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Cold hardiness of the fly pupal parasitoid *Nasonia vitripennis* is enhanced by its host *Sarcophaga crassipalpis*

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Abstract

Supercooling points (SCPs) and low temperature survival were determined for diapausing and nondiapausing larvae of the ectoparasitoid *Nasonia vitripennis*. Neither nondiapausing nor diapausing larvae could survive tissue freezing. The SCP profiles were nearly identical for nondiapause-destined (−27°C) and diapausing larvae (−25°C), but these values were not indicative of the lower limits of tolerance in either type of larvae: larvae were killed by chilling at temperatures well above the SCP. Diapausing larvae could withstand low temperature exposures 3–8 times longer than their nondiapausing counterparts. Low temperature survival was enhanced in diapausing and nondiapausing larvae by their encasement within the puparium of the host flesh fly, *Sarcophaga crassipalpis*: the LT_{50}s determined for nondiapausing and diapausing larvae enclosed by fly puparia were 2–3 times higher than values calculated for larvae removed from the puparia. Additional low temperature protection was gained through acquisition of host cryoprotectants during larval feeding: nondiapausing parasitoid larvae that fed on diapausing flesh fly pupae with high levels of glycerol were able to survive exposure to a subzero temperature 4–9 times longer than wasps reared on nondiapausing fly pupae that contained lower quantities of glycerol. Alanine may also contribute to the cold hardiness of *N. vitripennis*, as evidenced by the fact that larvae feeding on diapausing fly pupae both contained higher concentrations of alanine and exhibited greater cold hardiness. The results thus demonstrate that several critical features of cold hardiness in the wasp are derived from biochemical and physical attributes of the host.

Keywords: Cold tolerance; Cold shock injury; Supercooling points; Glycerol; Larval diapause; Ectoparasitoid

1. Introduction

Though cold tolerance has been studied in many insects, little is known about the effects of low temperatures on insect parasitoids. Many endoparasitic wasps depend on the physiological status of the host insect to regulate their own diapause (Schoonhoven, 1962; Brown et al., 1990; Polgar et al., 1991), and possibly the cold hardiness of these wasps is also host dependent. Parasitic wasps that feed externally (ectoparasitoids) are also influenced by the nutritional and biochemical conditions of the host (Vinson and Iwantsch, 1980; Rivers and Denlinger 1994a, 1995), but such factors seem to have a significantly reduced role in the development of these insects compared to their endoparasitic counterparts. Consequently, physiological features of ectoparasitoids, such as diapause, appear to be independent of host composition (Rivers and Denlinger, 1995). Consistent with this prediction is the apparent absence of diapause in at least some ectoparasitic pteromalids (Petersen and Meyer, 1983), suggesting that the development of these species is not synchronized with the diapause program of the host and therefore the capacity to survive adverse environmental conditions is host independent as well.

In this study, the pupal ectoparasitic wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) was used to examine cold tolerance characteristics in larvae, the developmental stage that normally overwinters. Facultative larval diapause in *N. vitripennis* is of maternal origin (Schneiderman and Horwitz, 1958) and regulated by photoperiod (Saunders, 1965), temperature

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(Saunders, 1966a), host deprivation (Saunders, 1966b), and host species (Saunders et al., 1970). The latter observation was challenged by Rivers and Denlinger (1995), who found that the incidence of larval diapause of *N. vitripennis* was not altered by the species, age, or physiological status of the host. These observational differences suggest that only certain species of fly hosts can induce larval diapause in this parasitic wasp, and implies that the cold-hardening response of *N. vitripennis* may also be host dependent when developing on specific fly hosts.

Here, we examine the cold tolerance of diapausing and nondiapausing larvae of *N. vitripennis* to subzero temperatures and determine the supercooling points in both of these developmental stages. The relationship between the cryoprotectant levels (alanine and glycerol) of host flesh flies, *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae), and the low temperature survival of nondiapausing wasp larvae is evaluated, and we compare host cryoprotectant levels to the cryoprotectant composition found in developing parasitoids. The unusual development of *N. vitripennis*, feeding on the exterior of its host but residing within the fly puparium, also provided an opportunity to evaluate the role of puparia from the flesh fly on the cold hardiness of diapausing and nondiapausing wasp larvae.

