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Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* fitch (diptera: tephritidae)

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Abstract Seasonal changes in metabolic rate and the potential for morphological development demonstrated that third-instar larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch, exhibit a distinct winter diapause. Metabolic rate (CO₂ production) was significantly lower from 15 October to 9 February than in early autumn (9 September) and spring (1 March) samples. The induction of diapause coincided with the development of cold-hardening, maximum larval mass, and gall senescence, but our experiments did not identify specific cues triggering diapause induction. We examined the influence of exposure to 0 °C and –20 °C on diapause development. Diapause development in larvae stored at 0 °C occurred at approximately the same rate as in nature. Until 15 December the larvae were in the refractory phase of diapause (incapable of morphological development, even at permissive temperatures), but afterward moved to the activated phase within which diapause intensity decreased until termination in February. Diapause development occurred in larvae collected during the winter and stored at –20 °C for periods of 1 week to 3 months. Diapause intensity decreased in frozen larvae through the winter but at a slower rate than in larvae stored at 0 °C.

Key words Diapause · Freezing · Cold · Goldenrod gall fly · Metabolic rate

Abbreviation *JH* juvenile hormone

Introduction

Diapause is a stage of arrested morphological development, common in insects during periods of adverse environmental conditions. Tropical insects typically enter diapause during the dry season, while many temperate insects do so during winter. Diapause involves a suite of neuroendocrine and other physiological changes manifested in a reduced metabolic rate and the cessation of morphological development (Tauber et al. 1986). A variety of cues signals entry into and termination of the diapause stage, and these cues may act individually or in combination depending on the species of insect and local conditions. The most common cues are temperature and photoperiod, but others include food availability or quality, moisture, oxygen, pH, and salinity (Danks 1987). A diapause stage allows insects to conserve energy during periods of unfavorable environmental conditions, and synchronizes reproduction both within a population and with environmental conditions that are optimal for reproduction (Mansingh 1971).

Diapause is a dynamic process which varies in intensity and “develops” so that the cues (and often the magnitude of the cues) necessary to terminate diapause change over time (Tauber et al. 1986; Danks 1987). The terminology used to describe the types and phases of diapause have taken many forms in the literature. Some authors have created new terms with operational definitions to describe biological responses resulting from experimental manipulation of animals in diapause (Tauber et al. 1986; Danks 1987). For simplicity, we use the general terminology proposed by Mansingh (1971) which is applicable to the responses of insects and other invertebrates both in the laboratory and in the field.

First, the general phrase “diapause development” refers to the physiological changes associated with progression through diapause. Diapause is preceded by a “preparatory phase” during which energy reserves are accumulated. The entry of an insect into diapause is referred to as “diapause induction.” Early in diapause,

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insects are typically in the “refractory phase” when they cannot terminate diapause and continue morphological development, even if exposed to optimal environmental conditions. Later in diapause, however, insects gradually move into the “activated phase” during which an insect is able to terminate diapause and continue development if environmental conditions are favorable. “Diapause intensity” changes throughout the phases mentioned above; it is typically measured as the duration of permissive conditions required for the insect to terminate diapause.

This study focuses on the winter diapause of the goldenrod gall fly, *Eurosta solidaginis* Fitch. This species, which ranges from Texas to southern Canada, forms galls on the stems of goldenrod plants. In the early summer (late May and early June in New York) newly emerged *E. solidaginis* mate and the short-lived female adults (9–10 days) lay eggs in the terminal buds of young *Solidago* spp. (Uhler 1951). Because the window of opportunity to mate and lay eggs is quite narrow, it is important that reproductive activity be synchronized with other members of the species and with the emergence of the host plant. Larvae feed and grow within the galls throughout the summer and into the fall. In late September the host plant begins to senesce and, about this time, larvae excavate an exit tunnel to the surface of the gall, then retreat to the center. The larvae overwinter as freeze-tolerant third-instar larvae, and do not pupariate and eclose until spring when temperatures increase and new host plants are available (Uhler 1951).

