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Changes in gut and Malpighian tubule transport during seasonal acclimatization and freezing in the gall fly *Eurosta solidaginis*

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Summary

Since few studies have examined cold tolerance at the organ level in insects, our primary objective was to characterize the functional responses of the gut and Malpighian tubules (MT) to seasonal acclimatization, chilling and freezing in larvae of the goldenrod gall fly *Eurosta solidaginis* Fitch (Diptera, Tephritidae). From September to December, hemolymph osmolality (455–926 mOsmol kg l^{-1}) and freezing tolerance increased markedly in field-collected larvae. Chlorophenol Red was readily transported into the lumen of the foregut, the posterior portion of the midgut, the ureter, the proximal region of the anterior pair of MT, and entire posterior pair of MT. Ouabain and KCN inhibited transport of Chlorophenol Red in the gut and MT. Transport was readily detected at 0°C and the rate of transport was directly related to temperature. The rate of fluid transport by the MT decreased steadily from a monthly high in September (10.7±0.8 nl min^{-1} for the anterior pair; 12.7±1.0 nl min^{-1} for the posterior pair) until secretion was no longer detectable in December; this decrease parallels entry into diapause for this species. Even in larvae that died following freezing for 40 days at –20°C, individual organ function was retained to a limited extent. Through the autumn, cholesterol concentrations in the hemolymph increased nearly fourfold. In contrast, the ratio of cholesterol to protein content (nmol mg l^{-1}) in the MT membrane remained relatively constant (22~24 nmol mg l^{-1} protein) during this period. Freezing of larvae for 20 days at –20°C caused a significant decrease in cholesterol levels in the hemolymph and the MT membranes compared to unfrozen controls. These results suggest that cholesterol plays a role in seasonal cold hardening and freeze tolerance in insects.

Key words: transport, cholesterol, epithelial membranes, freezing tolerance, cold tolerance, Malpighian tubule, gall fly, *Eurosta solidaginis*.

Introduction

Investigations of insect low temperature tolerance have focused extensively on ecological factors influencing winter survival and on mechanisms of cold hardiness at the organismal level, including regulation of supercooling, cryoprotectant accumulation, and proximal cues that trigger seasonal changes in cold tolerance (for reviews, see Lee and Denlinger, 1991; Leather et al., 1993). In contrast, relatively few studies have attempted to correlate underlying mechanisms of freezing injury and cold-hardening at the cellular or organ levels with levels of organismal tolerance (Bennett and Lee, 1997; Neufeld and Leader, 1998a; Yi and Lee, 2003). Organisms that survive chilling or freezing do so within specific temperature ranges (cf. –5 to –30°C) below which they die. Assessing the effect of freezing on specific organs is critical for understanding fundamental aspects of cold tolerance and factors determining the limits of freezing tolerance.

The goldenrod gall fly *Eurosta solidaginis* is a naturally freeze-tolerant insect that survives both intra- (as in the case of fat body cells) and extra-cellular (Salt, 1959; Lee et al., 1993; Bennett and Lee, 1997) freezing. During autumn, third instar larvae increase their cold-hardiness and become tolerant of extensive internal ice formation, partly owing to the accumulation of the cryoprotectants glycerol, sorbitol and trehalose (Baust and Lee, 1981; Storey et al., 1981; Lee and Hankison, 2003). Concurrently, the larvae acquire extreme resistant to desiccation through the deposition of large amounts of cuticular hydrocarbons and by metabolic depression associated with entry into diapause (Ramløv and Lee, 2000; Nelson and Lee, 2004).

Since Malpighian tubules (MT) and the gut constitute the primary system for ionoregulation, osmoregulation and excretion in insects (O'Donnell and Spring, 2000), these organs may be especially valuable models for studying the effects of freezing injury and cold-hardening. When stimulated, some MT can transport water and ions at rates higher than those of any other known tissue, resulting in them being called 'insect kidney tubules' (Meulemans and De Loof, 1992). The high rate of fluid secretion depends crucially on the activity of a V-ATPase located on the apical cell membrane

(Maddrell and O'Donnell, 1992). Along with a large amount of fluid passing through the cells, many small molecules, such as amino acids, sugars and ions also enter the lumen, but they may be reabsorbed during their passage through the tubules and the rectum (Maddrell and Gardiner, 1974; Bradley, 1985).

