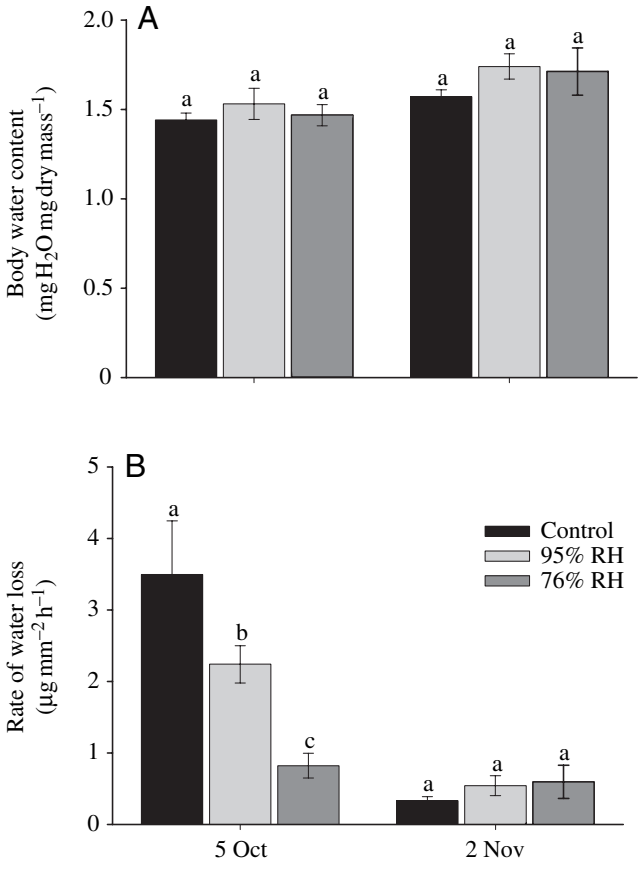

Fig. 7. The effects of moderate desiccation stress (95 or 76% RH) at 15°C for 10 days on body water content (A), and rates of water loss (B) on larvae collected on 5 October and 2 November 2001. Field group data was taken on larvae collected and analyzed on 3 and 30 October 2001 respectively. Values not sharing a letter are significantly different. Values are mean ± S.E.M.

We found no correlation between increased desiccation resistance and increased cold-tolerance early in the study. Between late September and 30 October, larvae exhibited a gradual increase in cold-tolerance (Fig. 3) that correlates well with other studies performed on this species in southwest Ohio (Lee and Hankinson, 2003) as well as in western Pennsylvania (Layne, 1991). This seasonal increase in cold-tolerance is due to the concomitant increase in cryoprotectants levels (Baust and Lee, 1981; Storey and Storey, 1992), as evidenced by hemolymph osmolality, which increased by 30% from 20 September to 30 October (Fig. 2C). However, cold-tolerance only gradually increased and hemolymph osmolality remained unchanged between 3 and 16 October when larval water loss rates decreased rapidly (Fig. 2D). In addition, larval rates of water loss were significantly lowered after being subjected to mild desiccation stress in early October (Fig. 7B), although larval cold-tolerance did not change (Fig. 6). Taken together, these data suggest that different mechanisms regulate desiccation resistance and cold-tolerance during this period.

In contrast to phase one, the second phase of increased desiccation resistance correlated closely with increases in hemolymph osmolality and suggests a link between desiccation resistance and cold-tolerance in *E. solidaginis* (Fig. 4). It is unlikely that the decrease in the rate of water loss during the second phase was caused by changes in respiratory water loss because *E. solidaginis* larvae remain in diapause, with a depressed metabolic rate until mid-January (Irwin et al., 2001). As mentioned previously, epicuticular hydrocarbons increase 40-fold in *E. solidaginis* from late summer to mid-winter (D. R. Nelson and R. E. Lee, 2004). Therefore, increased levels of epicuticular hydrocarbons may be partly responsible for the increased desiccation resistance between 16 October and 11 December. However, it is likely that most cuticular hydrocarbons were added prior to experiencing desiccating conditions as the gall tissue senesced and dried in early October.