2. Materials and methods

2.1. Insect rearing

Wasp larvae were reared as previously described (Rivers and Denlinger, 1994a). Nondiapausing larvae were produced by rearing adults at 25°C with a daily light:dark cycle of LD 15:9 h. Twenty to thirty females (3–7 days after emergence from host puparia) were placed in a Petri dish (15×100 mm) with 50 nondiapausing pupae (4 days after pupariation) of *S. crassipalpis* and a 50% honey solution. After 24 h, the adult wasps were removed and the parasitized hosts held at 25°C, LD 15:9 h. At this temperature, last instar (4th) larvae were present 5–6 days after egg deposition.

Diapausing larvae of *N. vitripennis* were generated by placing 20 females (3–7 days after emergence from host puparia) at 10°C in continuous darkness for 2 d. These females were then placed singly with 3 nondiapausing pupae (4 days after pupariation) of *S. crassipalpis* and held at 25°C in a LD 15:9 h light cycle for 24 h. Larval diapause was confirmed using the criteria of Schneiderman and Horwitz (1958).

A colony of *S. crassipalpis* was maintained as described by Denlinger (1972). Adults were held at 25°C with either a long-day (LD 15:9 h) or short-day (LD 12:12 h) daily light:dark cycle. To avert pupal diapause, larvae collected from LD 15:9 h adults were reared under a long-day regime at 25°C throughout development. Diapausing pupae were produced by rearing larvae collected from short-day adults at LD 12:12 h and 20°C. Pupal diapause was confirmed by removing the anterior portion of the puparium and looking for the developmental characteristics outlined by Fraenkel and Hsiao (1968).

2.2. Supercooling points

The supercooling points (SCPs) were determined as described previously (Lee and Denlinger, 1985). Diapausing and nondiapausing wasp larvae were positioned so that their integument made contact with cooper–constantan thermocouples. A cooling rate of ca. 1°C/min was maintained using a Neslab RE-8DD low temperature bath. The SCP was the lowest temperature recorded prior to release of the latent heat of fusion as body water freezes (Lee and Denlinger, 1985).

2.3. Hexane treatment

Exposure to hexane may be used as a tool to stimulate synchronous diapause termination in flesh fly pupae (Denlinger et al., 1980), an event which is followed by initiation of adult development and a rapid decline in the levels of glycerol (Lee et al., 1987). In this study, diapausing pupae were placed in a glass desiccator and exposed to hexane vapors for 2 h. This treatment terminated diapause in all individuals.

2.4. Low temperature survival

To examine intrinsic cold-shock tolerance (the ability to survive exposure to a subzero temperature in the absence of a preparatory cold-hardening treatment) (Chen et al., 1987), 5 parasitized fly puparia containing either diapausing (ca. 30–40 larvae/puparium) or nondiapausing larvae (ca. 40–60 larvae/puparium) of *N. vitripennis* were placed in thin-walled test tubes (13×100 mm) and capped with a cotton plug. The tubes were placed in a Lauda RM20 (Brinkmann) low-temperature glycerol bath preset at either −10°, −15°, −20°, or −30°C. After low temperature treatment, nondiapausing larvae were transferred to 25°C, LD 15:9 h and their developmental fate was recorded. Initial mortality was recorded at 6 h post-treatment. Larvae were considered dead if they did not move when touched several times, and mortality was again confirmed a few hours later by visible signs of necrosis (evident by a darkening of larval tissue and hemolymph, and loss of integument integrity).

Diapausing larvae were held at 25°C, LD 15:9 h for 48 h following each treatment and then were transferred to 10°C in continuous darkness for 3 months. *N. vitripennis* cannot terminate larval diapause without first experiencing several months at low temperatures below 15°C (Schneiderman and Horwitz, 1958). Following exposure to these conditions, diapausing larvae were

transferred to 25°C, LD 15:9 h to terminate diapause and their developmental fate was recorded.

Low temperature survival was also examined in diapausing and nondiapausing larvae that were removed from the host puparia ("naked" larvae). The opercula of parasitized fly puparia were removed and the exposed wasp larvae were transferred with forceps to thin-walled test tubes (10 larvae/tube). The naked larvae were then subjected to the low temperature treatments described above.