Overwintering third-instar larvae of *E. solidaginis* experience extreme variation in temperature and hydration state of the gall tissues. The galls are generally above the snowpack and the gall itself provides little insulation to the larva within. In fact, gall temperature closely follows ambient air temperature and therefore overwintering larvae may experience daily fluctuations as great as 29 °C and the extreme cold of winter (Layne 1991, 1993). Correspondingly, these larvae are extremely cold-tolerant (Salt 1959; Morrissey and Baust 1976; Lee et al. 1995) and highly resistant to desiccation (Ramløv and Lee 2000).

Previous reports have suggested the existence of diapause in overwintering *E. solidaginis* larvae, but none of these studies sought evidence for diapause by systematically measuring both metabolic rate and developmental potential (key indicators of diapause) through the entire autumn-to-spring transition. The first suggestion of diapause was made by Uhler (1951), who found that larvae collected prior to January 13 did not develop and emerge as adults when moved into a greenhouse. However, 18% of a sample collected on January 20, following 4 days of subzero temperatures, did develop to adulthood. Similarly, Bennett and Lee (1997) observed that only a small proportion of *E. solidaginis* larvae collected in November or December pupariated after placement at 23 °C following 24 h of freezing to either –10 °C or –20 °C. In contrast, almost 100% of the larvae collected in late January developed

to the adult stage after the same treatments. Also, January larvae demonstrated little change in oxygen consumption after 5 days at 15 °C, whereas the metabolic rates of March larvae increased significantly (Lee et al. 1995). The best evidence comes from measurements of oxygen consumption on seasonal (summer, autumn, and winter) samples of larval *E. solidaginis* (Layne and Eyck 1996). Summer larvae exhibited high metabolic rates and a high Q_{10} compared to the autumn and winter larvae. However, large changes in larval size and local weather during collecting prevented Layne and Eyck (1996) from attributing these patterns to diapause.

The study we present here critically tested for the existence of diapause in overwintering *E. solidaginis* using measures of metabolic rate and developmental potential and sought to describe the phenology of diapause development in this species. Because of this species' extreme cold tolerance, we also examined the effects of freezing on diapause development. To achieve our goals, we have provided a seasonal characterization of metabolic rate in this species, both throughout the winter and during diapause termination. We have used morphological development: (1) as an indicator of diapause development (including progression through the stages of diapause and changes in diapause intensity) through the fall and winter, and (2) to explore the effects of freezing on diapause development. Our results are considered within the framework of seasonal changes in the gall microenvironment and the physiological condition of the larvae.

Materials and methods

Seasonal changes in metabolic rate were measured in third-instar goldenrod gall fly larvae collected from Miami University's Ecology Research Center at 2–3 week intervals throughout the fall and winter of 1997–1998. Larvae were individually placed in glass respirometry chambers and the chambers were submerged in water inside a double-walled beaker. A refrigerated alcohol bath (Neslab, RTE-8DD) was used to control the temperature of coolant flowing through the beaker wall, thus allowing precise control of larval body temperature. CO₂ production of the larvae was measured using flow-through (40 ml·min⁻¹) respirometry (TR-3 system, Sable Systems) followed by analysis with DATACAN software (Sable Systems). CO₂ production was measured at 5 °C intervals from 10 °C to 20 °C with 1 h equilibration at each temperature before measurements were made. Larvae were weighed (± 0.01 mg) following the experiments to allow expression of metabolic rates as microliters CO₂ produced per gram fresh weight per hour. Larvae were then dried to constant mass and weighed to allow calculation of water content. The plant galls were also weighed, dried, and weighed again for calculation of water content.

The same protocol and apparatus described above were used to measure metabolic rates for the diapause termination experiment except that metabolic rate was only measured at 23 °C. (Preliminary experiments demonstrated that 23 °C was above the developmental threshold for larvae collected in late winter.) We used elevation of metabolic rate as an indicator that a larva had broken diapause and was continuing development. Because several days were required for January larvae to break diapause, each point on the graph represents a different group of eight larvae. In contrast, because measurements were made over only a period of hours, the February sample represents a single group of eight larvae measured repeatedly.

