The Journal of Experimental Biology 205, 2115–2124 (2002)
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JEB4053

Extreme resistance to desiccation and microclimate-related differences in cold-hardiness of gall wasps (Hymenoptera: Cynipidae) overwintering on roses in southern Canada

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Accepted 2 May 2002

Summary

Four species of cynipid wasp of the genus *Diplolepis* that induce galls on roses (*Rosa* species) in southern Canada and two species of inquiline cynipid associated with these galls were studied for their cold-hardiness and resistance to water loss and for possible links between these adaptations. Mid-winter-acclimated supranivean *D. spinosa* and *Periclistus pirata* had lower supercooling points (–38 to –40 °C) and higher hemolymph osmolalities (1760–1849 mosmol kg^{-1}) than subnivean *D. polita, D. gracilis, D. radicum* and *Periclistus* sp. (–31 to –32 °C and 977–1464 mosmol kg^{-1}, respectively). During a simulated transition from summer/fall to mid-winter conditions, the glycerol concentration of *D. spinosa* more than tripled, reaching a final value of 0.98 mol l^{-1}, while its supercooling point decreased by 13 °C from the initial value of –27.4 °C; however, glycerol concentration and supercooling point did not change for the subnivean species. The permeability of the cuticle of all species was extremely low (0.33–1.00 μg h^{-1} cm^{-2} mmHg^{-1} at 5 °C and 0 % relative humidity; 1 mmHg=0.133 kPa), even compared with that of desert species; however, there was no difference in cuticular permeability between supranivean and subnivean prepupae. Transition temperatures ranged between 32.3 and 34.6 °C; below 30 °C, temperature had little effect on rates of water loss for all species (Q_{10}=1.13–1.87).

Key words: desiccation, cold-hardiness, water loss, permeability, overwintering, gall wasp, *Diplolepis* sp., *Periclistus* sp., hibernaculum.

Introduction

Many overwintering insects in temperate and polar regions must endure cold and severely desiccating conditions, but most studies of adaptations for winter survival have focused primarily on survival at low temperature, with little consideration of water conservation. However, several authors have emphasized that certain adaptations affect not only cold-hardiness but also resistance to water loss (Ring and Danks, 1994; Block, 1996; Danks, 2000), suggesting that both behavioral and physiological adaptations for survival at low temperatures also promote water conservation. Many insects avoid variable and extremely harsh conditions by overwintering beneath leaf litter or snow, where conditions are relatively mild and stable, near 0 °C and 100 % relative humidity (RH) (Marchand, 1996); however, others are exposed to the coldest of ambient winter temperatures and must also resist drying conditions (Danks, 1991).

Most insects cannot survive internal ice formation and are termed freeze-intolerant. The seasonal accumulation of glycerol and other low-molecular-mass polyols and sugars, termed cryoprotectants, promotes increased cold-hardiness and resistance to water loss (Lee, 1991). These substances colligatively increase the insect's hemolymph osmolality, which enhances the ability of its body fluids to supercool (i.e. to remain in the liquid state below the melting point). These substances also act in a similar colligative manner to reduce the vapor pressure deficit between the insect's hemolymph and the environment and, thus, decrease its rate of water loss. Other insects achieve the same result by decreasing their body water content, which concentrates their hemolymph (Ring, 1981; Rickards et al., 1987).

Insects used in studies of adaptation to cold have been collected in a variety of habitats and associated environmental conditions such as in plant stems, under bark and rocks, in soil and within plant galls (Leather et al., 1995). Several species of gall-inducing insect, including the tephritid *Eurosta solidaginis* (Fitch) (Lee et al., 1995), the olethreutid moth *Epiblema scudderiana* (Clemens) (Rickards et al., 1987) and cynipids of the genus *Diplolepis* (Rickards and Shorthouse, 1989; Shorthouse et al., 1980; Sømme, 1964), have been used in cold-hardiness studies, partly because it is easy to collect large numbers in mid-winter. Wasps of the genus *Diplolepis*, all of which induce galls on wild roses (Shorthouse, 1993), are particularly useful for comparative studies of cold-hardiness because some species

overwinter above the snow (supranivean) while others overwinter below it (subnivean).

Approximately 30 species of nearctic *Diplolepis* are known, and each induces galls of a distinctive size and shape on the leaves, stems, buds or tips of stems arising from rhizomes (Shorthouse, 1993). All species are univoltine, and galls are initiated in the spring; larvae feed on specialized nutritive cells lining larval chambers, and they overwinter within their galls as freeze-intolerant prepupae (Sømme, 1964; Rickards and Shorthouse, 1989). Diapause ends and development resumes in early spring. The pupal stage lasts for approximately 15 days before the adult exits its gall and searches for oviposition sites (Brooks and Shorthouse, 1997).

Galls of some species of *Diplolepis* are inhabited and structurally modified by inquiline cynipids of the genus *Periclistus* (Brooks and Shorthouse, 1997, 1998; Shorthouse, 1998). The life cycles of *Periclistus* are similar to those of *Diplolepis* spp. except that *Periclistus* spp. kill the inducer with their ovipositors as they lay their eggs into developing *Diplolepis* galls. *Periclistus* spp. larvae then feed on nutritive cells they induce from tissues of the host gall (Shorthouse, 1998).

Depending upon the location of the gall on the host rose, overwintering *Diplolepis* and *Periclistus* prepupae may experience very different microclimatic conditions. Prepupae in ground-level galls on shoots or rhizomes are insulated from harsh conditions by overlying snow. Prepupae in leaf galls may occupy similar subnivean hibernacula as rose leaves abscise and fall to the ground before winter. In contrast, prepupae in stem galls may be supranivean and exposed to extreme cold and desiccation above the snowpack.

