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ORIGINAL PAPER

Differences in cold tolerance, desiccation resistance, and cryoprotectant production between three populations of *Eurosta solidaginis* collected from different latitudes

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Abstract Possible links between cold-tolerance and desiccation resistance were examined between larvae of the goldenrod gall fly collected from Michigan, southern Ohio, and Alabama locations as their host plant senesced. After acclimation to 5°C, Michigan-collected larvae were more cold-tolerant (25% survival after a 96 h exposure to −40°C) than larvae from Ohio (10% survival) and Alabama (0% survival). Increased cold-tolerance was partially linked to higher concentrations of the cryoprotectant glycerol (Michigan: 500 ± 30 mmol; Ohio: 270 ± 20; Alabama: 220 ± 20). Moreover, cryoprotectants may have functioned to reduce rates of overall and cuticular water loss for Michigan larvae, 0.10 ± 0.01 and 0.037 ± 0.003 μg mm^{-2} h^{-1}, respectively, values that were 40-44% lower than those for Ohio and Alabama larvae and may represent a link between desiccation resistance and cold-tolerance. After acclimation to 20°C, Alabama-collected larvae had metabolic rates that were 40% lower than those from Ohio and Michigan that averaged 0.100 ± 0.006 μl of CO_2 produced g^{-1} h^{-1}. The lower metabolic rate of Alabama-collected larvae at 20°C likely resulted in reduced respiratory transpiration that may represent a mechanism to maintain water balance at the higher overwintering temperatures they typically experience.

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Introduction

Many insects overwintering in temperate and polar regions must not only survive low temperature but also extremely desiccating conditions. To increase cold-tolerance, insects produce high concentrations of low-molecular-mass polyols, sugars and amino acids, termed cryoprotectants. Freeze tolerant insects use cryoprotectants to reduce the amount of body water frozen at a given temperature (Lee 1991; Zachariassen 1991; Storey and Storey 1992), thereby reducing cellular dehydration, which is considered to be the primary mode of damage caused by extracellular ice formation (Mazur 1984). Cryoprotectants may also lower rates of water loss by colligatively reducing the vapor pressure gradient between an insect's hemolymph and environmental water vapor (Ring and Danks 1994; Bayley and Holmstrup 1999; Sjursen et al. 2001) or possibly through non-colligative actions by binding water when desiccating conditions are more severe (Williams et al. 2004). The hypothesized link between cryoprotectant production and increased desiccation resistance led some authors to suggest that certain adaptations that increase cold-hardiness may also influence, or arose originally to promote, desiccation resistance (Ring and Danks 1994; Block 1996; Danks 2000). Yet, links between cold-hardiness and desiccation resistance remain unclear.

Temperate insects experience a large variation in the severity of winter conditions based on the location, elevation, and latitude of their hibernaculum (Danks 2004). Consequently, an insect's level of cold-hardiness varies depending on the severity of low temperatures it typically

experiences. On a local scale, stem galling cynipid wasps that overwinter above the snow pack in southern Canada experience more severe cold and are more cold-hardy than congeners in the buffered and mild subnivean space (Williams et al. 2002). On a global scale, Addo-Bediako et al. (2000) found that cold tolerance generally increases with higher latitudes across insect taxa. However, only a few studies have examined intraspecific differences in desiccation resistance and cold tolerance of wide ranging insects that experience different overwintering temperatures (Bennett et al. 2005; Klok and Chown 2005).

The goldenrod gall fly, *Eurosta solidaginis* (Fitch), ranges throughout much of North America, from southern Texas to southern Canada (Uhler 1951). Gall flies overwinter as freeze-tolerant, third instar larvae in dried stem galls on goldenrod plants (*Solidago* spp.). Larvae overwinter above the snowpack where they are subjected to winter extremes as dried gall tissue offers little protection against low temperature and desiccation stress (Uhler 1951; Layne 1993). Gall fly larvae likely experience much milder overwintering conditions in the southern portion of their range compared to the extremely low temperatures that occur at higher latitudes of the northern United States and Canada.

Throughout their range, *E. solidaginis* larvae seasonally increase their cold tolerance by producing high levels (~1 molar) of the cryoprotectants glycerol and sorbitol (Lee 1991). However, larvae collected from the southern portion of their range are less cold tolerant and produce considerably less glycerol under common garden conditions (Baust and Lee 1982), suggesting that lower levels of cold tolerance in southern larvae may be the result of genetic differences between the populations. Latitudinal variation in desiccation resistance for *E. solidaginis* larvae is unknown. However, larvae collected from southeast Ohio seasonally lower rates of water loss by increasing epicuticular lipids (Nelson and Lee 2004), by reducing respiratory transpiration as larvae enter diapause (Williams and Lee 2005), and possibly through the production of cryoprotectants (Williams et al. 2004).

A previous study focused on linking seasonal changes in levels of cold-tolerance, desiccation resistance and cryoprotectant production in single populations of *E. solidaginis* larvae (Williams et al. 2004). In contrast, the purpose of this study was to (1) determine if mid-winter collected larvae from northern latitudes were more cold-tolerant than larvae collected from a southern population and (2) to determine if cold-tolerance is positively linked to higher levels of resistance to desiccation. To investigate these questions we measured survival after freezing, hemolymph osmolality, cryoprotectant concentration, body water content, rate of total water loss, rate of cuticular water loss, metabolic rate, lipid content, and glycogen content of *E. solidaginis* larvae taken from three widely separated sites in Michigan, Ohio, and Alabama. More southerly collected larvae likely experience higher overwintering temperatures. Therefore, we measured several of the above parameters after acclimating larvae to a relatively high overwintering temperature.