To analyze seasonal changes in diapause development, galls were collected on the 15th day of November, December, January and February, 1997–98. On each collection date, 15 larvae were removed from galls, weighed, placed in individual wells of a tissue culture tray (one well filled with water to increase humidity), and placed at 23 °C. Larvae were kept in darkness to avoid the potentially confounding effects of photoperiod. The remaining galls from each collection were subdivided into two groups and stored in darkness at either 0 °C (unfrozen) or –20 °C (frozen). Sub-samples of 15 larvae were first held at 4 °C for 24 h, then transferred to 23 °C (as described above) at intervals of 1 week, 2 weeks, 1 month, 3 months, and 5 months after collection. Once moved to 23 °C, larvae were checked daily and the date of pupariation, date of adult emergence, and sex of the adult were recorded. We present time to pupariation as an indicator of diapause intensity, and the percent reaching adulthood as an indicator of developmental potential (i.e., refractory vs. activated phases of diapause). Almost every individual that pupariated reached adulthood.

Ambient air temperatures (maximum and minimum daily temperatures) were obtained from a weather station at Miami University's Ecology Research Center approximately 100 m from the site of collection.

Respirometry data were analyzed by ANOVA followed by Bonferroni multiple comparisons. All values presented in the text are mean \pm SE, and a significance level of $\alpha = 0.05$ was used in all statistical comparisons.

Results

Our measures of seasonal changes in metabolic rate indicate that third-instar larvae of *E. solidaginis* exhibit a distinct and stable diapause stage (Fig. 1A). CO₂ production at any given temperature was significantly depressed from 15 October to 9 February in comparison to the initial and final samples. The measures before initiation of diapause (9 September) and following diapause (1 March) were not significantly different. The changes in metabolic rate cannot be attributed to changes in body weight because larval weight and water content changed little through the periods of diapause induction and diapause termination (Figs. 1B, C). Diapause initiation was temporally correlated with gall drying (an indicator of host plant senescence) (Fig. 1B), peak larval weight (Figs. 1C and 5), and the first frost of winter (2 October) (Fig. 1D). These factors did not seem to be related to the termination of diapause near 1 March (Figs. 1 and 5).

As the winter progressed, the larvae stored at 0 °C progressed into the activated phase of diapause; that is, the proportion capable of development to adulthood increased (Fig. 2). Larvae collected on 15 November did not develop to adults when placed in a permissive temperature and were thus judged to be in the refractory phase of diapause. However, after 3 months or 5 months at 0 °C, these larvae had progressed to the activated phase as many were able to continue development to the adult stage: 73% after 3 months, 68% after 5 months. December-collected larvae showed a similar pattern: none developed initially but as the exposure to 0 °C increased from 1 week to 1 month, the proportion reaching adulthood increased. After 1 month, about 80% reached adulthood, but this proportion continued to rise slowly, reaching 100% after 5 months. The