In a parallel set of experiments, the importance of host cryoprotectants to the cold hardiness of developing wasp larvae was assessed by rearing *N. vitripennis* on pupae of *S. crassipalpis* containing different levels of glycerol and alanine, and then subjecting naked, nondiapausing parasitoid larvae to a −20°C low temperature treatment as described above. Following treatment, the wasp larvae were placed at LD 15:9 h and 25°C, and their developmental fate was recorded.

The exposure time required to kill 50% of the wasps (LT_{50}) at a given temperature was determined for each subzero temperature treatment by the method of Finney (1971). LT_{50}s were calculated on the basis of mortality that occurred in any subsequent life stage of the wasp.

2.5. Glycerol and alanine determination

Whole-body glycerol content was determined using an enzymatic assay (Sigma Chemical Co. #337-40A). Samples of 5 wasp larvae (late-4th instars) were homogenized in perchloric acid which was then neutralized with sodium bicarbonate to pH 7.0. Glycerol levels were determined spectrophotometrically by measuring sample absorbance of light (520 nm), and expressed in mM units based on the water content of wasp larvae determined by Yoder et al. (1994).

Total body alanine levels were monitored as described by Rivers and Denlinger (1994b). Briefly, samples of 3 late-4th instar larvae were homogenized in 25 mM sodium phosphate buffer (pH 7.4), centrifuged (2486***g***), the supernatant deproteinized with 6% trichloroacetic acid and high speed centrifugation (10,000***g*** at 4°C), and the final supernatant was adjusted to pH 3.5 using 5 M potassium carbonate. Alanine concentrations were determined using the reverse enzymatic reaction described by Williamson (1974) for alanine dehydrogenase (EC 1.4.1.1) (Sigma).

The concentration of alanine in fly pupae serving as hosts for *N. vitripennis* was also determined as previously described (Rivers and Denlinger, 1994b). Alanine concentration was expressed in mM units based on the fly water masses determined by Adedokun and Denlinger (1985).

3. Results

3.1. Supercooling point determinations and freeze susceptibility

Tissue freezing was lethal to all larvae regardless of diapause status. Exposure for even a few minutes to −30°C, a temperature below the SCPs of diapausing (−25.0°C±2.0) and nondiapausing larvae (−27.3°C±0.3) was lethal. Diapausing larvae were able to withstand exposure to −30°C slightly longer than nondiapause-destined larvae: a 5 min exposure killed 100% of the nondiapausing larvae while a 15 min exposure was required to kill all of the diapausing larvae.

3.2. Low temperature tolerance

Low temperatures above the supercooling point were also lethal if the duration of exposure was sufficiently long. This type of chilling injury increased as treatment temperature decreased and length of exposure increased (Fig. 1). Nondiapausing larvae were far more susceptible to chilling injury (Fig. 1 A–C) than diapausing larvae (Fig. 1 D–F) at each of the temperatures tested. This is reflected in LT_{50} values (survival time for 50% of population) for diapausing larvae that are 3–8 times higher than those determined for nondiapausing larvae at each temperature (Fig. 1).

Among the nondiapausing parasitoids, the severity of the low temperature was also reflected in the ultimate developmental stage attained by the wasp larvae. Increased severity (lower temperature or increased time at the same low temperature) caused higher mortality in the larval stage, whereas many parasitoids (>82%, n=225) exposed to less extreme conditions progressed to the pupal or to the pharate adult stage before dying. Such differences were not as apparent among diapausing larvae. While increasing exposure time did result in increased mortality at the larval stage, differences in the stage of mortality at −10°, −15°, and −20°C were far less pronounced for diapausing larvae than for nondiapausing larvae.

Differential mortality between male and female larvae was not observed, and all broods (clutches enclosed within a single fly puparium) were heavily female-biased at each low temperature: the mean±SEM sex ratio (males/total number of adult wasps) was 0.20±0.11 for nondiapausing larvae and 0.23±0.05 for diapausing larvae (combination of data from all 3 low temperatures, n=3582 broods for nondiapausing and 5400 broods for diapausing larvae). In comparison, larvae reared under long-day conditions (LD 15:9 h and 25°C) throughout development also produced female-biased clutches (0.21±0.9, n=895), as expected for *N. vitripennis* (Werren, 1984).