Materials and methods

Insect collection

All galls were collected from goldenrod (*Solidago altissima*) within 2 weeks of plant senescence in the fall of 2004. Galls were collected in mid-October from a northerly site consisting of two fields of goldenrod ~1 square kilometer in area, near Allendale, Michigan (42°97′ 02″N, 85° 95′ 03″W) and a centrally located site consisting of three fields of ~1.5 square kilometers in area at the Ecology Research Center of Miami University, near Oxford, Ohio (39° 31′ 57″N, 84° 43′ 23″W). In mid-November a southern population was collected near Auburn, Alabama (32° 61′ 59″N, 85° 26′ 01″W). Due to low gall numbers and high rates of parasitism, galls were collected from multiple sites within a 10 km radius of Auburn. The Michigan collection site was ~455 km north of the Ohio site and ~1,182 km north of the Alabama collection sites. After collection the larvae were removed from their galls and weighed. To standardize for size between populations, only larvae with a mass between 46 and 56 mg were used in the below experiments with average masses of 52.9 ± 1.0 (mean ± SEM) mg for Michigan collected larvae, 51.1 ± 1.1 mg for Ohio collected larvae, and 53.0 ± 1.1 mg for Alabama collected larvae. After mass determination larvae were held in a dark refrigerated incubator at 5°C over a saturated solution of sodium chloride to produce a relative humidity of 75%. To ensure that all analyses were performed during the coldest periods of winter while the larvae were in the state of diapause, larvae were held in the above conditions for 2–8 weeks prior to data collection.

Measures of cold-tolerance

Larval cold-tolerance was assessed by measuring survival after exposure to a low subzero temperature. Fifty larvae per collection site were cooled at 1°C min^{-1} from 5°C until reaching −40°C. After a 96-h exposure to −40°C, larvae were warmed to 5°C at 1°C min^{-1} and then returned to 5°C and 75% RH. Survival measurements were taken 24 h, 48 h, 2, 6, and 16 weeks after the low temperature exposure by determining if larvae moved in response to being gently touched with a blunt probe. After the 16-week survival assessment, all larvae were placed at 23°C, allowed to develop, and the number of larvae which pupated, eclosed,

and became viable adults was recorded. Adults were judged to be viable if they had fully formed wings and were able to right themselves within 1 min of being turned on their dorsal side.

Hemolymph osmolality and cryoprotectant concentrations

Hemolymph osmolality ($n = 10$ per collection site) was determined by drawing 7–10 µl of hemolymph into a capillary tube through a small incision in the larval cuticle. The hemolymph was then analyzed in a Wescor Vapro 5520 Hemolymph Osmometer (Logan, UT, USA). To measure cryoprotectant concentrations, larvae ($n = 10$ per collection site) were frozen at −80°C until whole body measurements of glycerol were performed by enzymatic assay (Sigma Chemical Co., St. Louis, MO, USA, no. 337) as described by Hølmstrup et al. (1999). Sorbitol concentration was measured on the same individuals using the enzymatic assay described in Bergmeyer et al. (1974).

Measures of desiccation resistance

To determine the overall rate of water loss, larvae ($n = 10$ per collection site) were weighed to ±0.01 mg to obtain a fresh mass. Larvae were then re-weighed after being desiccated over Drierite (W.A. Hammond Drierite Co., OH, USA) at 4% RH and 5°C until they lost ~5% of their fresh mass (120–240 h). Cuticular surface area was estimated from initial wet mass using an equation previously determined by Williams et al. (2004): $y = 0.912x + 4.204$, $r^2 = 0.804$, where y = surface area in mm^2 and x = mass in mg. Body water content was determined by placing the desiccated larvae in an oven at 65°C until a constant dry mass was obtained.

Hemolymph content was assessed by obtaining the fresh mass of the larvae prior to tearing open the cuticle with forceps and removing the hemolymph by gently blotting the larval body cavity with Kimwipes® that were slightly wetted with a glycerol solution (~900 mOsm) that was isosmotic to larval hemolymph as described in Folk et al. (2001). The hemolymph-free carcass was then re-weighed before being placed in an oven at 65°C until a constant dry mass was obtained. Hemolymph content was determined as the difference between initial wet mass and the hemolymph-free carcass mass.

Rate of cuticular water loss (µg mm^{-2} h^{-1}) was measured to determine the relative contributions of respiratory and cuticular components of overall organismal water loss. Rate of cuticular water loss was assessed by weighing larvae ($n = 10$) before and after exposure to 4% RH at 5°C as described above, however, prior to testing, the spiracles of each larva were topically blocked with a small amount of Thomas Scientific Lubriseal stop cock grease (Swedesboro, NJ, USA), to eliminate respiratory water loss. Although this method maybe invasive, diapausing *E. solidaginis* do not naturally respire cyclically or change breathing patterns when exposed to pure oxygen so other, less invasive, measures of cuticular water loss rates were not available (Lighton et al. 2004; Johnson and Gibbs 2004).

Metabolic reserves

Since desiccation-selected *Drosophila melanogaster* preferentially store metabolic fuels in the form of glycogen, which when catabolized release higher levels of water (Gibbs et al. 1997; Folk et al. 2001), we measured glycogen content on ten larvae per population using a calorimetric micromethod (Kemp and Kits Van Heijningen 1954); glycogen concentration was expressed in glucosyl units. Lipid content was also determined on ten larvae per population using the chloroform/methanol procedure described by Teitz (1970).