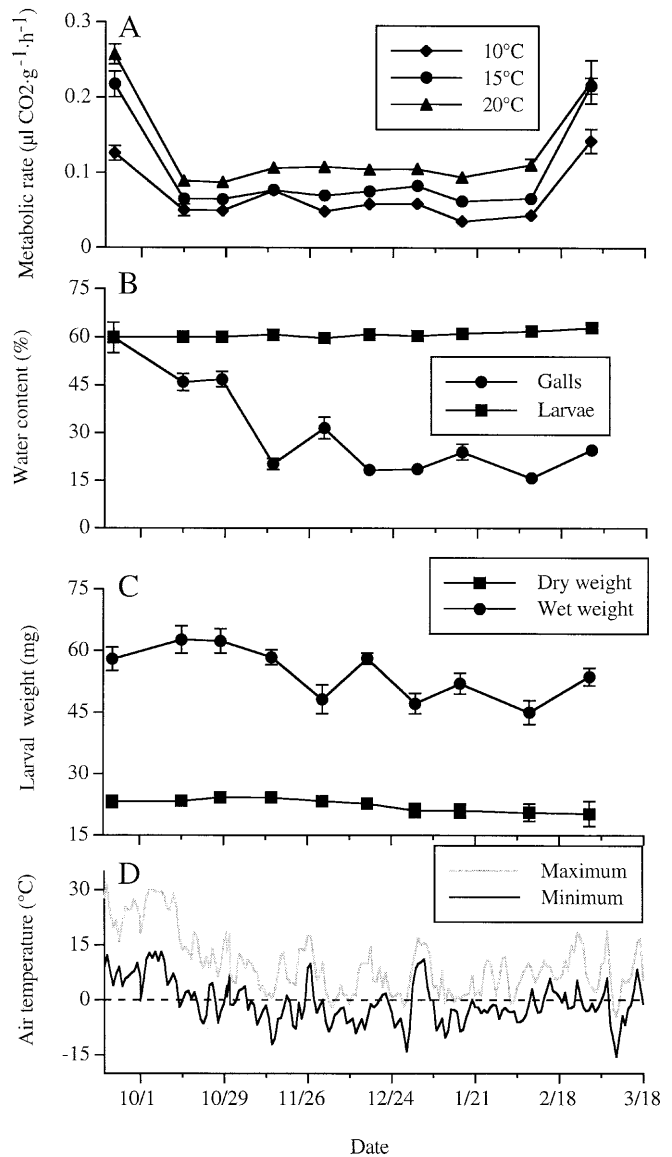
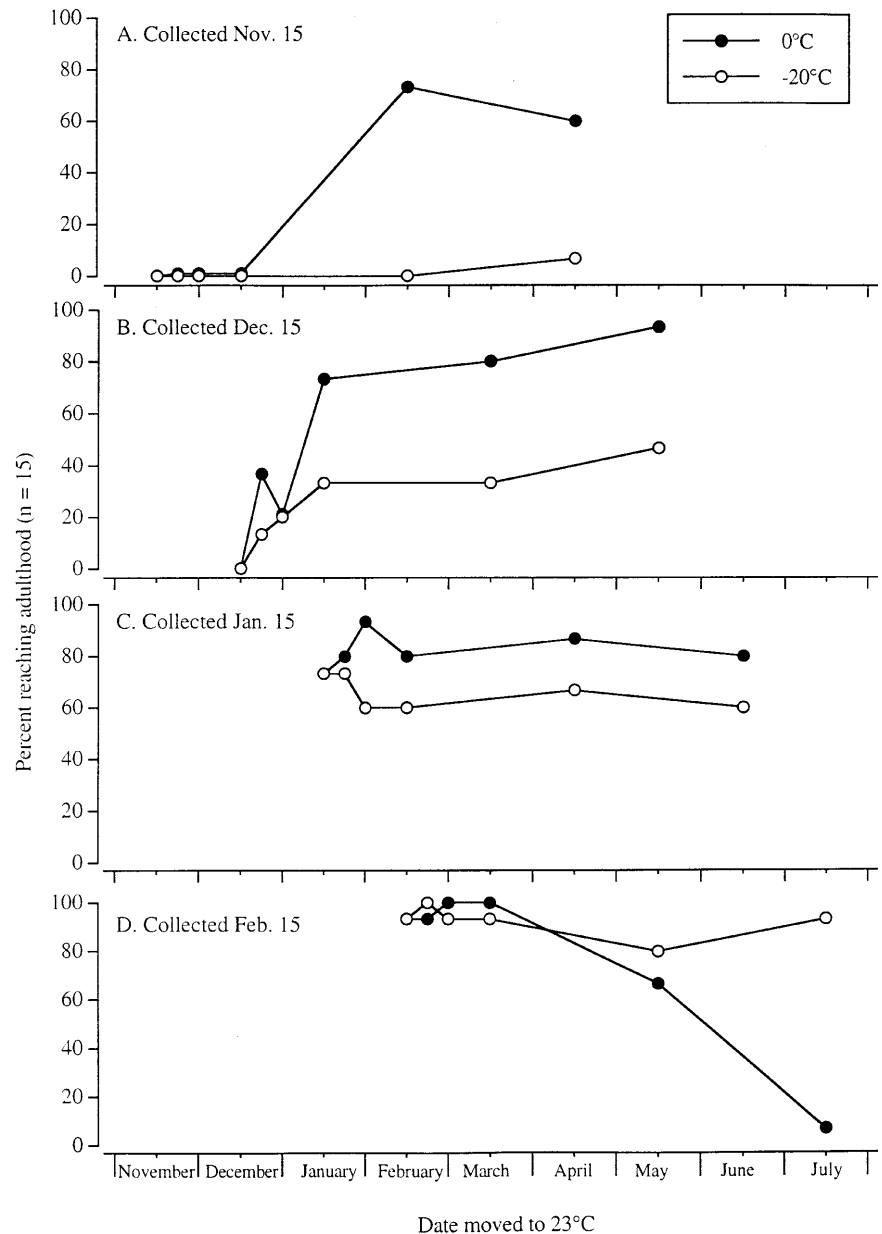