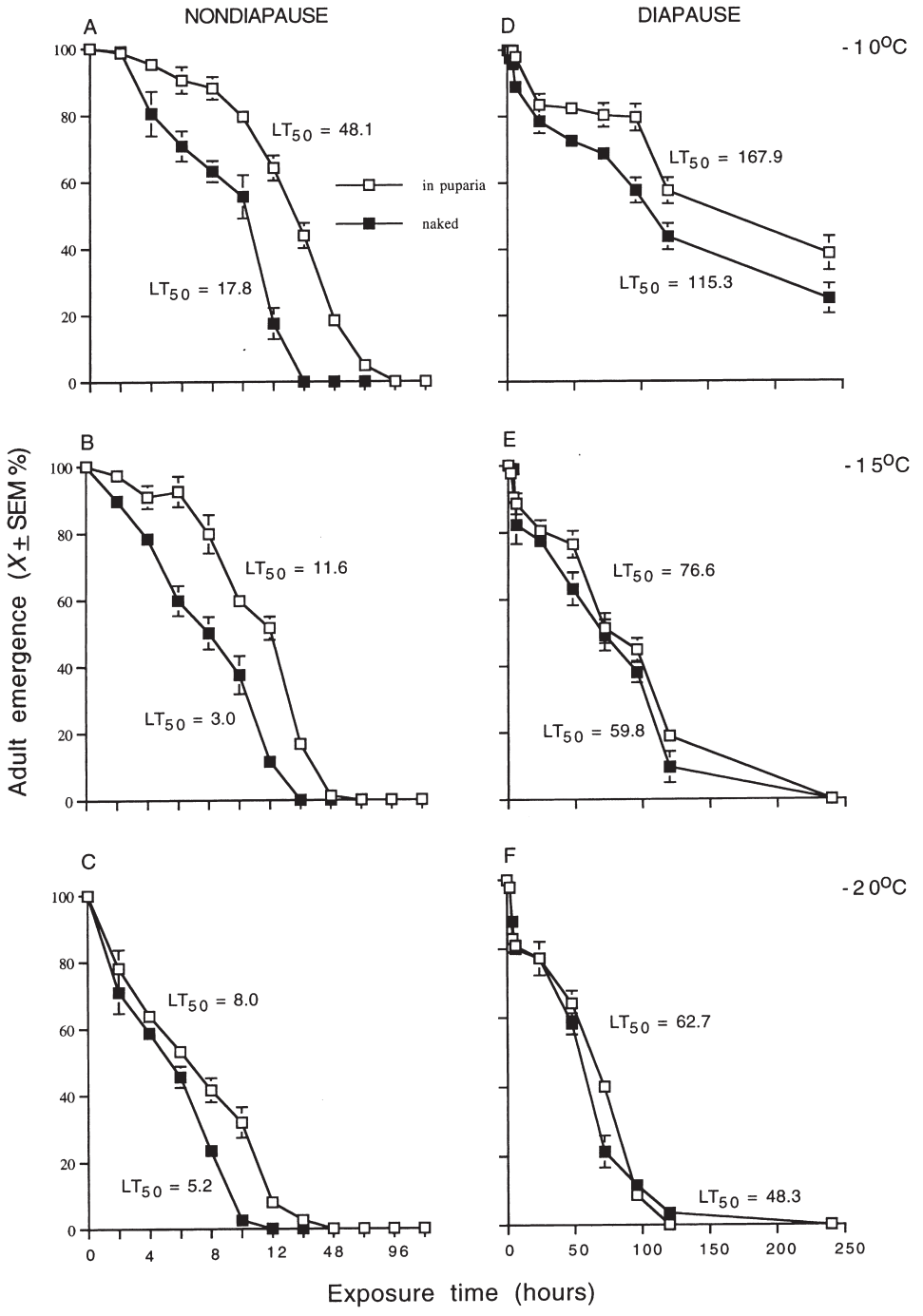

Fig. 1. Low temperature survival of nondiapausing and diapausing larvae of *N. vitripennis* either encased in host puparia or removed from the host puparia (naked). For each time point, n=50–128 wasp larvae. LT_{50} values are expressed in hours.