Measures of metabolic rate

To correlate rates of water loss and metabolism, we measured CO_2 emission. Larvae ($n = 8$ per collection site) were weighed and individually placed into small glass respirometry chambers kept within a temperature controlled bath held at 5°C in late morning to account for possible diurnal effects on metabolism. It is extremely invasive to sex third instar larvae of the goldenrod gall fly, thus gender effects could not be taken into account. After the larvae had equilibrated to the chamber for 1 h, CO_2 was measured using a flow-through (50 ml min^{-1}) respirometer (TR-3 model, Sable Systems, Las Vegas, NV, USA). Metabolic rate data was converted into the units of microliters of CO_2 emitted per gram fresh mass per hour using DATACAN software (Sable Systems).

Effect of 20°C acclimation on cold-hardiness and desiccation resistance

Alabama-collected larvae likely experience higher overwintering temperatures. Therefore, we measured several of the above parameters after acclimating larvae from all three collection sites to a relatively high overwintering temperature that they would all likely experience in nature. We used the previously described techniques to measured hemolymph osmolality, cryoprotectant levels, overall rate of water loss, rate of cuticular water loss, hemolymph volume, body water content, and metabolic rate on larvae that were acclimated to 20°C for 1 week. In contrast to previous methods, measurements of total rate of water loss, rate of cuticular water loss, and metabolic rate were assessed at 20°C in these experiments.

Statistical analysis

To compare normally distributed data sets within temperature treatments, larvae from the three collecting sites were analyzed using a one-way ANOVA followed by a Bonferroni multiple comparisons test with significant differences determined at $\alpha = 0.025$. Data for Michigan and Ohio larval rates of water loss at 20°C failed a Kolmogorov–Smirnov test for normality ($P > 0.05$) and thus values within this temperature treatment were compared using the non-parametric Kruskall–Wallis exam followed by a Dunn's multiple comparison test with significant differences determined at $\alpha = 0.05$. *T*-tests were used to determine differences between 5 and 20°C temperature treatments from a single collection site. A one-way ANOVA followed by a Tukey's multiple comparison exam, after the proportions were angularly transformed, was used to determine differences in survival between larvae at each assessment period. Significance for ANOVA followed by a Bonferroni multiple comparisons test was determined at $\alpha = 0.025$, while significance for the remaining analyses were set at $\alpha = 0.05$. All data are reported as mean ± SEM.

Results

Measures of cold-tolerance

Northern larvae were more cold tolerant than ones from southern populations. Survival after −40°C exposure was significantly lower ($P < 0.05$) for larvae collected in Alabama compared to Ohio and Michigan larvae (Fig. 1a, b). Two weeks after the low temperature treatment, only 29 of 50 Alabama larvae responded to tactile stimulation, compared to 94 and 98%, respectively, of larvae in the Ohio and Michigan groups. The pattern of lower survival for Alabama larvae continued after being placed at room temperature as no larvae in the Alabama group eclosed (Fig. 1b). In contrast, Ohio larvae demonstrated moderate survival as 16% eclosed and 10% developed into viable adults. Survival was the greatest for Michigan-collected larvae ($P < 0.05$) which had the highest rates of eclosion (43%) and adult development (25%) (Fig. 1b).

Larvae collected from Michigan and Ohio responded to tactile stimulation much sooner than Alabama larvae. For example, 96% of the larvae from Michigan and Ohio that responded to tactile stimulation did so within 48 h of being removed from the low-temperature exposure, while only 10% of larvae collected from Alabama responded at this time, even though 58% were responsive after 2 weeks (Fig. 1a).

As with cold tolerance, levels of cryoprotectants varied among larval populations (Fig. 2). Michigan-collected

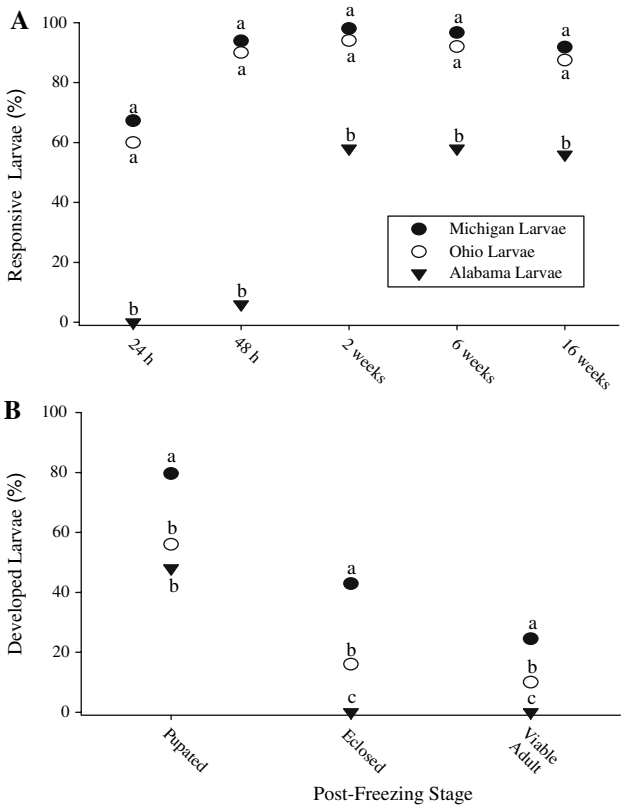

Fig. 1 **a** Percent responsive and **b** percent developed larvae of *Eurosta solidaginis* ($n = 50$ per collection site) collected from Michigan, Ohio, and Alabama after exposure to −40°C for 96 h. Larvae were held for 16 weeks at 5°C and judged to be responsive if they moved after tactile stimulation. After that period, larvae were transferred to 23°C and larvae were determined to be alive if they pupated, eclosed, or became fully formed adults. At a given testing period or developmental stage, values not sharing the same letter were significantly different using a one-way ANOVA followed by Tukey's multiple comparison procedure after the proportions were angularly transformed