Most dipteran MT contain four tubules, two oriented anteriorly (MTA) and two oriented posteriorly (MTP) in the abdomen (Meulemans and De Loof, 1992; Mugnano et al., 1996). Each pair of tubules forms a common ureter that opens at the junction between the midgut and hindgut (Waterhouse, 1950). In *E. solidaginis* larvae, the anterior pair of tubules has two morphologically distinct parts, proximal and distal. The distal region contains numerous clear spherules of $Ca_3(PO_4)_2 \cdot xH_2O$ while the proximal region lacks crystals, and is yellow-green in color (Mugnano et al., 1996; Yi and Lee, 2003). The calcium phosphate spherules have an ice-nucleating function that promotes freeze tolerance by limiting the capacity of larvae to supercool (Mugnano et al., 1996). However, little is known about the epithelial function of the MT in relation to the seasonal acquisition of freeze tolerance and entry into diapause.

Cholesterol is an important component of biological membranes and the precursor for the biosynthesis of steroid hormones in insects and other animals (Waterman, 1995). This molecule is believed to have dual roles in preserving membrane fluidity, which is essential for cell survival and function, in response to changes in environmental temperature: at high temperature cholesterol functions to make membranes less fluid, while at low temperature it serves to maintain membrane fluidity by preventing the acyl chains of lipids from binding to each other and rigidifying the membrane (Crockett, 1998). Although mechanisms involving cholesterol in the modulation of membrane structure and function have been proposed (Yeagle, 1991; Crockett, 1998), little is known about their role in insects.

Our primary objective was to characterize functional responses of the gut and MT to seasonal acclimatization and freezing tolerance in overwintering larvae of the goldenrod gall fly. Since little research has been done with these organs in *E. solidaginis* larvae, we began this project by characterizing the basic structure and transport functions of these organs.

Materials and methods

Insect collection

Galls on goldenrod plants *Solidago altissima* containing third instars of *Eurosta solidaginis* Fitch (Diptera, Tephritidae) were collected from fields at the Miami University Ecology Research Center located near Oxford, OH, USA during the autumn of 2001 and were stored under ambient field temperatures until used for experiments (Bennett and Lee, 1997).

Supercooling point

The supercooling point (SCP) was identified as the lowest body temperature recorded immediately prior to the spontaneous release of the latent heat of fusion as water froze within the insect (Lee, 1991). The SCP was measured by positioning a thermocouple on the larval surface within a 1.5 ml plastic tube placed in a glass test tube (1.6 cm×15.0 cm) suspended in a refrigerated bath (Neslab, model RTE-140, Portsmouth, NH, USA). Temperatures were recorded on a chart recorder connected to a multichannel thermocouple recorder (Omega, model RD3752, Stamford, CT, USA). Larvae (N=10) were cooled from ≈22° to –20°C at a rate of 1°C min^{-1}.

Assessing freeze tolerance

Three groups of 30 larvae from each collection were placed individually in a 0.5 ml plastic tube and all tubes were kept in a large plastic container. Because larvae greatly increased their tolerance during the course of the study it was necessary to increase the severity of the freezing treatment to induce freezing injury and assess cold tolerance. Consequently, after field collection larvae were frozen at either –20°C or –80°C for various periods as follows: 48 h for larvae collected in September, 10 days for October, 20 and 40 days for November and 2 months for December. Larvae were checked for survival every 30 min for 5 h under a dissecting microscope after they were removed from the freezers and thawed at 22°C. Movement in response to tactile stimuli was used to identify viable larvae.

Tissue dissection and measurement

The larval gut and MT were dissected in Coast's solution (Coast, 1988). Larvae were pinned dorsal-side uppermost in a silicone elastomer-filled (Sylgard 184, Dow Corning, Midland, MI, USA) Petri dish. A midline incision allowed removal of the fat body to expose the gut and MT. The entire gut (foregut, midgut and hindgut) and the attached MT were removed from the body. The length of each tissue was measured, and the cells and crystals in the MT were counted under a dissecting microscope. For fluid secretion assays, the MT was dissected free from the gut and quickly transferred to a fluid secretion assay system (Xu and Marshall, 2000). For experiments concerning transmembrane transport of ions, the gut-MT complex was transferred into 1.0 ml of 0.5 mmol l^{-1} Chlorophenol Red–Coast's solution in a tissue culture dish (35 mm×10 mm, Corning, NY, USA).