3.3. Role of the host's puparium

To test the effect of the host's puparium in protecting the parasitoid larvae from chilling injury, larvae were exposed to low temperatures either within the host puparium or after being removed from the puparium (Fig. 1). For both diapausing and nondiapausing larvae, the LT_{50}s for "naked" larvae (unprotected by the host's puparia) were significantly shorter than for larvae enclosed within the puparium (Fig. 1). Among the "naked" larvae, all mortality occurred in the larval stage: none succeeded in developing into pupae or pharate adults. In contrast, parasitoids encased within fly puparia were more likely than "naked" larvae to show some progression in development following low temperature exposure. For example, 94% (n=278) of the larvae enclosed in host puparia developed into pharate adults when exposed to −10°C for <24 h, whereas only 7%

of the naked larvae reached this developmental stage under the same conditions. As the severity (lower temperature or increased length at the same low temperature) of exposure conditions increased, parasitoids positioned along the periphery of the puparium died more quickly than those located in the center of the intrapuparial space, and none of the wasps from the periphery exhibited signs of continued development following low temperature treatment.

3.4. Influence of host's diapause status

For nondiapausing wasp larvae, protection from chilling injury was also acquired by feeding on diapausing hosts which had high levels of cryoprotectants. Wasp larvae which developed on diapausing pupae of *S. crassipalpis* displayed LT_{50} values that were 4–9 times higher than parasitoids reared on nondiapausing pupae or on flies that had been treated with hexane vapors to terminate pupal diapause (Fig. 2). An analysis of host cryprotectant levels confirmed that tissues extracted from diapausing flesh flies contained far more glycerol than those from nondiapausing or hexane-treated pupae (Table 1). Parasitoids that developed on these hosts contained corresponding levels of glycerol: larvae of *N. vitripennis* that fed on diapausing pupae contained 4-fold more glycerol than parasitoids that consumed nondiapausing hosts (Table 1).

To determine if wasp larvae were accumulating other host cryoprotectants while feeding, we analyzed parasitoid and host tissues for alanine, the amino acid that is elevated to the highest levels in flesh flies during diapause (Johnson and Denlinger, unpublished) and following injection of venom by *N. vitripennis* (Rivers and Denlinger, 1994b). Alanine levels were relatively high in all host stages examined but levels observed in diapausing and nondiapausing hosts that were parasitized did not differ significantly (Table 1). The concentration of alanine extracted from parasitoids, however, was highest among those wasp larvae which were most cold tolerant, i.e. the wasp larvae reared on diapausing fly pupae (Table 1, Fig. 2). Thus, the host source of alanine was not higher in the parasitized diapausing fly pupae than in nondiapausing hosts, but the wasp larvae reared on the diapausing pupae accumulated higher stores of alanine.

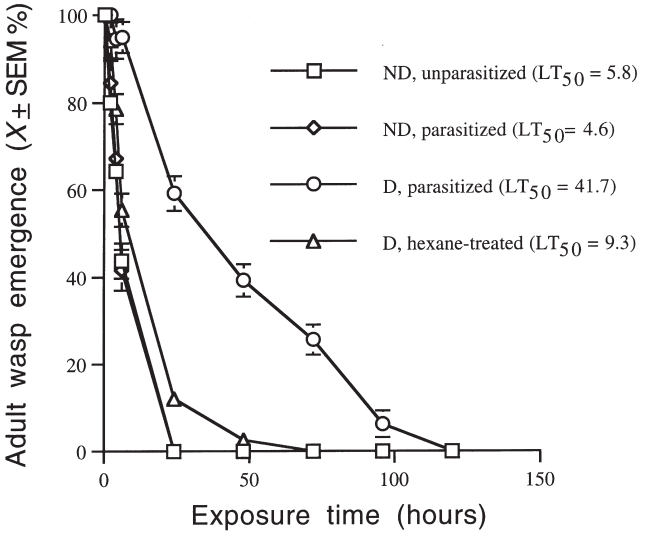

Fig. 2. Adult emergence of nondiapausing *N. vitripennis* that were reared on hosts with different concentrations of glycerol and alanine, and then subjected to −20°C as 4th instar larvae removed from the host puparia. ND=nondiapausing hosts, D=diapausing hosts. For each time point, n=75 wasp larvae. LT_{50} values are expressed in hours.