The manner in which the elevated cryoprotectant concentrations could have affected water loss rates is unknown, however it is unlikely that it is was due to a colligative reduction in the water potential deficit between the

insect's hemolymph and environmental water vapor (Edney, 1977; Williams et al., 2002). In response to desiccating conditions, the springtail *F. candida* rapidly synthesizes osmolytes, predominantly myoinositol and glucose, which colligatively lowers its hemolymph water activity and consequently reduces or even eliminates organismal water loss (Bayley and Holmstrup, 1999; Sjursen et al., 2001). The production of these solutes can reduce water loss colligatively only because the desiccating conditions the springtails experience are quite mild (A_v ~0.984) with a water potential deficit between the insect's hemolymph and environmental water vapor of only ~17 bars (Bayley and Homlstrup, 1999). By contrast, gall fly larvae experience much drier conditions during winter. For example, the water potential deficit between larval hemolymph and their environment of 14,400 bars (simulated in the water loss trials of Fig. 2D) is commonly experienced by these insects in mid-winter. Between 16 October and 11 December, larvae increased their hemolymph osmolality by 302 mOsmol kg^{-1} (Fig. 2C); this increase in solutes would reduce the water potential deficit between the hemolymph and the environment by only ~7 bars. Such a small reduction of the water potential deficit through colligative actions of increased solutes would have a negligible effect on rates of water loss over that period.

Multiple lines of evidence indicate that carbohydrates influence arthropod water relations in a non-colligative manner. Trehalose, glycerol and sorbitol can protect cell membranes against severe desiccation stress and increase organismal tolerance to desiccation (Crowe et al., 1984; Bryszewska and Epand, 1988; Crowe, 2002; Oliver et al., 2002). Certain cryoprotectants, like glycerol and sorbitol, are effective at binding water (Storey et al., 1981; Storey, 1983). Bound water differs markedly from bulk water as it is less likely to freeze than bulk water and is also highly resistant to removal when dried at biologically relevant temperatures (see references in Danks, 2000; Block, 1996, 2003). Intracellular bound water may increase post-freeze survival for freeze-tolerant organisms (Storey et al., 1981; Storey, 1983), however, little is known about the effect of extracellular bound water.

The insect cuticle, a multi-layered structure with a single basal layer of epidermal cells, is the primary barrier to organismal water loss (Hadley, 1994). The cuticle also functions as the main barrier by which freeze-susceptible insects resist inoculative freezing (Somme, 1982). Several insects seasonally increase their resistance to inoculative freezing (Duman, 2001). Winter-acclimated larvae of the beetle, *Dendroides canadensis*, resist inoculative freezing better than summer larvae, in part because they produce antifreeze proteins that adhere to the epidermis (Olsen et al., 1998). Antifreeze proteins lower the non-equilibrium freezing point of a solution without affecting the melting point (Duman et al., 1991; Duman, 2001). Recently, Duman (2002) found that the cryoprotectant glycerol interacts synergistically with antifreeze proteins to increase their activity, apparently by stabilizing the protein.

We speculate that cryoprotectants associate with proteins on the surface of the epidermal layer and, thereby, enhance resistance to desiccation. Although no antifreeze proteins are known to be produced by *E. solidaginis,* they do produce a novel, dehydrin-like protein during natural cold hardening (Pruitt and Shapiro, 2001). Dehydrins are a family of proteins produced in response to desiccation. Certain dehydrins are localized to leaf epidermal tissue in cold acclimated barley (Bravo et al., 2003) and may interact with low molecular mass cellular components (see references in Allagulova et al., 2003). Thus, it is possible that an epidermal proteins associates with cryoprotectants in *E. solidaginis*. Glycerol and sorbitol substantially increase the amount of bound water in *E. solidaginis* during natural cold-hardening; 10 to 20% of total body water is bound due to sorbitol and glycerol in mid-winter larvae (Storey et al., 1981; Storey, 1983). Taken together, bound water associated with epidermal cryoprotectants may collectively thicken the cuticular barrier, resulting in decreased rates of water loss (Fig. 4).

In summary, hemolymph osmolality and cold-tolerance of *E. solidaginis* larvae steadily increased during the autumn. An initial rapid decrease in seasonal rates of water loss was correlated with drying of the gall tissue surrounding the larvae that was probably caused by decreased respiratory water loss as larval metabolism fell upon entering diapause. Later in the autumn, cryoprotectant accumulation may have affected water conservation through non-colligative actions.

We thank Jim Oris, Alan Cady and Jack Vaughn for help in the design of this project, Robert Schaefer for aide with statistical analysis, and two reviewers who added clarity to the paper. Support for this project was provided by the National Science Foundation IBN# 0090204 and IBN# 00416720.