larvae had significantly higher concentrations of glycerol ($P < 0.05$) after acclimation at 5°C (500 ± 30 mmol) than Ohio and Alabama larvae (Fig. 2a). However, after acclimation to 20°C larvae from all three collection sites had similar glycerol levels (~270 ± 24 mmol). Although 20°C acclimation had no effect compared to the 5°C treatment for Ohio and Alabama larvae, Michigan larvae at 5°C had higher glycerol levels than those at 20°C. Unlike glycerol, sorbitol concentrations did not differ ($P > 0.05$) among the collection groups acclimated at 5°C, averaging 328 ± 63 mmol (Fig. 2b), and between those acclimated at 20°C, averaging 39 ± 17 mmol (Fig. 2b). However, sorbitol concentrations were significantly higher within all populations acclimated at 5°C ($P < 0.05$) compared to those acclimated at 20°C.

Larvae collected in Michigan and Ohio averaged ~170 mOsm kg^{-1} higher hemolymph osmolality than those from Alabama, which averaged 777 ± 16 mOsm kg^{-1} when

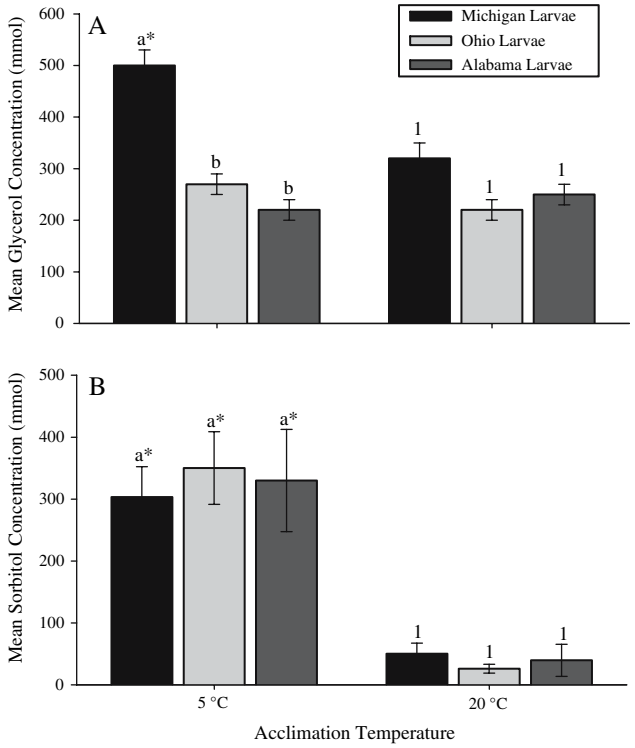

Fig. 2 **a** Mean glycerol concentration ($n = 10$) and **b** mean sorbitol concentrations ($n = 10$ per data point), for larvae of *Eurosta solidaginis* collected from Michigan, Ohio, and Alabama and acclimated to either 5 or 20°C. Means (±SEM) of larvae acclimated at 5°C not sharing the same letter or means of larvae acclimated at 20°C not sharing the same number were significantly different when analyzed with a one-way ANOVA followed with a Bonferroni multiple comparisons test. Values in the 5°C temperature treatment with an *asterisk* indicate a significant difference between 5 and 20°C data from larvae collected at the same site when using a *t*-test

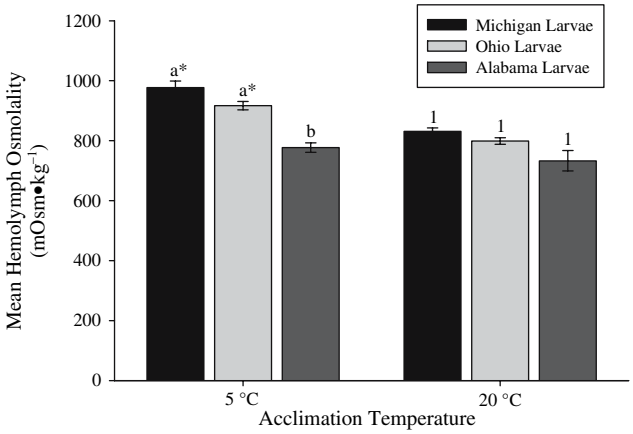

Fig. 3 Mean hemolymph osmolality ($n = 10$) for larvae of *Eurosta solidaginis* collected from Michigan, Ohio, and Alabama and acclimated to either 5 or 20°C. Means (±SEM) of larvae acclimated at 5°C not sharing the same letter or means of larvae acclimated at 20°C not sharing the same number were significantly different when analyzed with a one-way ANOVA followed with a Bonferroni multiple comparisons test. Values in the 5°C temperature treatment with an asterisk indicate a significant difference between 5 and 20°C data from larvae collected at the same site when using a *t*-test