Table 1
Glycerol and alanine concentrations in larvae of *N. vitripennis* reared on pupae from *S. crassipalpis* differing in physiological status

	Concentration (mM)	
Species	Alanine	Glycerol
Host		
Sarcophaga crassipalpis		
ND pupae, nonparasitized	12.1±5.2	28[a]
ND pupae, parasitized	26.2±3.8	–
D pupae, parasitized	20.6±2.7	56[a]
D pupae, hexane-treated	18.1±4.6	28[a]
Parasitoid		
Nasonia vitripennis larvae reared on:		
ND pupae, nonparasitized	16.7±3.1	–
ND pupae, parasitized	14.3±2.0	4.8±0.6
D pupae, parasitized	31.5±1.5	18.0±3.0
D pupae, hexane-treated	13.8±0.9	–

[a] Values determined by interpolation from Fig. 3 and 4, Lee et al., 1987. Late third instar, nondiapausing larvae of *N. vitripennis* reared either on diapausing or nondiapausing pupae of *S. crassipalpis* were used in glycerol and alanine assays. Nonparasitized hosts were flies that were not naturally parasitized by *N. vitripennis*, but which received 20 eggs transferred from a parasitized pupae of the same age and physiological status. Hexane-treated diapausing pupae were generated by exposing flies (40 days into diapause at 20°C) to hexane vapors for 2 h, followed by incubation at L15:D9, 25°C for 48 hours.

4. Discussion

In temperate climates, *N. vitripennis* utilizes a facultative larval diapause to avoid the harsh environmental conditions of winter (Schneiderman and Horwitz, 1958). The short days of late summer in combination with cool night time temperatures serve as environmental cues that program the wasp for diapause (Saunders, 1965; Saunders, 1966a). Entry into this physiological state, however, does not ensure winter survival. The overwintering sites of *N. vitripennis* are typically fly puparia buried only a few centimeters below the soil surface, and consequently these insects are exposed to environmental temperatures that may plunge far below zero. In this study, we demon-

strated that nondiapausing and diapausing larvae of *N. vitripennis* are intolerant of tissue freezing, and chilling injury and death were observed at temperatures well above the SCPs for both larval stages. Thus, winter mortality for this species apparently is largely due to non-freezing temperatures. For *N. vitripennis* offspring, cold hardening presumably results not only in a lowering of the lethal temperature, but also increases the wasp's tolerance to prolonged exposure to non-freezing temperatures.

Cold-hardening refers to the physiological and biochemical processes which result in enhanced tolerance of subzero temperatures (Zachariassen, 1985). Accumulation of antifreeze compounds and the seasonal lowering of the supercooling point are usually associated with cold-hardening in insects intolerant of freezing. Such seasonal adaptations have not been reported for *N. vitripennis*, but several lines of evidence suggest that the mechanisms of cold-hardening used by this wasp are closely related to those described for two species of natural fly hosts, *S. bullata* and *S. crassipalpis*:

(a) the SCP of *N. vitripennis* (−25°C) is nearly identical to that of *S. crassipalpis* (−23°C; Lee and Denlinger, 1985);
(b) a loss of body water occurs in both wasps and flies (Yoder and Denlinger, 1991; Yoder et al., 1994) prior to entry into diapause; and
(c) cold-hardening of *N. vitripennis* and *S. crassipalpis* is associated with accumulation of glycerol and alanine (Lee et al., 1987; Kukal et al., 1991).

In some insects preparing to enter winter dormancy, a reduction in body water precedes an increase in cold hardiness (Sømme, 1982). This physiological phenomenon appears to be part of the diapause syndrome of *N. vitripennis*: diapausing larvae contain 10% less water than nondiapause-destined larvae (Yoder et al., 1994), and these larvae could also withstand low temperature exposure 3–8 times longer than their nondiapausing counterparts. Water loss is thought to enhance cold hardiness through a redistribution of body water that passively elevates extracellular cryoprotectant levels (Sømme, 1982). Such a passive mechanism alone, however, cannot account for the cold tolerance displayed by diapausing larvae in this study nor under field conditions, but may serve to reduce the energy costs associated with acquisition of cold hardiness (Hochachka and Somero, 1984).