The *Diplolepis* complex and its *Periclistus* inquilines are unique among gall-inducers in that groups of species overwinter in distinctly different sites. Here, we report on the cold-hardiness and resistance to desiccation of the supranivean galler *D. spinosa* and the inquiline *P. pirata*, found in the gall of *D. nodulosa* (Brooks and Shorthouse, 1997, 1998), the subnivean leaf gallers *D. polita*, *D. gracilis* and an unnamed *Periclistus* inquiline, found in the gall of *D. polita* (Shorthouse, 1998), and the shoot tip galler *D. radicum* found at ground level. We examined cold-hardiness and desiccation-resistance in prepupae of these species exposed to a simulated autumn-to-winter transition and in winter-acclimated individuals by measuring supercooling points, glycerol concentrations, hemolymph osmolalities, resistance to water loss, transition temperatures, the effects of treatment with various solvents on rates of water loss and the ability to absorb atmospheric water vapor.

Materials and methods

Field temperatures

Microclimatic conditions experienced by these species were measured by placing temperature data loggers (Onset Computer Corp., MA, USA; model WTA08, range –39 to 75 °C) in a typical gall collecting site near Sudbury, Ontario, Canada. Temperature loggers were spaced 25 cm apart in a vertical transect starting at ground level and reaching a height of 125 cm above the ground. Double sheets of 20 cm×25 cm rectangles of 1/2 inch (1.27 cm) plywood with a 3 cm airspace between each rectangle were used to protect the data loggers against direct insolation. Temperature data collected from 50 to 125 cm above the ground are not reported in this study because they did not differ substantially from temperatures recorded 25 cm above the ground.

Gall collections

Maturing galls containing prepupae of *Diplolepis* spp. and *Periclistus* spp. were collected from mid-August to the beginning of October 1999 at four sites in southern Canada. Galls of *D. radicum* and *D. polita* and those containing *P. pirata* and *Periclistus* sp. were collected near Sudbury, Ontario, Canada. Galls containing *D. spinosa* were found at two sites; those collected near Medicine Hat, Alberta, Canada, were termed *D. spinosa* AB and those collected within the Cypress Hills Provincial Park south of Maple Creek, Saskatchewan, Canada, were termed *D. spinosa* SK. Galls of *D. gracilis* were collected within the Douglas Provincial Park northwest of Moose Jaw, Saskatchewan, Canada.

Galls were held at 15 °C until all samples had been collected. In late October, prepupae were removed from their galls, placed individually in culture plates and held in an incubator at 15 °C and 65 % RH in the dark. To simulate winter conditions, all species were transferred on 11 November 1999 to desiccators containing saturated solutions of NaCl (75 % RH) and held at 5 °C.

Desiccation measurements after cold acclimation

After 2 months at 5 °C, the specimens were weighed to ±0.1 μg before and after desiccation using a Mettler Toledo UMT2 balance. Animals were held in ELISA plastic well plates while desiccated over Drierite (W. A. Hammond Drierite Co., Ohio, USA) with an RH of 0 % (1.5×10^{-2} % RH; Toolson, 1980) until a measurable water loss of 2–5 % of fresh mass was detected (Hadley, 1994).

The surface area of the prepupae was estimated by using the equation of the best-fitting line derived from individuals of known mass and surface area. The surface area was calculated for five individuals of each species by making a small incision on the insect's cuticle, expressing the internal contents and then flattening them on millimeter-squared paper. Equations for the six species were, $y=5.65x-1.19$ for *D. gracilis*, where y is predicted surface area in mm^2 and x is the mass of the organism in mg, $y=2.59x+2.99$ for *D. polita*, $y=1.41x+8.51$ for *D. radicum*, $y=1.54x+11.52$ for *D. spinosa*, $y=3.41x-2.07$ for *P. pirata* and $y=2.98x+1.10$ for *Periclistus* sp. The strength of linear association, as measured by r^2, for these regression equations ranged from 0.78 to 0.91, averaging 0.86. Meeh's formula ($S=kW^{0.667}$, where S is surface area, k is a constant and W is mass; Wigglesworth, 1945; Hadley, 1994) for estimating surface area was also used. In all species, Meeh's formula estimated a 4–10 % larger surface area compared with the best-

fitting line method; it was not used for any subsequent calculations.

The difference in vapor pressure, ΔP, between the hemolymph of the animal and the surrounding air was calculated using the formula:

$$\Delta P = \{[55.556/(55.556 + O)] - (RH/100)\}P_w,$$

where P_w is the standard vapor pressure of pure water at a given temperature (Lundheim and Zachariassen, 1993), O is the body fluid osmolality of the organism being desiccated and RH is the relative humidity to which the prepupae were exposed. ΔP is in mmHg (1 mmHg=0.133 kPa) in the cuticular permeability equation.

Body fluid osmolality was determined using the psychrometric vapor pressure depression technique described by Hølmstrup and Westh (1994). For each species, five groups of 2–5 individual prepupae, with a combined mass of approximately 10 mg, were placed in the sample holder, lanced open with fine probes to expose their hemolymph and quickly inserted into a Wescor C-52 sample chamber (Logan, UT, USA). Samples were allowed to equilibrate for 1 h before osmolality was determined using a Wescor HR33T dew-point microvoltmeter operated in the dew-point mode.

The temperature at which there is an abrupt and dramatic increase in the rate of cuticular transpiration is termed the critical transition temperature. This was determined by exposing live prepupae to temperatures of 5, 20, 27, 33, 40 and 45 °C over Drierite until a measurable water loss of 2–5 % of fresh mass was detected (Hadley, 1994). Eight individuals of each species were used for the 5 °C trial, while five specimens were used in the remaining temperature treatments.

To determine whether water loss was under physiological control, five prepupae of each species were killed by exposure to cyanide for 24 h at 22 °C and desiccated over Drierite at 5 °C. Total body water was measured by placing eight individuals of each species in an oven at 65 °C for 24 h followed by a final weighing to determine dry mass.