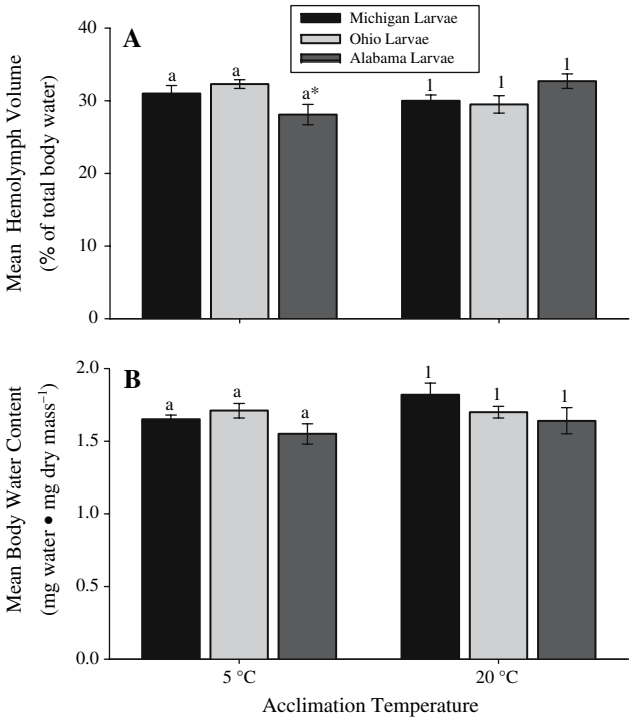

Fig. 4 **a** Mean hemolymph volume ($n = 10$), and **b** mean body water content ($n = 10$) for larvae of *Eurosta solidaginis* collected from Michigan, Ohio, and Alabama and acclimated to either 5 or 20°C. Means (±SEM) of larvae acclimated at 5°C not sharing the same letter or means of larvae acclimated at 20°C not sharing the same number were significantly different when analyzed with a one-way ANOVA followed with a Bonferroni multiple comparisons test

acclimated to 5°C (Fig. 3). In contrast, hemolymph osmolalities did not differ among collection groups after acclimation to 20°C, averaging 788 ± 19 mOms kg^{-1}.

Body water, glycogen, and lipid content

Body water contents did not differ among larval groups ($P > 0.05$) regardless of collection site or acclimation temperature, ranging between 1.6 and 1.8 mg water mg dry $mass^{-1}$ or 60.5 and 64.3% body water (Fig. 4b). Similarly, hemolymph volume did not differ among collection groups acclimated at 5°C, averaging 30.5% of total water volume, or among those acclimated at 20°C (30.7%) (Fig. 4a). Alabama larvae acclimated at 20°C did, however, have a higher hemolymph volume ($P < 0.05$) than larvae acclimated at 5°C.

Larval glycogen contents were similar among the three collection sites with values ranging between 57 and 81 mg glycogen g^{-1} dry mass, nor did lipid content differ among populations (Table 1). However, these data suggest a trend with Alabama larvae having the lowest levels of glycogen and lipid compared to the more northern Ohio and Michigan larvae.

Table 1 Mean lipid content ($n = 10$) and glycogen content ($n = 10$), for larvae of *Eurosta solidaginis* collected from Michigan, Ohio, and Alabama

Collection site	Lipid content (mg lipid g dry mass^{-1})	Glycogen content (mg glycogen g dry mass^{-1})
Michigan	580 ± 45	72 ± 7
Ohio	547 ± 55	81 ± 8
Alabama	470 ± 12	57 ± 7

Means (±SEM) within a column were not significantly different when analyzed with a one-way ANOVA followed with a Bonferroni multiple comparisons test

Measures of rates of water loss

Overall rates of water loss were ~40% lower ($P < 0.05$) for larvae collected in Michigan (0.10 ± 0.01 μg mm^{-2} h^{-1}) compared to Ohio and Alabama larvae when acclimated to, and desiccated at, 5°C (Fig. 5a). In contrast, rates of overall water loss did not differ among collection groups ($P > 0.05$) when acclimated and tested at 20°C (Fig. 5a). Within each population, rates of water loss were significantly higher ($P < 0.05$) for larvae acclimated and tested at 20°C compared to those at 5°C (Fig. 5a).

Similar to rates of total water loss, Michigan larvae at 5°C had a ~44% lower rate of cuticular water loss (0.037 ± 0.003 μg mm^{-2} h^{-1}) compared to the Ohio and Alabama larvae (Fig. 5b). In contrast, rates of cuticular water loss did not differ among populations at 20°C, averaging 0.215 ± 0.019 μg mm^{-2} h^{-1} (Fig. 5b). Unsurprisingly, rates of cuticular water loss significantly increased, ~3.1 fold ($P < 0.05$), for all larval groups acclimated at 20°C compared to those at 5°C (Fig. 5b).

Carbon dioxide production ranged from 0.017 to 0.028 μl g^{-1} h^{-1} among larvae at 5°C. CO_2 levels were significantly lower ($P < 0.05$) for larvae collected in Ohio compared to those from Michigan (Fig. 5c). Michigan-and Ohio-collected larvae had significantly higher rates of CO_2 production (~4.4 fold; $P < 0.05$) at 20°C compared to those at 5°C (Fig. 5c). Production of CO_2 also increased significantly for Alabama-collected larvae at 20°C compared to those at 5°C ($P < 0.05$), however, CO_2 release only increased by 2.6-fold (Fig. 5c).