Fluid secretion by MT

In vitro fluid secretion by the MT was assayed by a procedure modified from those of Ramsay (1952), Spring and Hazelton (1987) and Xu and Marshall (2000) in a Coast's solution saturated with oxygen by bubbling air through it at room temperature. An isolated tubule was placed in 0.5 ml Coast's solution that was covered with mineral oil in a Petri dish. The open (ureter) end of the MT was pulled out into the oil and wrapped around a pin. Secreted fluid formed a droplet in the oil. The droplet was then removed from the tubule every 30 min with a fine hair and its diameter was measured with an ocular graticule (Spring and Hazelton, 1987). The volume was

calculated based on the assumption that it was spherical (Xu and Marshall, 2000). For each tubule, the rate of secretion, expressed as nl min^{-1}, was determined by the cumulative volume secreted during a period of 30 min to 2 h.

Transepithelial transport

Chlorophenol Red is a pH sensitive dye that can be seen accumulating in the MT lumen. The dye is carried in the water that follows the active transport of potassium (Yurkiewicz, 1983; Pritchard and Miller, 1993). To test the epithelial function of both the gut and MT, the gut-MT complex from November-collected larvae was isolated and incubated in 1.0 ml of 0.5 mmol l^{-1} Chlorophenol Red–Coast's solution in a tissue culture dish. To examine the effect of temperature on active transport, tissues were dissected immediately from newly collected larvae and incubated under various temperatures or from larvae held frozen at –20°C for 40 days and then thawed at 22°C. The effects of metabolic (0.01 mol l^{-1} KCN) and membrane (0.01 mol l^{-1} ouabain) inhibitors on transport were also tested. Preliminary measurements indicated that pH varied by 0.2 units or less among the different tissues, which did not cause color changes of the Chlorophenol Red, and thus did not interfere with the dye transport assay.

Membrane preparation

MT dissected from control and freeze-treated groups were either immediately homogenized in an ice-cold homogenization buffer containing 250 mmol l^{-1} sucrose and 5 mmol l^{-1} imidazole, pH 7.4 (Al-Fifi et al., 1998) or stored in 100 µl of the same buffer containing 10% DMSO (dimethyl sulfoxide) at –80°C overnight. Membrane preparations were conducted with a modified procedure based on the methods described by Al-Fifi et al. (1998), Crockett and Hazel (1995), Fogg et al. (1991) and Sørensen (1981, 1993). Briefly, tissues were first homogenized in a glass homogenizer with the homogenization buffer (1 mg tissue in 10 µl buffer), and then processed with an Ultrasonic Processor (Cole-Parmer Instrument Co., Vernon Hill, IL, USA) for 10 s, three times, with an Amplitude setting at 40. All processes were carried out on ice. The homogenate was centrifuged at 600 ***g***, 4°C in an Eppendorf centrifuge for 10 min to remove nuclei. The supernatant was then centrifuged at 135,000 ***g*** in a Beckman L5-50 B Ultracentrifuge for 20 min at 4°C to yield a supernatant and a membrane fraction. The membrane fraction was suspended by homogenization in an appropriate volume of homogenization buffer containing 10 mmol l^{-1} $MgCl_2$ and 0.1 mol l^{-1} choline chloride for cholesterol and protein assays.

Biochemical assays for cholesterol and proteins

A specific enzymatic cholesterol assay system provided by DCL (Diagnostic Chemicals Limited, Oxford, CT, USA) was used in hemolymph and the membrane preparations of MT, using a single reagent and a standard cholesterol calibrator (Sigma, St Louis, MO, USA). The assay includes coupled enzymatic reactions in which hydrogen peroxide (H_2O_2) is produced from the sequential action of cholesterol esterase and cholesterol oxidase on cholesterol and its esters. The H_2O_2 oxidatively couples with 4-aminoantipyrine and *p*-hydroxybenzoate in the presence of peroxidase to produce the chromogen, quinoneimine, measured at 505 nm. We reduced the volumes of both sample and reagent to suit our purpose, i.e. in separate test tubes, 10 µl buffer (as blank), calibrator (as standard) or unknown samples were mixed respectively with 1.0 ml of reagent in a 1.5 ml semi-micro cuvette. After incubation at room temperature for 20 min, absorbance of the mixture (OD value) was measured at 505 nm using the reagent blank as a background. Cholesterol content was calculated by division of the OD value of an unknown sample by the OD value of the calibrator, and then multiplying by a factor of 4.6 (concentration of the calibrator in mg/100 ml).