References

Ahearn, G. A. (1970). The control of water loss in desert tenebrionid beetles. *J. Exp. Zool.* **53**, 573-595.

Allagulova, C. R., Gimalov, F. R., Shakirova, F. M. and Vakhitov, V. A. (2003). The plant dehydrins: structure and putative functions. *Biochem. Moscow* **68**, 945-951.

Baust, J. G. and Lee, R. E. (1981). Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginsis*. *J. Insect Physiol.* **27**, 485-490.

Baust, J. G. and Lee, R. E. (1982). Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* **28**, 431-436.

Bayley, M. and Holmstrup, M. (1999). Water vapor absorption in arthropods by accumulation of myoinositol and glucose. *Science* **285**, 1909-1911.

Bell, R. A., Nelson, D. R. and Borg, T. K. (1975). Wax secretion in non-diapausing and diapausing pupae of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* **21**, 1725-1729.

Block, W. (1996). Cold or drought – the lesser of two evils for terrestrial arthropods? *Eur. J. Entomol.* **93**, 325-339.

Block, W. (2003). Water or ice? The challenge for invertebrate cold survival. *Sci. Prog.* **86**, 77-101.

Bravo, L. A., Gallardo, J., Navarrete, A., Olave, N., Martinez, J., Alberdi, M., Close, T. J. and Corcuera, L. J. (2003). Cryoprotective activity of a cold-induced dehydrin purified from barley. *Physiol. Plant.* **118**, 262-269.

Bryszewska, M. and Epand, R. M. (1988). Effects of sugar alcohols and disaccharides in inducing the hexagonal phase and altering membrane-

properties – implications for diabetes-mellitus. *Biochim. Biophys. Acta* **943**, 485-492.

Coudron, T. A. and Nelson, D. R. (1981). Characterization and distribution of the hydrocarbons found in diapausing pupae tissues of the tobacco hornworm, *Manduca sexta* (L). *J. Lipid Res.* **22**, 103-112.

Crowe, L. M. (2002). Lessons from nature: the role of sugars in anhydrobiosis. *Comp. Biochem. Physiol. A* **131**, 505-513.

Crowe, L. M., Mouradian, R., Crowe, J. H., Jackson, S. A. and Womersley, C. (1984). Effects of carbohydrates on membrane stability at low water activities. *Biochim. Biophys. Acta* **769**, 141-150.

Danks, H. V. (1987). *Insect Dormancy: An Ecological Perspective*. Ottawa: Biological Survey of Canada (Terrestrial Artropods).

Danks, H. V. (2000). Dehydration in dormant insects. *J. Insect Physiol.* **46**, 837-852.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.* **63**, 327-357.

Duman, J. G. (2002). The inhibition of ice nucleators by insect antifreeze proteins is enhanced by glycerol and citrate. *J. Comp. Physiol. B* **172**, 163-168.

Duman, J. G., Xu, L., Neven, L. G., Tursman, D. and Wu, D. W. (1991). Hemolymph proteins involved in insects subzero-temperature tolerance: ice nucleators and anti-freeze protiens. In *Insects at Low Temperature*, (ed. R. E. Lee and D. L. Denlinger), pp. 94-127. New York: Chapman and Hall.

Edney, E. B. (1977). *Water Balance In Land Arthropods*. New York: Springer-Verlag.

Gibbs, A. G. (1998). Water-proofing properties of cuticular lipids. *Am. Zool.* **38**, 471-482.

Hadley, N. F. (1994). *Water relations of terrestrial arthropods*. San Diego: Academic Press.

Hegdekar, B. M. (1979). Epicuticular wax secretion in diapause and non-diapause pupae of the Bertha armyworm *Mamestra configurata*. *Annu. Entomol. Soc. Am.* **72**, 13-15.

Hølmstrup, M. and Westh, P. (1994). Dehydration of earthworm cocoons exposed to cold – a novel cold-hardiness mechanism. *J. Comp. Physiol. B* **164**, 312-315.

Irwin, J. T., Bennett, V. A. and Lee, R. E., Jr (2001). Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera: Tephritidae). *J. Comp. Physiol. B* **171**, 181-188.

Layne, J. R. (1991). Microclimate variability and the eurythermic nature of goldenrod gall fly (*Eurosta solidaginis*) larvae (Diptera, Tephritidae). *Can. J. Zool.* **69**, 614-617.