Cold hardening by a passive mechanism also depends on the presence of cryoprotectants in the body fluids of overwintering larvae of *N. vitripennis*. No information is available concerning the ability of diapausing wasp larvae to synthesize their own antifreeze compounds. However, the possibility exists that these wasp larvae cold harden by sequestering host-derived cryoprotectants. Venom from *N. vitripennis* induces a rapid decline in glycogen and trehalose reserves, and a 3-fold elevation in alanine levels in nondiapausing, pharate adults of *S. bullata* (Rivers and Denlinger, 1994b). These biochemical adjustments are very similar to metabolic changes associated with cold-hardening in many insects: glycogen provides the carbon source for the synthesis of glycerol (Mansingh and Smallman, 1972; Storey et al., 1981)), and elevated titers of alanine accompany increased cold hardiness in *Diatraea grandiosella*, *Ostrinia nubilalis* (Morgan and Chippendale, 1983) and *S. crassipalpis* (Kukal et al., 1991). Developing wasp larvae completely consume (or nearly so) the fly host by the end of the larval feeding period (Rivers and Denlinger, 1995). Thus, host cryoprotectants are readily available to *N. vitripennis*.

In this study, we have shown that nondiapausing larvae of *N. vitripennis* reared on hosts with high levels of glycerol contain higher levels of this antifreeze compound than wasp larvae reared on flies with lower quantities of cryoprotectant. The cold tolerance of *N. vitripennis* correlates closely with this pattern of cryoprotectant accumulation: wasp larvae containing high levels of glycerol exhibited greater cold tolerance. The overwintering strategy of this parasitoid appears to include sequestering host antifreeze compounds during feeding, thereby decreasing the energetic costs of its own cold-hardening.

Though alanine levels were also high in the most cold-tolerant stages of *N. vitripennis*, elevation of this amino acid is most likely not through accumulation of host alanine. The alanine composition of all fly hosts used in this study (with the exception of unparasitized, nondiapausing pupae) was nearly identical, and indicates that new synthesis must account for the elevated levels of alanine in nondiapausing wasp larvae. The fact that the alanine content was nearly identical in nondiapausing wasp larvae that differed tremendously in cold tolerance suggests that this amino acid is not the primary cryoprotectant used by *N. vitripennis*.

Additional low temperature protection was provided by puparia of *S. bullata*. Both nondiapausing and diapausing wasp larvae could tolerate longer durations at subzero temperatures when encased by host puparia than when exposed as naked larvae. A similar protective effect has recently been noted for diapausing eggs of the evergreen bagworm, *Thyridopteryx ephemeraeformis* (Haworth) (Lepidoptera: Psychidae) when deposited in the silken-bag of the mother (Rivers and Yoder, unpublished data). In the case of *T. ephemeraeformis*, the protection afforded by the adult bags is attributed to the presence of an unstirred air-space within the bag, which probably serves as a buffer to changes in ambient temperatures that results in slower rates of cooling within the bag than outside. Of course this explanation is only pertinent to larvae of *N. vitripennis* under field con-

ditions where ambient air temperatures fluctuate widely throughout the long duration of winter (Guzman and Petersen, 1986).

Under laboratory conditions, perhaps the most significant factor influencing the increased survival of encased wasp larvae was the "insulation" effect created by larvae positioned along the periphery of the intrapuparial space. Wasps huddled in the center of the unstirred air-space and surrounded by siblings, were more likely to survive extreme temperature conditions than those wasps on the periphery and in direct contact with the host puparia. In this scenerio, wasps located along the outer regions of the intrapuparial environment could function as an insulating layer for their siblings. The layering of wasp larvae within the host puparium may simply lead to differential cooling rates from outside to inside, resulting in wasps located in the center of the intrapuparial space chilling at a slower rate than parasitoids found at the periphery.

The ability of filth fly parasitoids to survive harsh winter conditions is a major limitation inhibiting the successful use of these wasps in biological control programs aimed at muscoid flies (Guzman and Petersen, 1986). The results obtained in this study suggest that other pteromalids may demonstrate cold hardiness comparable to *N. vitripennis* and that cold tolerance could be enhanced in these parasitoids by rearing them on hosts with enriched levels of cryoprotectants.

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