Fig. 1 Seasonal changes in third-instar *E. solidaginis* larvae and their environment: **A** metabolic rate (CO₂ production), **B** water content of galls and larvae, **C** fresh and dry larval mass, and **D** ambient air temperature at the collection site. Data are presented as mean \pm SE

proportion of January- and February-collected larvae that reached adulthood also reached a plateau at 80–100%, with the exception of the 3-month and 5-month exposures to 0 °C. These groups exhibited high mortality, likely due to depletion of energy reserves (after 3 months, larval mass was 18% lower in the 0-°C group than in the –20-°C group) during this unnaturally long “winter” exposure that extended into the summer months.

Directly corresponding to the seasonal progression in the proportion of larvae reaching adulthood was a decrease in the number of days required for pupariation after transfer from 0 °C to 23 °C (Fig. 3); this pattern indicates a reduction in diapause intensity and

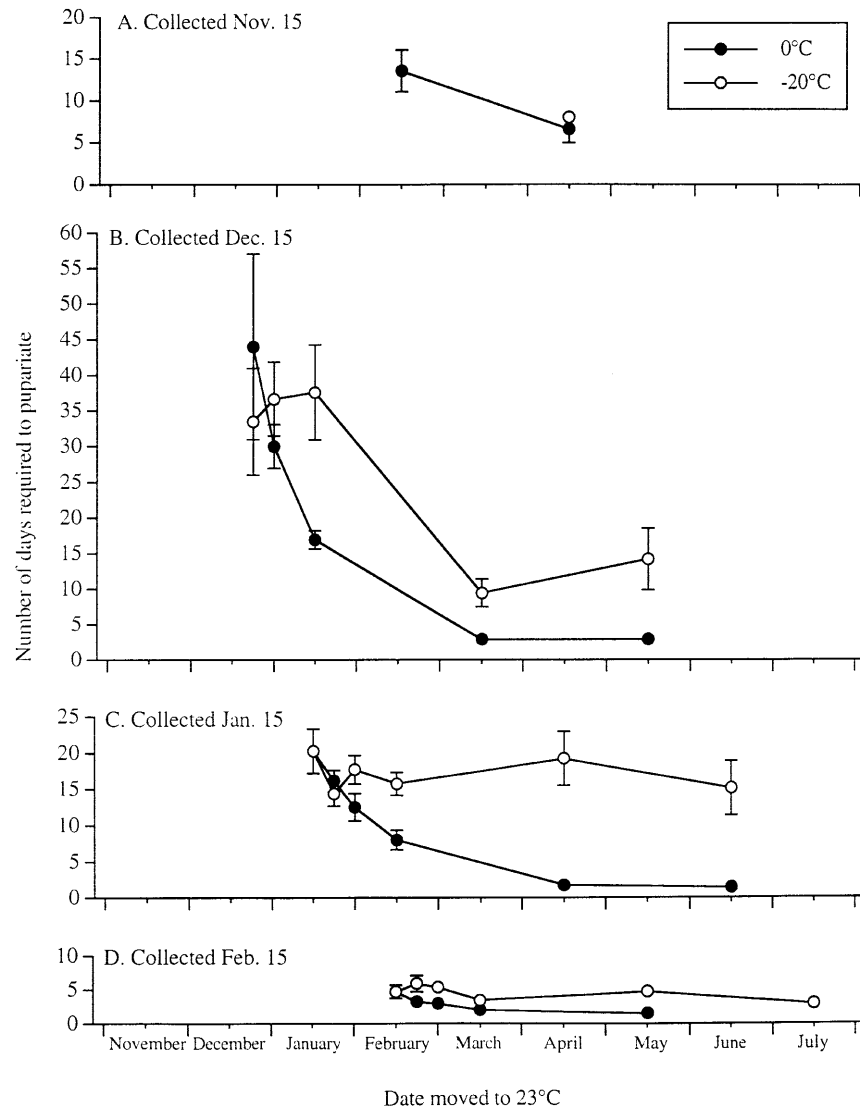
Fig. 2A–D Proportion of *E. solidaginis* larvae ($n=15$) stored at 0 °C or –20 °C that continued development and successfully emerged as adults. Four collection periods are represented: 15 November (A), 15 December (B), 15 January (C), and 15 February (D). The ability to continue development and reach adulthood indicates a shift from the refractory to activated phase of diapause. The first sample of each collection date was transferred directly from the field to 23 °C