Layne, J. R. (1993). Winter microclimate of goldenrod spherical galls and its effects on the gall inhabitant *Eurosta solidaginis* (Diptera, Tephritidae). *J. Therm. Biol.* **18**, 125-130.

Layne, J. R. and Medwith, R. E. (1997). Winter conditioning of third instars of the gall fly *Eurosta solidaginis* (Diptera:Tephritidae) from western Pennsylvania. *Physiol. Chem. Ecol.* **26**, 1378-1384.

Lee, R. E. and Hankison, S. J. (2003). 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). *J. Insect Physiol.* **49**, 385-393.

Nelson, D. R. and Lee, R. E. (2004) Accumulation of cuticular lipids and increased desiccation resistance in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera: Tephritidae). *Comp. Biochem. Physiol. B*. **138**, 313-320.

Nicolson, S. W. and Louw, G. N. (1982). Simultaneous measurement of evaporative water-loss, oxygen-consumption, and thoracic temperature during flight in a carpenter bee. *J. Exp. Zool.* **222**, 287-296.

Oliver, A. E., Hincha, D. K. and Crowe, J. H. (2002). Looking beyond sugars: the role of amphiphilic solutes in preventing adventitious reactions in anhydrobiotes at low water contents. *Comp. Biochem. Physiol. A* **131**, 515-525.

Olsen, T. M., Sass, S. J., Li, N. and Duman, J. G. (1998). Factors contributing to seasonal increases in inoculative freezing resistance in overwintering fire-colored beetle larvae *Dendroides canadensis* (Pyrochroidae). *J. Exp. Biol.* **201**, 1585-1594.

Pruitt, N. L. and Shapiro, C. (2001). Evidence for a cryoprotective protein in freeze tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Am. Zool.* **41**, 1561-1561.

Ramløv, H. and Lee, R. E. (2000). Extreme resistance to desiccation in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *J. Exp. Biol.* **203**, 783-789.

Ring, R. A. and Danks, H. V. (1994). Desiccation and cryoprotection – overlapping adaptations. *Cryo-Lett.* **15**, 181-190.

Rojas, R. R., Lee, R. E. and Baust, J. G. (1986). Relationship of environmental water-content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis* (Fitch). *Cryobiol.* **23**, 564-564.

Sjursen, H., Bayley, M. and Holmstrup, M. (2001). Enhanced drought tolerance of a soil-dwelling springtail by pre-acclimation to a mild drought stress. *J. Insect Physiol.* **47**, 1021-1027.

Somme, L. (1982). Supercooling and winter survival in terrestrial arthropods. *Comp. Biochem. Physiol. A* **73**, 519-543.

Storey, K. B. (1983). Metabolism and bound water in overwintering insects. *Cryobiol.* **20**, 365-379.

Storey, K. B., Baust, J. G. and Buescher, P. (1981). Determination of water "bound" by soluble subcellular components during low-temperature acclimation in the gall fly larva, *Eurosta solidagensis*. *Cryobiol.* **18**, 315-321.

Storey, K. B. and Storey, J. M. (1992). Biochemical adaptations for winter survival in insects. In *Advances in Low-Temperature Biology*, vol. 1 (ed. P. L. Steponkus), pp. 101-140. London: JAI Press.

Tauber, M. J., Tauber, C. A. and Masaki, S. (1986). *Seasonal Adaptations of Insects*. New York: Oxford University Press.

Uhler, L. D. (1951). Biology and ecology of the goldenrod gall fly, *Eurosta solidaginis* (Fitch). New York: New York State College of Agriculture.

Williams, J. B., Shorthouse, J. D. and Lee, R. E. (2002). Extreme resistance to desiccation and microclimate-related differences in cold-hardiness of gall wasps (Hymenoptera: Cynipidae) overwintering on roses in southern Canada. *J. Exp. Biol.* **205**, 2115-2124.

Yoder, J. A., Denlinger, D. L., Dennis, M. W. and Kolattukudy, P. E. (1992). Enhancement of diapausing flesh fly puparia with additional hydrocarbons and evidence for alkane biosynthesis by a decarbonylation mechanism. *Insect Biochem. Mol. Biol.* **22**, 237-243.

Zachariassen, K. E. (1991). The water relations of overwintering insects. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 47-63. New York: Chapman and Hall.