To investigate the effects of solvents on the rate of water loss, live prepupae (five per species) were gently washed with hexane, methanol/chloroform (1:2), acetone or water for 1 min. Specimens were then carefully blotted with filter paper and air-dried for 5 min before being weighed and exposed to 0 % RH at 20 °C for 12–24 h to obtain a water loss rate.

To ascertain whether these species were capable of absorbing atmospheric water vapor, individuals (five per species) were desiccated at 0 % RH and 20 °C until they lost 5–10 % of their original body mass. The specimens were then placed over a saturated sodium phosphate solution (95 % RH) at 20 °C for 72 h and reweighed.

Measurements of cold tolerance after cold acclimation

The cold-hardiness of the six species was assessed by measuring their supercooling points and glycerol concentrations. Prepupae (eight per species) were placed in submerged vials in an alcohol bath and cooled at 1 °C min^{-1} until an exotherm, indicating the supercooling point, was detected by a copper–constantan thermocouple placed near the animal. During supercooling point determinations, prepupae were cooled to as much as 5 °C below their supercooling point. Prepupae were then warmed at 1 °C min^{-1} to 5 °C, where they were held for 24 h before being judged to be alive if they responded to tactile stimulation. Glycerol concentration was measured on the same individuals by enzymatic assay (Sigma no. 337), as described by Hølmstrup et al. (1999).

We believe that the supercooling point is a good measure of cold tolerance because preliminary data collected for five of the six cynipid species, *D. gracili*s being the absent group, had survival rates of above 50 % for 10 prepupae per species held within 5 °C above their mean supercooling point for 24 h. Furthermore, no individual survived freezing during supercooling point determination.

Accumulation of resistance to cold and desiccating events

The rhizome shoot galler *D. radicum*, the stem galler *D. spinosa* SK and the leaf galler *D. gracilis* were used to examine changes in rates of water loss and parameters related to cold hardening of the prepupa from late fall to mid-winter conditions through exposure to a constant low temperature. These species were selected because they belong to the same genus and they include representatives that overwinter in both supranivean and subnivean hibernacula. Acclimating procedures were followed as described above, with prepupae extracted from the gall and directly transferred from 15 to 5 °C on 11 November 1999. Using the techniques described above, measurements of rates of water loss, percentages of total body water, glycerol concentrations and supercooling points were taken after 0, 20, 40 and 62 days of exposure to 5 °C. Day zero corresponds to the day when the holding temperature was lowered on 11 November.

Statistical analyses

Analyses of the data included one-way analysis of variance (ANOVA) followed by a Fisher's protected least significant difference (PLSD) test (Sokal and Rohlf, 1995). These analyses were used to identify differences within a species over time when examining the accumulation of resistance to cold and desiccation as well as between species when examining measures of cold-hardiness and desiccation-resistance in mid-winter-acclimated individuals. A significance level of α=0.05 was used for all tests, and values are presented as means ± S.E.M. Linear regression analysis was used to estimate the surface area of the cynipid prepupae, as stated above, and also in estimating the critical transition temperature of mid-winter-acclimated individuals.

Results

Abiotic measurements

Weather data taken from Sudbury (Ontario, Canada) airport indicated that the winter of 1999–2000 was relatively mild compared with the previous 30 years. Between October and April, monthly air temperatures were 2.3 °C warmer than the

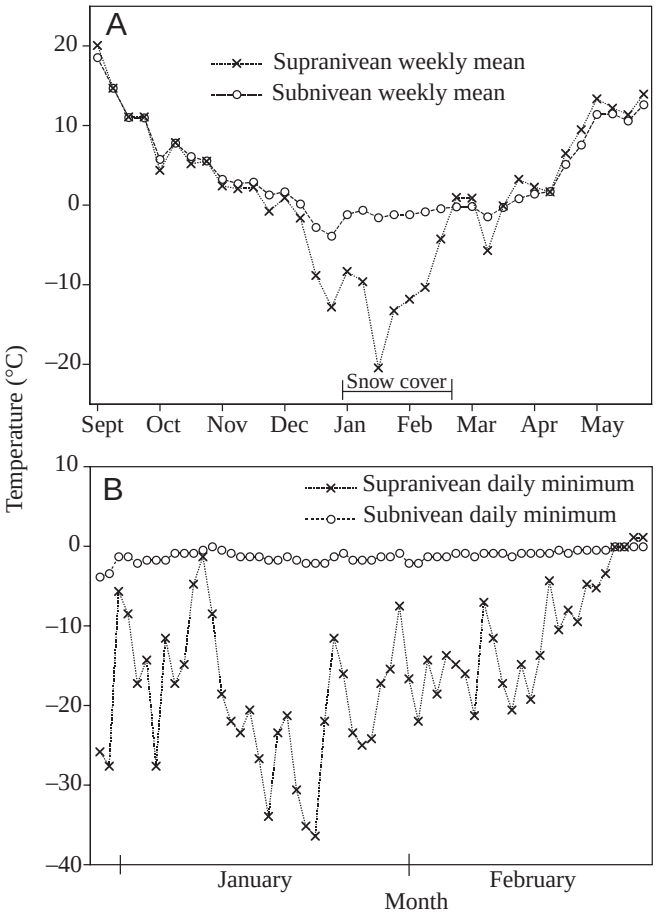

Fig. 1. Weekly mean temperatures from September to May 1999–2000 (A) and daily minimum temperatures from 30 December to 26 February 1999–2000 (B) near Sudbury, Ontario, Canada. Snow cover indicates continuous days of at least 10 cm of snowpack, the period represented in B. Subnivean temperatures were recorded at the ground surface while supranivean temperatures were recorded 25 cm above the ground.

30-year average of –4.7 °C. November and January were the only two months to reach average levels of snow fall.