progression through the activated phase of diapause. November-collected larvae exposed to 0 °C for 3 months required 13.6 ± 2.5 days to pupariate but those held for 5 months required only 6.6 ± 1.6 days. The trend is more pronounced with the December-collected larvae where the number of days required to pupariate fell from 44 ± 13 days after 1 week at 0 °C to 2.9 ± 0.5 days after 3 months and 5 months at 0 °C. Similar decreases in the number of days to pupariation were also evident in the January- and February-collected larvae. In fact, the trend lines for time to pupariation in January- and February-collected larvae closely matched the pattern seen in the December-collected larvae (Fig. 3). The similarity between these patterns indicates that diapause development occurred at a similar rate at 0 °C as it did under field conditions.

Diapause development also occurred in larvae frozen at –20 °C. The November-collected larvae did not exhibit an increase in the proportion reaching adulthood, even after 5 months of exposure. However, the December-collected larvae held at –20 °C exhibited trends similar to, but lagging behind, those observed in the 0-°C treatment group. The proportion reaching adulthood increased but only to a plateau of ~40%, not the 80–100% seen in the 0-°C group (Fig. 2B). Time to pupariation also lagged behind the 0-°C group. Only after 3 months at –20 °C did the number of days required to pupariate decrease, and then it fell from 37.6 ± 6.7 days (at 1 month) to 9.4 ± 4.4 days (3 months) and 14.1 ± 4.3 days (5 months) (Fig. 3B). Although this was a substantial decrease, the time to pupariation at 3 months and 5 months remained long compared to the

Fig. 3A–D Number of days at 23 °C required for *E. solidaginis* larvae stored at 0 °C or –20 °C to pupariate. Again, four collection periods are represented: 15 November (A), 15 December (B), 15 January (C), and 15 February (D). Sample sizes vary depending on the proportion continuing development as illustrated in Fig. 2. Data are presented as mean \pm SE



2.9 \pm 1.8 days required by the 0-°C group after 3 months and 5 months (Fig. 3B).

Measures of metabolic rate indicated that larvae collected 16 January were in diapause and required 10–13 days to terminate diapause and resume morphological development (Fig. 4). In contrast, larvae collected 19 February increased their metabolism and resumed development immediately upon warming (Fig. 4), and thus were not in diapause.

Discussion

The presence of a distinct diapause stage in overwintering third-instar *E. solidaginis* is evident from the long and stable period of reduced metabolic rate throughout the winter and the inability to continue development at permissive temperatures. In regions such as southwest Ohio where winters are relatively mild and unseasonably warm weather is common (Fig. 1D), gall temperatures often reach > 25 °C (Layne 1993; Abrahamson and Weis

1997). High rates of metabolism would be detrimental during these periods, especially because adult *E. solidaginis* lack functional mouth parts and all adult nutritional reserves, including those required for reproduction, must be obtained from larval feeding the previous summer and fall. Therefore, there would be strong selection for a winter diapause to improve energy conservation in *E. solidaginis* (e.g., Irwin and Lee 2000). Diapause would also prevent premature development and emergence during unseasonably warm winter weather, thus preventing adult emergence before host plants are available. Reduced metabolic rate during winter may also enhance freeze tolerance by reducing the accumulation of toxic by-products of anaerobic metabolism while the animal is frozen (Rojas et al. 1989).