Protein contents in the MT membrane preparations were determined by a Bio-Rad standard procedure (Bradford, 1976) with a reduced volume (1.02 ml), using BSA as the standard.

Hemolymph osmolality

Hemolymph was collected individually from 10 larvae with a 10 µl glass capillary micropipette (Drummond Scientific Co., Broomall, PA, USA). Osmotic concentration of the hemolymph was determined with a Wescor 5500 vapor pressure osmometer (Wescor, Logan, UT, USA).

Statistical analysis

SigmaPlot was used to conduct *t*-tests and ANOVA post hoc tests used Statview from SAS Institution. A value of $P<0.05$ between groups was considered as a significant difference. Values are reported as mean ± S.E.M., $N=10$.

Results

Seasonal growth and cold-hardening

Larval mass increased by 71% from 36.3±2.5 mg in early September to a maximum of 62.1±1.2 mg on October 9 (Fig. 1). After this time, the body mass decreased to ≈50 mg in December. During this same period, the larval SCP remained more or less constant from September (–10.5±1.1°C) to December (–9.7±0.1°C), although values for individual larvae ranged from –6.0 to –15.0°C.

Through the autumn, hemolymph osmolality and cold-hardiness increased steadily (Fig. 2). The hemolymph osmolality, which was used as a measure of cryoprotectant accumulation (Baust and Lee, 1981), increased twofold from 455±16 mOsmol l^{-1} in September to 926±27 mOsmol l^{-1} in December (Fig. 2A). During this period the larvae also acquired a high level of freezing tolerance. In September, no larvae survived freezing for as little as 48 h at –20°C (Fig. 2B). In contrast, by October 80% and 33% survived following 10 days of freezing at –20 and –80°C, respectively, and by Nov. 13 all larvae survived freezing for 20 days at –20°C and 83% survived at –80°C.

Freezing for extended periods reduced survival rates and increased the recovery time upon thawing. For example, the survival rate for November-collected larvae frozen for 40 days

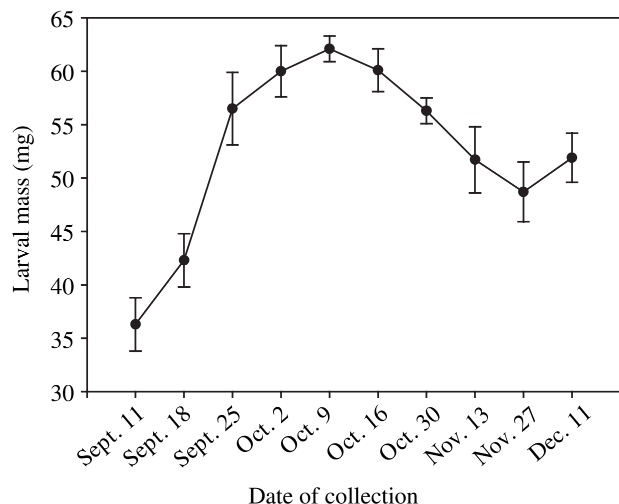

Fig. 1. Seasonal changes in body mass of larvae collected from September to December.