Even with the milder conditions, differences between supranivean and subnivean microclimates were readily apparent from a vertical temperature transect located near the study site, which was 25 km south of Sudbury airport. From September to late December and from late February to the end of May, weekly mean temperatures at ground level were only 1.1 °C higher than temperatures recorded 25 cm above the ground (Fig. 1A). However, during the coldest period of the winter, from late December to late February, there was a continuous snowpack of at least 10 cm. This snow cover resulted in weekly mean temperatures for the subnivean microclimate that were 9.1 °C higher than for the supranivean microclimate. During this period, subnivean temperatures never fell below –2.8 °C, with a daily temperature range averaging only 0.4 °C (Fig. 1B). In contrast, supranivean temperatures were below –20 °C on 14 different days and had an average daily temperature range of 9.9 °C.

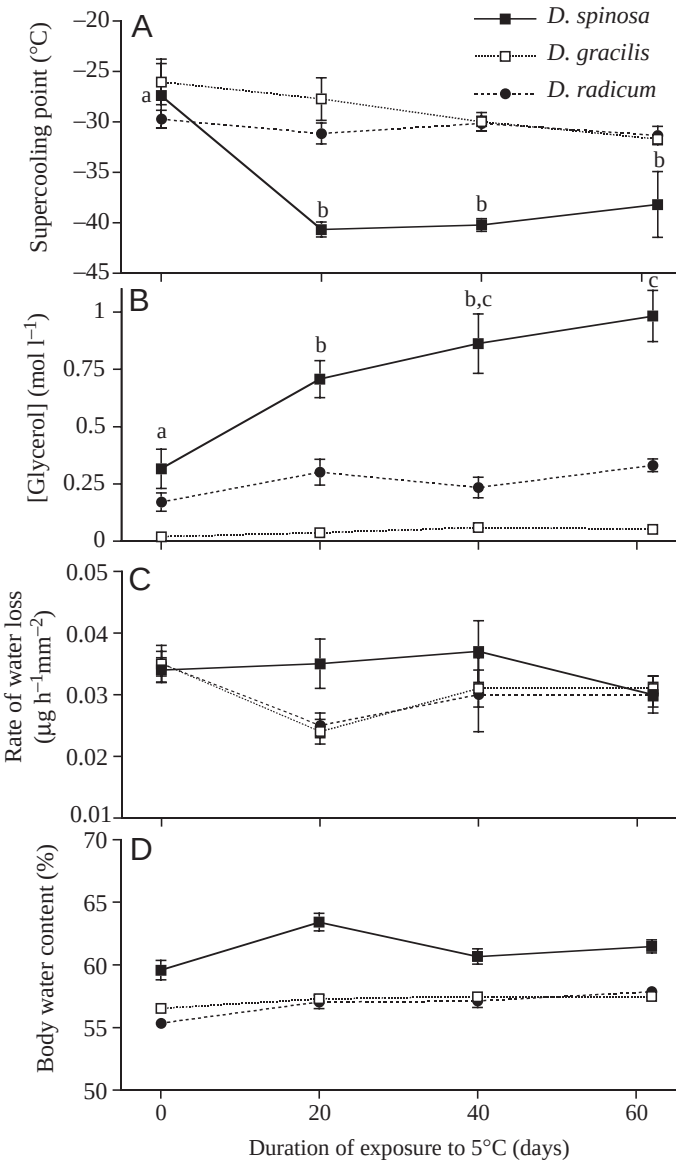

Fig. 2. Supercooling point (A), glycerol concentration (B), rate of water loss (C) and body water content (D) for *Diplolepis spinosa*, *D. gracilis* and *D. radicum* prepupae during winter acclimation at 5 °C. Data points that have, but do not share, letters are significantly different. Values are means ± S.E.M. (*N*=8). Some S.E.M. are within the size of the symbol.

Time course of cold-tolerance and desiccation-resistance during cold acclimation

Throughout the 62 days of cold acclimation at 5 °C, *D. spinosa*, *D. gracilis* and *D. radicum* supercooled extensively within the range –26 to –40 °C (Fig. 2A). These species were judged to be freeze-intolerant because no individuals survived supercooling point determinations. During cold-acclimation, supercooling points remained unchanged for *D. gracilis* and *D. radicum*, species that overwinter in subnivean hibernacula. In contrast, *D. spinosa*, which overwinters in supranivean hibernacula, significantly reduced its supercooling point by more than 13 °C from day 0 (–27.4±3.0 °C; *N*=8) to day 20

(P<0.05). Because of this large decrease, *D. spinosa* had significantly lower supercooling points than *D. gracilis* and *D. radicum* from day 20 until the end of the study (P<0.05).

Decreases in supercooling points were paralleled by increases in glycerol concentrations for *D. spinosa* (Fig. 2B). Levels of the cryoprotectant glycerol significantly increased more than threefold during cold-acclimation, reaching a final value of 0.98±0.11 mol l^{-1} (N=8, P<0.05). At the beginning of the acclimation period, *D. radicum* already contained considerable amounts of glycerol (0.17±0.04 mol l^{-1}; N=8) which was significantly higher than was found in *D. gracilis* (P<0.05). However, neither of these species increased their glycerol level during cold-acclimation.

Body water content remained relatively constant for all species during cold-acclimation (Fig. 2D). The supranivean *D. spinosa* had a significantly higher body water content (P<0.05) than the subnivean *D. gracilis* and *D. radicum* throughout the acclimation period. Similarly, there were no significant changes in the rates of water loss (P>0.05) between or among the three species tested (Fig. 2C). *D. spinosa* averaged 0.034 mg h^{-1} mm^{-2} of water loss compared with 0.030 mg h^{-1} mm^{-2} for both *D. gracilis* and *D. radicum*.