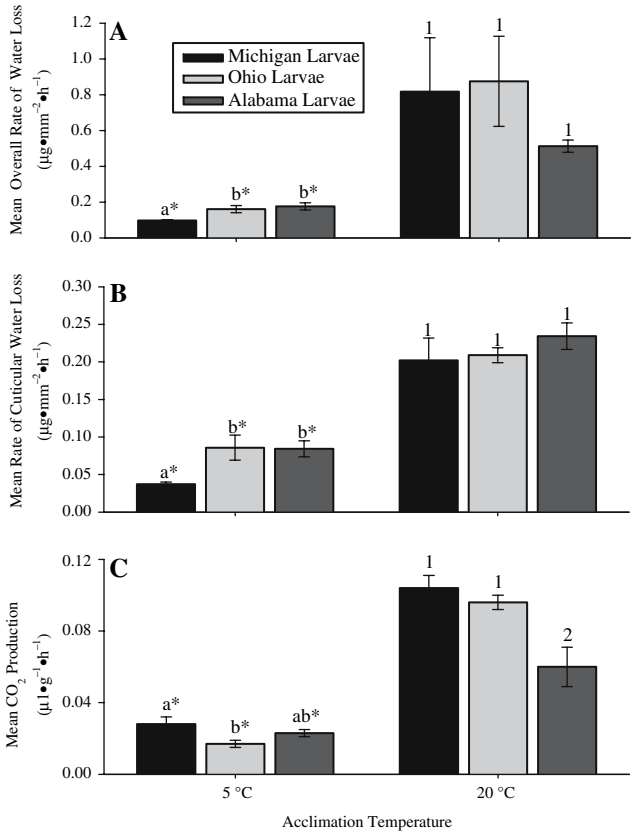

Fig. 5 **a** Mean total rate of water loss ($n = 10$), and **b** mean rate of cuticular water loss ($n = 10$), and **c** mean CO_2 production ($n = 8$) for larvae of *Eurosta solidaginis* collected from Michigan, Ohio, and Alabama and acclimated to either 5 or 20°C. Means (±SEM) of larvae acclimated at 5°C not sharing the same letter or means of larvae acclimated at 20°C not sharing the same number were significantly different when analyzed with a Kruskall–Wallis exam followed by a Dunn's multiple comparison test (mean water loss rate at 20°C) or a one-way ANOVA followed with a Bonferroni multiple comparisons test (all remaining values). Values in the 5°C temperature treatment with an asterisk indicate a significant difference between 5 and 20°C data from larvae collected at the same site when using a *t*-test

Discussion

Levels of cold-hardiness

Larvae collected from Michigan were more cold-hardy than larvae from either Ohio or Alabama when using adult development and viability as the determinant (Figs. 1a, b). There is no standard protocol for assessing insect cold tolerance and the ability to survive low temperatures can vary greatly depending on biotic factors such as age or acclimation status of the insect, as well as the methodology (*cf* cooling rate, exposure temperature, and duration) (Denlinger and Lee 1998). However, from the parameters used in this study, it appears that adult development was the most ecologically relevant assessment of cold-hardiness. For example, Michigan and Ohio larvae had the same levels of cold-hardiness when movement in response to tactile stimulation was used (Fig. 1a), even though Michigan larvae typically experience much lower overwintering temperatures (Fig. 6a, b). However, when using adult viability as the determinant, the cold-hardiness of Michigan larvae was significantly greater than those from Ohio. In addition,

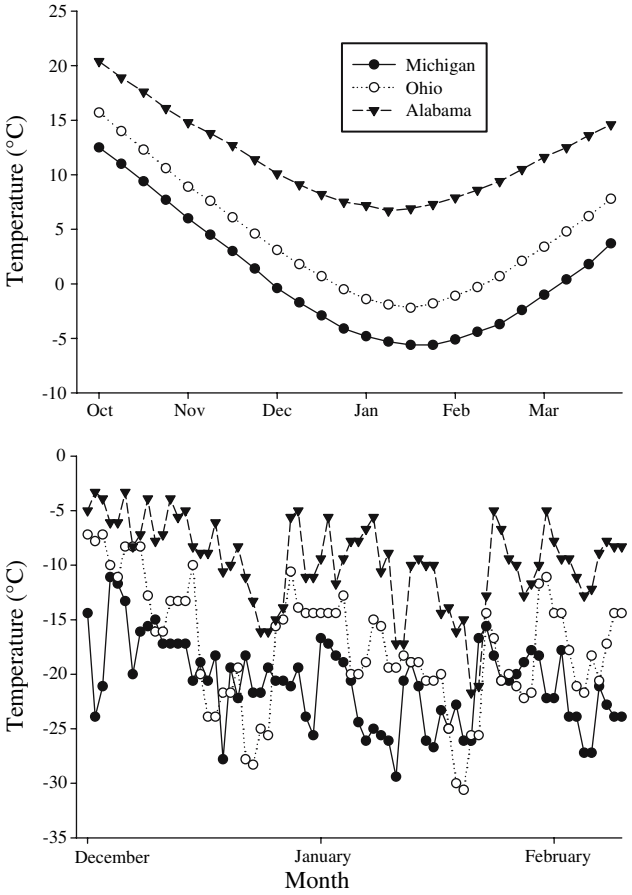

Fig. 6 **a** October to March weekly mean air temperature from 1971 to 2000 from weather stations located within 15 km of the larval collection sites. **b** Lowest daily minimum temperature from December to mid-February, the coldest part of the winter, between 1971 and 2000 from weather stations located within 15 km of the larval collection sites. Michigan temperature data were recorded from the weather station located at the Grand Rapids airport, Ohio temperature data were recorded at the Fairfield weather station, and Alabama temperature data were recorded at the Auburn Agronomy weather station. All weather station data can be accessed on the National Oceanic and Atmospheric website: http://www.ncdc.noaa.gov/oa/climate/climateresources.html

Alabama-collected larvae, which rarely experience subzero temperatures in winter (Fig. 6a, b), had the same pupation rate as Ohio larvae (Fig. 1b). Yet, when using adult viability as the determinant, Alabama had the lowest level of cold-hardiness (Fig. 1b).

The fact that response to external stimuli varied considerably compared to adult development in all larvae suggests this method of assessing cold-tolerance should be avoided. For example, 2 weeks after removal from the low temperature exposure, 58% of Alabama larvae responded to external stimuli (Fig. 1a). If this were the only assessment of cold-tolerance for these larvae, then they would be considered relatively tolerant to the low temperature exposure. However, the assessment of cold-hardiness was drastically different when using the more ecologically relevant determinant of cold-tolerance, adult development. No larvae from Alabama developed into an adult, suggesting that the low-temperature exposure was excessively severe for these larvae (Fig. 1b).