Several physiological and environmental events are correlated with the induction of diapause in this species. The first frosts occur around the time of diapause induction (compare Figs. 1A and 1D). These cold nights, possibly in combination with photoperiod (Danks 1987), may have triggered the induction of diapause. In other

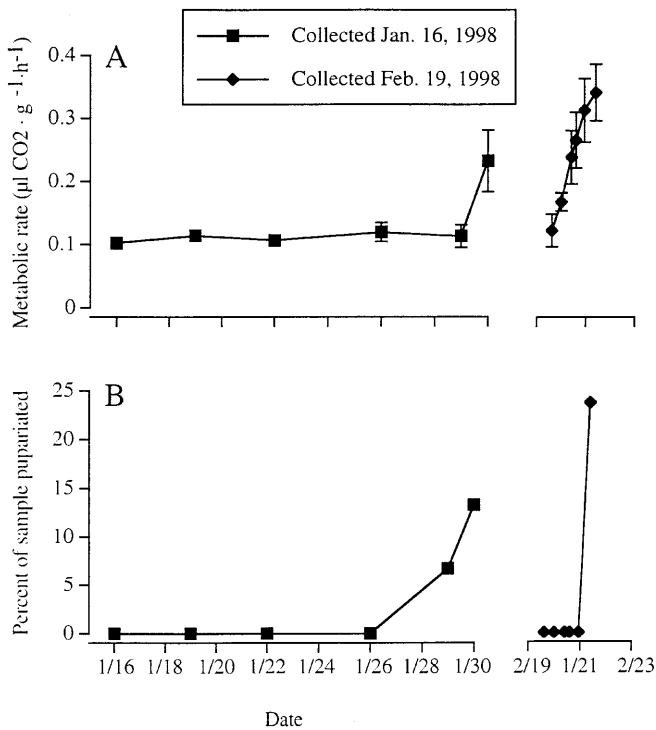


Fig. 4 Termination of diapause (as indicated by elevation of metabolic rate) in *E. solidaginis* after transfer to 23 °C (A), and the corresponding proportion of larvae that had pupariated (B). Metabolic rate data are presented as mean \pm SE

phytophagous species, diapause induction is often stimulated by host plant senescence (Danks 1987) and, indeed, gall senescence is also correlated with diapause induction in *E. solidaginis* (Fig. 1B). During this time larval body weight is also at its peak (Fig. 5). Mansingh (1971) suggests that insects are best served by entering diapause when energy reserves are at their maximum because reducing metabolic rate at this time optimizes energy conservation through the winter. Although the cold nights, gall senescence, and peak larval weight all occur around the time of diapause induction (see summary on Fig. 5), more experiments are required to identify which specific cues, or combination of cues, may be responsible for diapause induction in *E. solidaginis*.

Denlinger (1991) identifies some endocrinological and physiological links between diapause induction and the development of cold-hardiness in insects, but is careful to point out that one cannot assume the presence of cold-hardiness on the basis of diapause or vice versa. Indeed, our data suggest that there is incomplete linkage between diapause and cold-hardiness in *E. solidaginis* (Fig. 5). Diapause induction (between 9 September and 15 October; Fig. 1) is closely correlated with the development of freeze tolerance to -25 °C in this species (between 26 September and 10 October; Bennett and Lee 1997) and the accumulation of the cryoprotectant glycerol (Hamilton et al. 1986; Fig. 5). The accumulation of glycerol during the fall occurs as the gall senesces (Rojas et al. 1986) and is stimulated by juvenile hormone (JH);