Comparison of cold-tolerance and desiccation-resistance for cold-acclimated cynipid prepupae

Aspects of cold-tolerance and desiccation-resistance were examined for six species of cynipid prepupae after 2 months of exposure to 5 °C. The mean mass of the prepupae ranged widely from 1.5 mg for *Periclistus* sp. to 9.1 mg for *D. spinosa* AB, while body water contents were between 53.3 and 62.3 % for all species (Table 1). Statistically, body water content separated the prepupae into three groups. The two populations of *D. spinosa* had significantly higher body water contents than did *D. polita*, *D. gracilis* and *D. radicum*. In turn, these three species had significantly higher water contents than the two *Periclistus* species (P<0.05).

Table 1. *Mass and body water content for six species of cynipid prepupae acclimated to mid-winter conditions at 5 °C*

Species	Site of gall inducement	Mass (mg)	Body water content (%)
Supranivean galls			
Diplolepis spinosa SK	Stem	9.0±0.4	61.5±0.5[a]
D. spinosa AB	Stem	9.1±1.0	62.3±1.0[a]
Periclistus pirata	Stem	2.6±0.1	53.3±0.4[b]
Subnivean galls			
Periclistus sp.	Leaf	1.5±0.1	55.0±0.9[b]
D. polita	Leaf	3.0±0.2	59.3±1.1[c]
D. gracilis	Leaf	2.3±0.1	57.5±0.3[c]
D. radicum	Shoot	6.1±0.2	57.9±0.3[c]

Values are means ± S.E.M., N=50.

Values for body water content not sharing a superscript letter are significantly different (P<0.05).

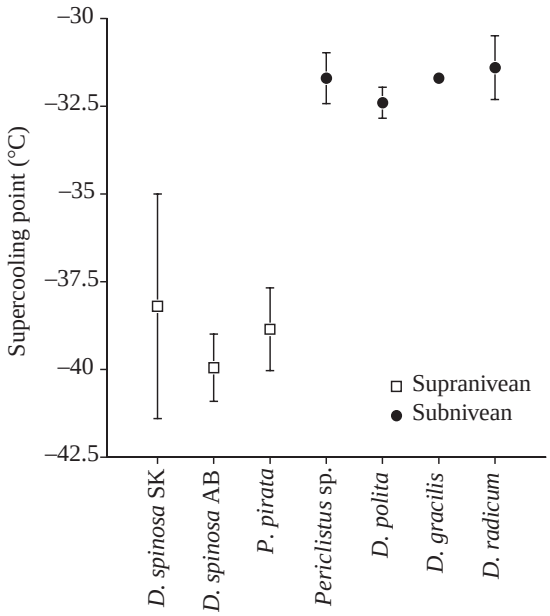

Fig. 3. Supercooling point (mean ± S.E.M., N=8) for cynipid prepupae overwintering in supranivean and subnivean galls. S.E.M. values for *D. gracilis* are within the symbol.

In addition to prepupae tested during cold acclimation *Diplolepis spinosa* AB, *P. pirata*, *Periclistus* sp. and *D. polita* prepupae also supercooled extensively and were unable to survive freezing (Fig. 3). There was no significant difference in the mean supercooling points among the supranivean species, with values ranging between –38 and –40 °C. However, their supercooling points were significantly lower (P<0.05) by 6–8 °C than those of all four subnivean species.

Within each genus, hemolymph osmolality was significantly higher (P<0.05) for species overwintering in supranivean hibernacula compared with those overwintering beneath the snow (Fig. 4). The supranivean inquiline *P. pirata* had the highest overall mean osmolality at 1849±65 mosmol kg^{-1} (N=5), and the leaf gall inquiline *Periclistus* sp. had the lowest overall osmolality at 977±84 mosmol kg^{-1} (N=5). The *D. spinosa* populations averaged 1796 mosmol kg^{-1} compared with the subnivean *Diplolepis* species, which had hemolymph osmolalities that did not exceed 1464 mosmol kg^{-1}.

As with hemolymph osmolality, concentrations of glycerol showed similar trends between supra- and subnivean species within each genus (Fig. 4). The supranivean *D. spinosa* had substantial levels of glycerol that were 5–7 times higher than that of any subnivean species (P<0.05). Likewise, glycerol levels for the supranivean *P. pirata* (300 mosmol kg^{-1}) were significantly higher than those of its subnivean *Periclistus* congener (P<0.05).

Cuticular permeabilities for the *Diplolepis* species were extremely low and did not differ markedly among themselves, ranging between 0.33 and 0.54 μg h^{-1} cm^{-2} $mmHg^{-1}$ (Table 2). However, the two *Periclistus* species had significantly higher cuticular permeabilities at 5 °C and 0 % RH (0.71 μg h^{-1} cm^{-2} $mmHg^{-1}$ for *P. pirata* and

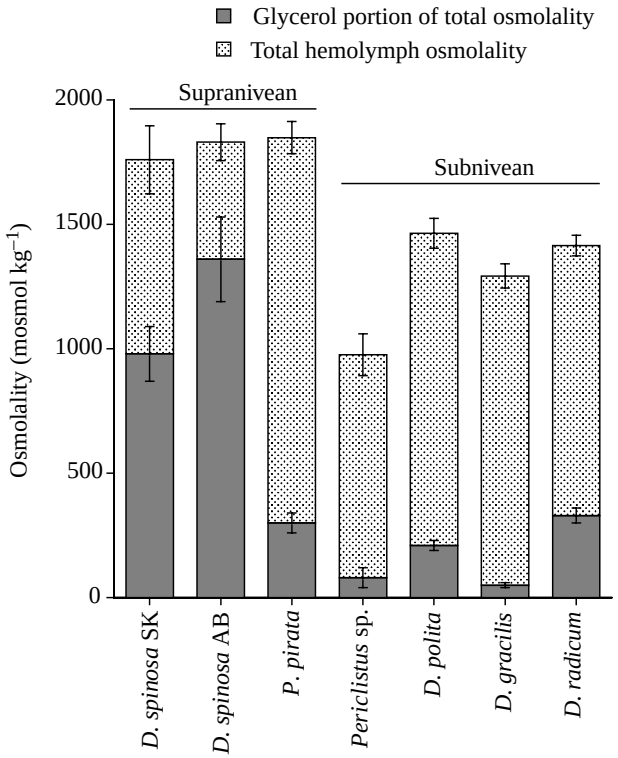

Fig. 4. Total hemolymph osmolality and the glycerol component of total osmolality for the six cynipid species examined under mid-winter conditions. Values are means ± S.E.M. (N=5–8).