Mechanisms of cold-tolerance

Higher levels of cold-hardiness for Michigan larvae were likely due, at least in part, to higher concentrations of cryoprotectants that can mediate the effects of extracellular ice formation and cellular dehydration (Lee 1991). As reported previously (Baust and Lee 1982), the most northerly population of *E. solidaginis*, in this case the Michigan larvae, had higher concentrations of glycerol than the lower-latitude populations. In contrast to previous reports, Michigan larvae had lower concentrations of glycerol after acclimation to a higher temperature (20°C) (Storey et al. 1981; Baust and Lee 1982; Storey et al. 1986). However, few studies have examined the glycerol content of mid-winter *E. solidaginis* larvae acclimated at the relatively high temperature of 20°C, and one report suggested that glycerol levels vary in mid-winter for field collected larvae (Baust and Lee 1981). Concentrations of the cryoprotectant sorbitol did not differ between larval groups acclimated at 5°C and were similar to levels reported for Texas and Minnesota collected larvae (Baust and Lee 1982). Sorbitol is anabolized into glycogen at temperatures above 10°C (Storey et al. 1981) consistent with the observed lower sorbitol levels for all larvae acclimated to 20°C.

Cryoprotectants function in a variety of ways to increase levels of insect freeze tolerance; however the exact mechanism(s) by which cryoprotectants influenced larval survival in this study is unknown. Elevated glycerol concentration could have reduced ice content to a greater extent in Michigan larvae by colligatively lowering the melting point of its hemolymph (Lee 1991; Denlinger and Lee 1998) or non-colligatively by binding more water (Storey 1983; Block 1996) than in those from Ohio and Alabama. Higher concentrations of the membrane-penetrating glycerol could have reduced cellular dehydration to a greater extent in Michigan larvae during freezing (Zachariassen 1991). Lastly, higher concentrations of cryoprotectants may have positively influenced cold-tolerance of Michigan larvae by stabilizing membranes and proteins during freezing and thawing (Carpenter and Crowe 1988; Crowe et al. 1990). Therefore, it is likely that higher concentrations of glycerol conferred increased cold-tolerance in the Michigan larvae.

However, cryoprotectants are unable to explain all differences in cold tolerance between larval populations. For example, Ohio and Alabama larvae had similar concentrations of cryoprotectants (Fig. 3a, b), yet those from Ohio were more cold tolerant than those from Alabama (Fig. 1b).

Other physiological parameters that could account for the difference in cold tolerance between populations are type and saturation levels of lipid membranes (Bennett et al. 1997; Kostál et al. 2003; Lee et al. 2006) and the ability of membrane bound transporters to function during and after the low-temperature exposure. Michigan and Ohio larvae were able to respond to tactile stimulation sooner than Alabama larvae following the freezing exposure (Fig. 1a), which was likely the result of their ability to restore the electrochemical gradients across their membranes more quickly. Ion electrochemical gradients across a cell membrane are needed for many cell and tissue functions, such as neural impulses and muscular contraction. However, transporters which maintain the electrochemical gradient can be perturbed during the low temperatures and high hemolymph solute concentrations associated with severe freezing, allowing ions to equilibrate across the cell membrane (Morris and Clarke 1981). Upon thawing, re-establishment of the ion gradient can be a slow process and takes more than 24 h for the wood fly, *Xylophagus cinctus*, once removed from freezing temperatures (Kristiansen and Zachariasen 2001). We suggest that Michigan and Ohio larvae responded sooner to tactile stimulation following the low-temperature exposure because they had ion transporters that functioned more efficiently at the recovery temperature and established pre-freeze electrochemical gradients faster than those from Alabama. If ion transporters in the Michigan and Ohio larvae functioned more efficiently at low temperatures, then other membrane transporters that would directly affect cold-tolerance, such as sorbitol transporters, may also function more efficiently at low temperatures (Storey and Storey 1996). However, more studies of transporter function at low temperature are needed to justify this claim.

Resistance to desiccation stress

To survive desiccation stress, insects can either tolerate water loss and low body water levels or limit the rate at which they lose water to the environment (Danks 2000). Insects that tolerate water loss typically have higher levels of initial body water (Miller 1968; Block et al. 1990), and/or greater hemolymph volume (Folk et al. 2001). Desiccation tolerant insects may also have higher concentrations of glycogen which releases more water when metabolized than lipid (Gibbs et al. 1997). Body water content (Fig. 4b), hemolymph volume (Fig. 4a), and glycogen levels (Table 1) were the same in all larvae, suggesting that differences in water balance between the populations would exist primarily in the rate larvae lose water.

There were differences in desiccation resistance among collection groups as overall rates of water loss were ~40% lower for Michigan larvae than those from Ohio and Alabama at 5°C (Fig. 5a). Overwintering and dormant insects typically lose water only through cuticular and respiratory transpiration (Danks 2000). Rates of cuticular water loss were ~44% lower in Michigan larvae acclimated at 5°C compared to the other populations. Thus, it is likely that the lowered rates of cuticular water loss for the Michigan larvae were primarily responsible for their lower overall rate of water loss at 5°C (Fig. 5a, b).