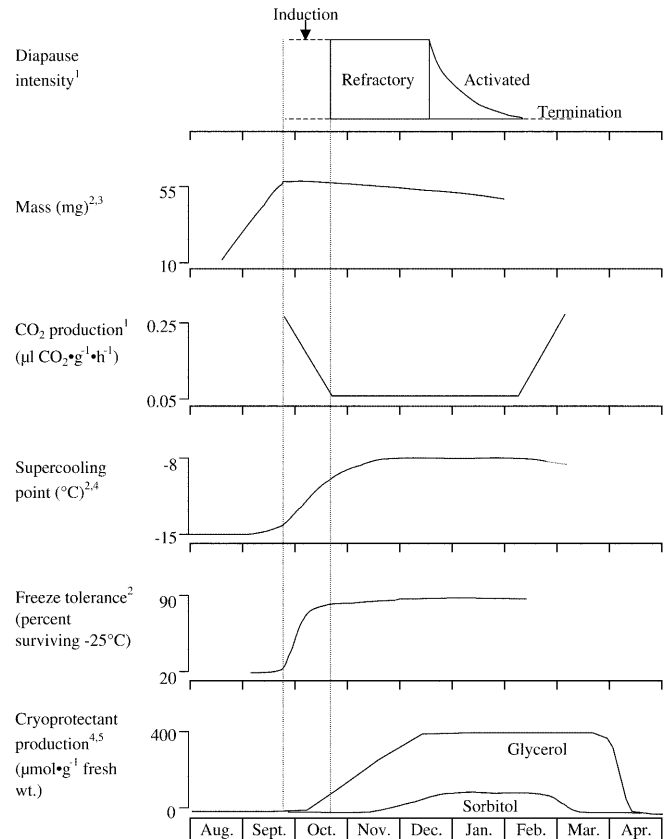


Fig. 5 Summary of seasonal changes in various parameters associated with diapause development and cold-hardiness of third-instar *E. solidaginis* larvae. Sources: ¹This study, ²Bennett and Lee 1997, ³Layne and Medwith 1997, ⁴Morrissey and Baust 1976, ⁵Storey and Storey 1986

Hamilton et al. 1986). JH also plays a role in diapause regulation: in many insect species diapause induction is associated with an increase in JH titers (see reviews by Tauber et al. 1986; Danks 1987). Thus, increases of JH associated with diapause induction may induce cold-hardiness through glycerol production. In addition, JH plays a role in regulation of supercooling, another adaptation associated with cold hardiness (Rojas et al. 1987). However, other aspects of cold-hardiness are not linked to diapause. The degree of freeze tolerance improves as the winter progresses (Bennett and Lee 1997), at least in part because of the accumulation of additional cryoprotectants (e.g., sorbitol; Fig. 5), but this increase is in response to low temperature (Baust and Lee 1982), not JH (Hamilton et al. 1986).

Diapause development occurred in all of our temperature treatments of the overwintering larvae. The first larvae successfully pupariated 1 week after the 15 December collection, and by 15 January almost all larvae were in the activated phase. The cues that brought about this transition are still unclear. Photoperiod probably did not play a role as these larvae were placed in darkness upon collection. Freezing is not required for transition to the activated phase because even those larvae that had never experienced freezing

(either in nature or during exposure to 0 °C in the laboratory) developed to adulthood. We observed large changes in diapause intensity throughout the winter but these changes were not reflected in our measure of metabolic rate; CO₂ production remained low and consistent through both the refractory and activated phases of diapause (Fig. 1A).

Low temperatures have marked effects on diapause development in other insects. Way (1960) found that exposure to -22 °C and -24 °C accelerated post-warming hatching of diapausing *Leptohylemyia coarctata* (Muscidae) eggs and attributed it to inhibition by cold of a hormone normally produced during diapause. However, these eggs were supercooled, not frozen; freezing is lethal to eggs of this species. Similarly, both Goettel and Philogène (1980) and Bell (1983) found that chilling reduced time to pupation of lepidopteran larvae (*Pyrrharctica isabella* and *Ephestia elutella*, respectively). We did not observe accelerated diapause development in *E. solidaginis* following freezing to -20 °C compared to those held at 0 °C. In fact, diapause development was slower in frozen larvae.

Diapause development occurred in larvae frozen at -20 °C. These larvae moved into the activated phase at approximately the same time as those at 0 °C (although in smaller numbers); and diapause intensity also decreased over time (Figs. 2 and 3), albeit at a slower rate than observed in the 0-°C group. Thus, *E. solidaginis* larvae frozen to -20 °C, in which approximately 65% of the body water was frozen (Lee and Lewis 1985), underwent at least some of the physiological events normally associated with diapause development (e.g., changes in hormone titers). Although this is the first report of diapause development in a frozen insect, other physiological changes have been observed in frozen *E. solidaginis* larvae. Freezing to -5 °C and -30 °C increases the activity of some enzymes, particularly those involved in cryoprotectant synthesis (Storey et al. 1981), resulting in increases in cryoprotective glucose in frozen larvae (Storey and Storey 1985). Although our results suggest that complex physiological processes continue in frozen *E. solidaginis*, further research is required to understand the environment experienced by the cells of a frozen insect and how these conditions influence endocrine responses.

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