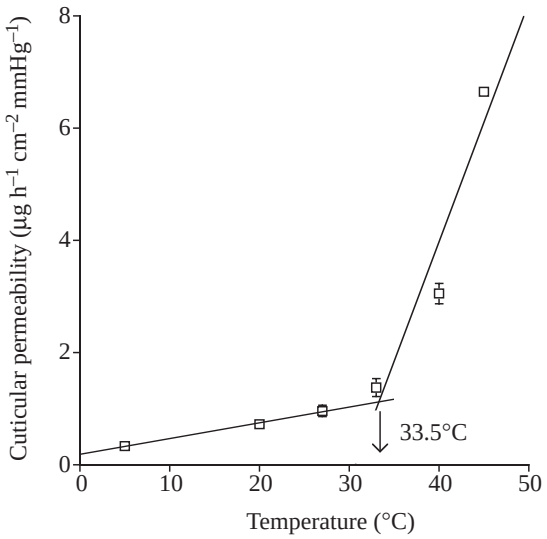

Fig. 5. Effects of temperature on water permeability for prepupae of *Diplolepis spinosa* collected at Medicine Hat, Alberta, Canada. The transition temperature for this species was 33.5 °C. The equation for the regression line below the transition temperature is y=0.028x+0.18 (r^2=0.99), where y is cuticular permeability (μg h^{-1} cm^{-2} mmHg^{-1}) and x is temperature (°C), and the equation for the regression line above the transition temperature is y=0.426x–13.08 (r^2=0.91). Values are means ± S.E.M. (N=5–8).

1.00 μg h^{-1} cm^{-2} mmHg^{-1} for *Periclistus* sp.) than the *Diplolepis* species (P<0.05). To distinguish cuticular *versus* respiratory contributions to water loss, we also determined the cuticular permeability of dead insects. Rates of water loss increased dramatically, by 1.5- to twofold, for dead individuals, suggesting that a substantial component of the resistance to water loss is under physiological/ventilatory control.

To assess further the role of the cuticle in desiccation-resistance, the integuments of the prepupae were treated with water and three organic solvents (Table 3). Washing with acetone did not increase water loss rates significantly compared with control values (washed with water) for any species (P>0.05). However, washing with hexane increased water loss rates significantly for all but two test species, *D. spinosa* SK and *Periclistus* sp., while washing with the methanol/chloroform (1:2) mixture increased water loss rates significantly compared with control values for all species in the study (P<0.05).

Table 2. *Cuticular permeability for six species of cynipid prepupae and the lowest values found in the literature for other species*

Species	Permeability (μg h^{-1} cm^{-2} mmHg^{-1})	Stage	Conditions	Habitat	Remarks	Reference
Diplolepis spinosa SK	0.48±0.05	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Diplolepis spinosa AB	0.33±0.02	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Periclistus pirata	0.71±0.04	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Periclistus sp.	1.00±0.05	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Diplolepis polita	0.54±0.04	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Diplolepis gracilis	0.49±0.03	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Diplolepis radicum	0.47±0.03	Prepupa	5 °C, 0 % RH	Mesic to xeric	Live	Present study
Eurosta solidaginis	5.12	Larva	20 °C, 4 % RH	Mesic to xeric	Live	Ramløv and Lee (2000)
Tenebrio molitor	5	Larva	30 °C	Xeric	Dead	Mead-Briggs (1956)
Onymacris laeviceps	3.41	Adult	27 °C, 5 % RH	Xeric	–	Edney (1977)
Onymacris plana	0.75	Adult	27 °C, 5 % RH	Xeric	–	Nicholson et al. (1984)

Values are means ± S.E.M. (N=8).
1 mmHg=0.133 kPa.
RH, relative humidity.

Table 3. *The effect of washing with water (control), hexane, acetone and methanol/chloroform (1:2) on rates of water loss for prepupae of six cynipid species*

Species	Site of gall inducement	Rate of water loss ($mg\,h^{-1}\,cm^{-2}$)			
		Water	Acetone	Hexane	Methanol/ chloroform
Supranivean species					
Diplolepis spinosa SK	Stem	0.24±0.04[a]	0.29±0.07[a]	1.18±0.12[a]	6.64±1.64[b]
D. spinosa AB	Stem	0.14±0.02[a]	0.14±0.02[a]	0.81±0.07[b]	0.95±0.31[c]
Periclistus pirata	Stem	0.35±0.08[a]	0.60±0.10[a]	5.56±1.77[b]	16.6±0.52[c]
Subnivean species					
Periclistus sp.	Leaf	0.28±0.03[a]	0.26±0.06[a]	0.76±0.14[a]	10.6±2.24[b]
D. polita	Leaf	0.20±0.03[a]	0.26±0.05[a]	0.65±0.09[b]	0.64±0.41[b]
D. gracilis	Leaf	0.14±0.01[a]	0.32±0.03[a]	4.48±0.42[b]	8.76±0.47[c]
D. radicum	Shoot	0.23±0.01[a]	0.32±0.04[a]	5.85±3.48[b]	13.7±1.37[c]

Values are means ± S.E.M., N=5.
Values within a row not sharing the same superscript letter were significantly different.