Mechanisms of desiccation resistance

Lower rates of cuticular water loss for Michigan larvae acclimated at 5°C may be due to differences in epicuticular lipids that are the primary barrier to cuticular water loss (Hadley 1994; Gibbs 1998). However, cryoprotectants may have functioned to lower rates of cuticular water loss in Michigan larvae compared to Ohio and Alabama larvae. After *E. solidaginis* larvae enter diapause, cryoprotectant production, as measured by hemolymph osmolality, is strongly correlated with reductions in the rate of overall water loss (Williams et al. 2004). Williams et al. (2004) hypothesized that cryoprotectants may function in a non-colligative manner to reduce rates of water loss by binding water at the cuticular basement membrane which would increase the distance bulk water would have to travel to be lost to the environment. Michigan larvae acclimated at 5°C had much higher concentrations of the cryoprotectant glycerol (Fig. 2a), and also significantly lower cuticular water loss compared to Ohio and Alabama larvae. In addition, after the 7 day acclimation to 20°C, Michigan larvae had similar concentrations of glycerol and also similar rates of cuticular water loss as larvae collected from Ohio and Alabama. Thus, these data suggest that cryoprotectants may function to reduce rates of cuticular and overall rates of water loss for Michigan collected larvae acclimated to 5°C and may represent a link between cold-hardiness and desiccation resistance.

Resistance to desiccation stress at 20°C

Since larvae from Alabama typically experience much warmer temperatures during winter than those from Michigan and Ohio (Fig. 6a, b), we measured parameters associated with desiccation resistance after larvae from each population were acclimated to 20°C. It is likely that larvae from Michigan and Ohio rarely experience 20°C during the winter, as average daily maximum temperatures in December and January taken at the weather stations in Fig. 6 ranged between 3.9 to −1.7°C and 2.8 to 8.3°C, respectively (data not shown). Over the same time period, average daily maximum temperatures were much higher at the Alabama weather station, ranging between 12.2 and 16.1°C (data not shown). Therefore, *E. solidaginis* larvae in

Alabama likely experience 20°C during the mid-winter, especially when considering the effect of solar radiation that can warm the gall well above ambient air temperature (Layne 1991).

After acclimation to 20°C, larvae from Alabama had the same overall rate of water loss as those from Michigan (Fig. 5a). This is in contrast to the 5°C acclimation, in which Alabama larvae had much higher overall rates of water loss than Michigan larvae. The relative lowering of overall rates of water loss for Alabama larvae acclimated at 20°C appears to be the result of respiratory transpiration which increases relatively slowly with temperature. After acclimation to 5°C, larvae from Michigan, Ohio, and Alabama lost 62, 47, and 52% of their total water loss, respectively, through respiratory transpiration. We estimated respiratory transpiration by subtracting rates of cuticular water loss (Fig. 5b) from overall rates of water loss (Fig. 5a). After acclimation to 20°C, percentages of total rates of water loss attributed to respiratory transpiration was considerably higher for Michigan and Ohio larvae, averaging ~75%. In contrast, the percentage of total rates of water loss attributed to respiratory transpiration for Alabama collected larvae acclimated to 20°C remained relatively unchanged at 54%.

The lowered respiratory transpiration for Alabama larvae acclimated to 20°C was likely the result of a reduced metabolic rate (Fig. 5c). Overall rate of water loss is positively linked to metabolic rate in insects performing high metabolic activities, such as flight (Nicolson and Louw 1982) and in inactive insects that have such low rates of cuticular water loss that respiratory transpiration constitutes the majority of overall water loss (Zachariassen 1996; Williams and Lee 2005). Larvae of *E. solidaginis* from all three collection sites had extremely low rates of cuticular water loss (see references in Hadley 1994) which resulted in cuticular transpiration being the major avenue of overall water loss (see previous paragraph). Thus, it is likely that the lowered metabolic rate of Alabama larvae at 20°C would allow them to lower respiratory transpiration (Gibbs et al. 2003) compared to the other larvae.

If a lowered metabolic rate allowed Alabama larvae to reduce respiratory transpiration compared to other populations at 20°C, then it may represent an adaptive link between water conservation and energy conservation in overwintering insects. Levels of CO_2 production at 5°C indicated that larvae from all collection sites were in the state of diapause (Irwin et al. 2001). Diapause is defined as a genetically determined state of lowered metabolism and suppressed development induced by environmental factors which lasts longer than the adverse conditions (Tauber et al. 1986; Danks 1987). The lowered metabolic rate of diapausing insects is typically associated with conserving metabolic reserves needed for post-diapause development and activities (Tauber et al. 1986; Danks 1987). However, even in diapause, overwintering temperatures can influence metabolic reserves. Larvae of *E. solidaginis* and prepupae of cynipid wasps have lower potential fecundity when subjected to ecologically relevant, yet high overwintering temperatures (Irwin and Lee 2003; Williams et al. 2003). Alabama-collected larvae likely experience substantially higher overwintering temperatures compared the Michigan and Ohio populations (Fig. 6a, b) and undoubtedly, the lower metabolic rate at 20°C would conserve metabolic fuels during the winter. However, the lowered metabolic rate also would allow larvae to conserve body water by substantially lowering respiratory transpiration compared to the other larvae. If the respiratory transpiration of Alabama larvae increased at a similar rate as Michigan and Ohio larvae between 5 and 20°C, then total rates of water loss for Alabama larvae could be as much as 42% higher than what was measured.

In summary, Michigan-collected larvae likely experience the lowest overwintering temperatures and were more cold tolerant than the more southerly Ohio and Alabama larvae. Cold-tolerance was partially linked to higher concentrations of the cryoprotectant glycerol and possibly membrane transporters which function better at lower temperatures. Cryoprotectants also may have functioned to reduce rates of cuticular and overall water loss for Michigan larvae at 5°C. In contrast, Alabama larvae likely experience the highest overwintering temperatures and consequently were the less cold-tolerant than the other two populations. In addition, metabolic rate of Alabama-collected larvae was less affected by the high, 20°C, acclimation temperature which likely aides in maintaining metabolic reserves and water balance by reducing respiratory transpiration during the mild winters they typically experience.

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