Table 4. *Summary of the critical transition temperature, including regression line parameters and Q_{10} values derived from cuticular permeability measurements between 5 and 27 °C, for six cynipid wasp species*

Species	Site of gall inducement	Critical transition temperature (°C)	Increase in cuticular permeability from 5 to 27 °C		
			Equation	r^2	Q_{10}
Supranivean species					
Diplolepis spinosa SK	Stem	34.5	y=0.059x+0.08	0.87	1.87
D. spinosa AB	Stem	33.5	y=0.028x+0.18	0.99	1.62
Periclistus pirata	Stem	32.3	y=0.035x+0.80	0.99	1.30
Subnivean species					
Periclistus sp.	Leaf	32.9	y=0.028x+1.17	0.99	1.18
D. polita	Leaf	33.0	y=0.021x+0.45	0.97	1.31
D. gracilis	Leaf	34.6	y=0.007x+0.47	0.75	1.13
D. radicum	Shoot	33.7	y=0.048x+0.16	0.97	1.77

Cuticular permeability ($\mu g\,h^{-1}\,cm^{-2}\,mmHg^{-1}$) is represented by the y variable, while temperature (°C) is represented by x.
1 mmHg=0.133 kPa.

Cuticular permeability increased gradually as temperature increased (Table 4). Between 5 and 27 °C, the Q_{10} values ranged from 1.87 for *D. spinosa* SK to 1.13 for *D. gracilis*. The critical transition temperature, marked by a dramatic increase in cuticular permeability, was estimated graphically to be 33.5 °C for *D. spinosa* AB (Fig. 5). The same procedure was used to estimate transition temperatures for the remaining test groups, whose values ranged between 32.3 and 34.6 °C (Table 4).

To determine whether these species can absorb atmospheric water vapor, individuals from each species were desiccated until they had lost 5–10 % of their original wet mass; they were then exposed to 95 % RH at 15 °C for 72 h. Over this interval, no species showed a net gain in mass. In fact, there was a mean loss of 0.5–2 % of their body mass, suggesting that these organisms did not absorb water vapor under these conditions.

Discussion

Although roses are north temperate plants, fossil records from Colorado and Oregon (Sheperd, 1978) establish their presence on the North American continent for at least 32 million years. Little is known about the evolutionary history of the association between *Diplolepis* and roses and the association between *Diplolepis* and *Periclistus*, but it is possible that these associations began when roses grew in xeric habitats. As the roses dispersed into more northern habitats, the insects associated with them would have had to evolve the means to tolerate freezing temperatures. Wasps that were inducers of stem galls in the upper branches of roses would have found themselves in a precarious predicament – isolated within small chambers and subjected to the coldest and driest conditions of winter. In years of heavy precipitation, some of these stem gallers on the lower branches would have been protected by the insulative qualities of the snow, but those in

supranivean galls would have perished when ambient temperatures dropped below their supercooling point. Wasps in leaf galls would have been better protected than stem gallers as they would find themselves in the warmer subnivean leaf litter throughout the winter. Species of *Periclistus* that became associated with *Diplolepis* stem galls would likewise have to evolve more rigorous cold-hardiness than *Periclistus* associated with leaf galls.

The results obtained in the present study support the above scenario. All species of *Diplolepis* and *Periclistus* examined were highly cold-tolerant, with those species overwintering in supranivean galls having supercooling points 6–9 °C lower than those overwintering beneath the snow. Since the supercooling point approximates the lower lethal temperature for these species, the results suggest that supranivean species are more cold-tolerant than subnivean ones. Furthermore, the values we measured closely matched those of Rickards and Shorthouse (1989) for *D. spinosa* (–38 °C) also collected near Sudbury, Ontario, and Sømme (1964) for *D. radicum* (–33 °C) collected near Lethbridge, Alberta. Although our acclimation protocol exposed prepupae to somewhat milder conditions than they may experience in the field, it was sufficient to induce high levels of cold-tolerance.

The mean supercooling points for the cynipid species collected near Sudbury (*P. pirata*, *Periclistus* sp., *D. radicum* and *D. polita*) corresponded to the environmental temperatures recorded during the winter of 1999–2000. The daily minimum supranivean temperatures were below –30 °C on four occasions, with the lowest recorded winter temperature being –36.4 °C (Fig. 1B); however, even the lowest temperature was not below the mean supercooling point of the supranivean *P. pirata* (–39 °C). In contrast, subnivean species supercooled to approximately –32 °C, well below the minimum temperature recorded (–6.4 °C) at ground level, which occurred prior to the formation of the snowpack (Fig. 1A).

Increases in the supercooling capacity of the supranivean species were associated with increases in hemolymph osmolality. Theoretically, a 1000 mosmol kg^{-1} increase in solute concentration depresses the melting point by 1.86 °C and also decreases the supercooling point by 2–3 times that of the melting point (Duman et al., 1991). By this measure, the mean difference in hemolymph osmolality between the supranivean and subnivean species accounted for approximately 3 °C of the 7 °C difference in their mean supercooling points (Figs 3, 4).

Glycerol was the major solute in the hemolymph of the two populations of the supranivean *D. spinosa*, constituting 56 and 74 % of their overall osmolality (Fig. 4). However, the remaining five species all had low levels of glycerol ranging from 4 to 23 % of the overall osmolality. With such a large proportion of the hemolymph solutes unaccounted for by glycerol, other cryoprotectants such as sorbitol, mannitol or trehalose may constitute the remaining and major solutes for these species (Lee, 1991).

Compared with literature reports for other insects, these cynipid wasps had extremely low rates of water loss (Table 2). These rates are as low as those of the most xeric-adapted species, including the heavily sclerotized adult desert beetle *Onymacris laeviceps*, which Hadley (1994) reported as having the lowest known cuticular permeability. Such high resistance to desiccation for these cynipid species suggests that they experience extreme desiccation stress in winter. Consistent with this pattern is information for the stem-galling tephritid *Eurosta solidaginis*, whose northern range overlaps with that of the cynipid populations we studied (Lee et al., 1995) and which is also highly resistant to water loss (Ramløv and Lee, 2000), suggesting that insects in exposed supranivean hibernacula and in particular those within gall chambers experience extreme water stress.

Even though there were differences in cold-tolerance between the supranivean and subnivean species of cynipids, there were no differences in rates of water loss among them (Table 2). The similarity of their well-developed resistance to water loss implies that they experience similar desiccating conditions. Anatomical studies have shown that, during the late summer and early autumn, tissues of rose galls senesce and dry (Shorthouse, 1993; Brooks and Shorthouse, 1998) and that, once the larvae cease feeding, thick-walled sclerenchyma cells line the interior surface of all gall chambers. The formation of this layer of sclerenchyma undoubtedly provides galls with structural support and perhaps gives protection from parasitoids to the gall former. More importantly, the sclerenchyma layer might also prevent the absorption of water (Rickards and Shorthouse, 1989), which could benefit the prepupae by decreasing the chances of fungal penetration and/or inoculative freezing. We suspect that rose gall cynipids cannot obtain or do not come into contact with free water within their galls until the adults exit 8–9 months later. Consequently, rose gall cynipids may experience desiccation stress for much longer than the winter months. The extremely low rates of water loss, which were similar to mid-winter levels, for *D. spinosa*, *D. gracilis* and *D. radicum* prior to cold-hardening (Fig. 2) support this conclusion.

Several parameters related to water conservation reflect the xeric microhabitat and the extended period of dormancy of these wasps. At temperatures below the transition temperature, Q_{10} values for cuticular permeability were relatively low (Table 4), ranging from 1.13 and 1.87, compared with an average for insects of approximately 2 (Hadley, 1994). These Q_{10} values indicate that large diurnal and seasonal changes in temperature would have little effect on rates of water loss. Similarly, critical transition temperatures for all prepupae (32.3–34.6 °C) were at the low end of the range for terrestrial arthropods (40–60 °C; Hadley, 1994), possibly reflecting the relatively cooler conditions found in their northern climate. These rose gall wasps also appear to conserve water by regulating ventilatory losses. As reported for other insects (Hadley, 1994), rates of water loss increased dramatically for dead individuals, indicating that active physiological control of their spiracles was important for reducing ventilatory losses (Hadley, 1994). The removal of epicuticular lipids by organic solvents illustrated the importance of the cuticle in water conservation for these species. The average 11-fold increase in

water permeability due to hexane washing and 33-fold increase in permeability due to methanol:chloroform (1:2) washing were similar to values reported for the larvae of *E. solidaginis* (Ramløv and Lee, 2000).

Lundheim and Zachariassen (1993) concluded that the lower vapor pressure deficit of the frozen larvae of *Pytho depressus* and adults of the beetles *Upis ceramboides* beetles contributed to their lower rates of water loss compared with that of supercooled individuals. A reduction in the vapor pressure deficit between the insect's hemolymph and the surrounding air elicited by an increase in hemolymph osmolality was one factor that led Ring and Danks (1994) to suggest that adaptations primarily associated with cold-hardiness, such as increased hemolymph solute levels, might be even more important for water conservation.

As discussed above, the species with the highest hemolymph osmolalities had the greatest capacity to supercool and were therefore the most cold-tolerant. However, the effect of elevated hemolymph osmolalities on water conservation was less clear. Supranivean cynipids acclimated to mid-winter conditions had the highest hemolymph osmolalities (Fig. 4), but their rates of water loss were not significantly lower than those of subnivean species (Table 2). Edney (1977) contends that even a large increase in osmolality would have a minimal effect on reducing a large vapor pressure deficit and, thus, would have little effect on water loss. The approximate range of hemolymph osmolalities for the wasps in this study (977–1849 mosmol kg^{-1}) would create a corresponding range in vapor pressure deficits of only 6.42–6.33 mmHg (at 5 °C and 0 % RH), a difference of 1.4 %. Experimentally, differences in rates of water loss due to such small decreases in vapor pressure deficit would be difficult to demonstrate because of individual variability and because cuticular waterproofing may differ among the species. However, compared with typical hemolymph osmolalities for summer insects (300 mosmol kg^{-1}; Edney, 1977), it is possible that a three- to sixfold increase in hemolymph osmolality may have a significant effect on water conservation over the varied conditions and extended periods in which rose cynipids inhabit their galls. Furthermore, the fact that supranivean species within a genus had higher levels of glycerol in their hemolymph compared with subnivean species gives issue to the possibility that the well-known hydroscopic properties of this polyhydric alcohol (Crowe and Clegg, 1973) are important for water conservation.

We suggest that traits associated with water conservation may represent pre-adaptations that facilitated the evolution of an increased capacity for cold-tolerance. This, in turn, could have allowed for dispersal of these species from southern to northern climates. The prepupal stages of *D. spinosa* and *D. radicum* had relatively high glycerol concentrations (Lee, 1991) even prior to cold exposure (Fig. 2), suggesting that these solutes may be associated with water retention. The presence of biochemical pathways for synthesizing and accumulating glycerol and other polyhydric alcohols associated with water retention in warmer climates may have been selected for because quantitative increases in the production of these solutes would promote supercooling and cold-hardiness. Increased hemolymph solute levels together with other physiological and morphological factors that also promote supercooling and cold-hardiness, such as a relatively small body size compared with other insects (Lee and Costanzo, 1998), may have facilitated the radiation of these species northwards.

We thank Dr Joe Bagatto, Jonathan Leggo and Marilyn R. Shorthouse for assistance in collecting the galls and Scott Shreve who aided in the laboratory work. Dr Mercedes Ebbert, Dr Dennis Claussen, Dr A. John Bailer and two anonymous reviewers added to the clarity of the manuscript. This work was supported by Miami University's summer workshop program, NSF IBN-0090204 to R.E.L. and grants from NSERC and the Laurentian University Research Fund to J.D.S.

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