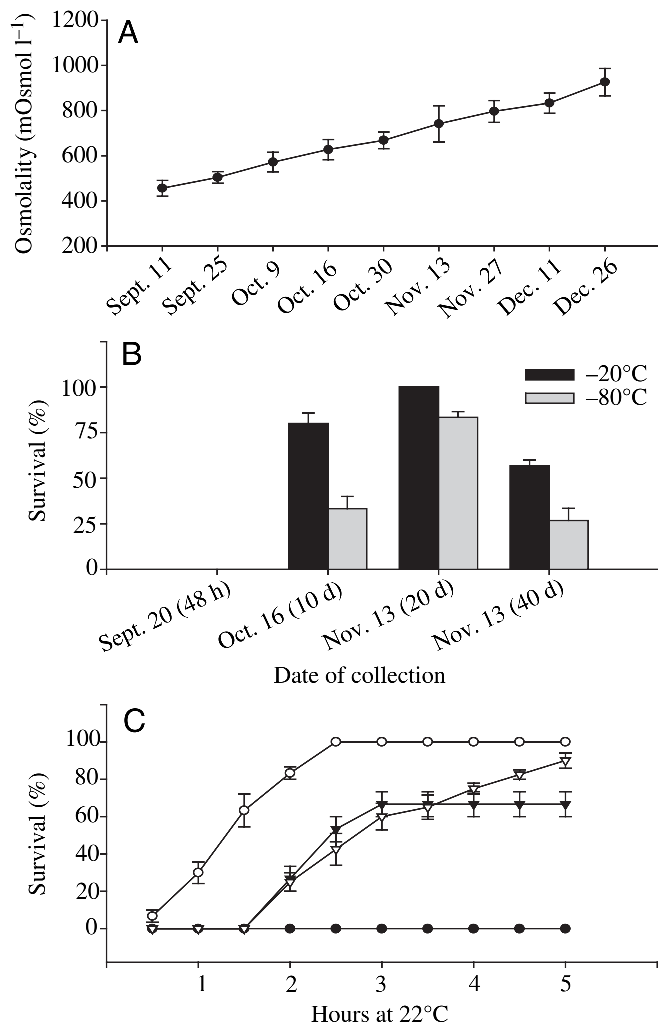

Fig. 2. Relationship between hemolymph osmolarity and freeze tolerance. (A) Seasonal increases of hemolymph osmolality (B) Freeze-tolerance to either –20°C or –80°C based on 2 h observation at 22°C (no larvae survived freezing on September 20). (C) The effect of observation/thawing time on percentage survival. Open circles, Nov. 13 (–80°C, 20 days); filled triangles, Nov. 13 (–80°C, 40 days); open triangles, Dec. 11 (–80°C, 2 months); filled circles, Sept. 25 (–80°C, 48 h).

was lower by 43% at –20°C and 56% at –80°C compared to that in the 20-day-frozen group (Fig. 2B). Depending on the given conditions, larvae that were frozen for longer periods required more time to recover at 22°C (Fig. 2C); November-collected larvae needed 2.5 h to reach a 100% rate of survival after freezing at –80°C for 20 days, while the December-collected larvae required 5 h before reaching a 90% rate of survival after being frozen for 2 months.

Transport in the gut and MT

Since transport processes in the gut and MT of *E. solidaginis* larvae have not previously been investigated, we began by characterizing their basic anatomical and physiological characteristics (summarized in Fig. 3) as a foundation for the studies reported later in this section. Within the gut, the midgut was the longest region (15.4±0.5 mm) compared to the foregut (6.0±0.5 mm) and hindgut (5.4±0.3 mm). In the MT, the length of the ureter was 1.2±0.1 mm and contained 49.7±2.0 epithelial cells. The distal crystal-containing region of the anterior pair of MT was 8.2±0.9 mm in length and contained 19.5±1.2 calcium phosphate spherules (see Mugnano et al., 1996). The proximal region of the anterior pair of tubules was 7.8±0.3 mm in length and contained 185±11 cells. The posterior pair of tubules was 7.6±0.4 mm long and contained 188±12 cells.

To investigate basic transport properties of the gut and MT we used Chlorophenol Red, a pH-dependent colored substrate commonly used to study organic anion transport systems (Pritchard and Miller, 1993). The amount of dye being transported from the bathing solution into the lumen can be estimated visually. This dye is a rosy red at a pH (6.4) and turns yellow at pH 4.8. We first examined *in vitro* transmembrane transport of Chlorophenol Red by the isolated gut and MT in November-collected larvae (Table 1). Chlorophenol Red was readily transported into the lumen of foregut, the posterior portion of the midgut, ureter, the proximal region of the anterior MT, and the posterior MT, but not the anterior portion of the midgut, the entire hindgut and the crystal-containing distal region of the anterior MT (Fig. 3; Table 1). When either 0.01 mol l^{-1} of KCN or ouabain was present in the solution, the expected inhibitory effect on transport was observed; this effect was especially distinct in the MT compared to the control values (Table 1). Although 0.01 mol l^{-1} solutions of both inhibitors were used, KCN showed a greater inhibitory effect on the ion transport by MT than did ouabain.

Effect of temperature on transport in the gut and MT

Incubation temperature had a significant effect on the rate of dye transport for *in vitro* preparations of the gut and MT (Table 2). At 22°C, the dye first appeared in the lumen of transporting regions of the gut and MT within 5 min and reached the highest level of coloration after 10 min of

incubation. However, at a lower temperature of 10°C, the dye was not secreted into the lumen until 10 min after incubation began, and 30 min were required to reach transport levels as high as that of the 10 min-incubation at 22°C, except in the foregut, which did not reach the highest level even after 30 min. At the lowest incubation temperature of 0°C, the rate of ion transport by each tissue slowed still more, however, maximal transport levels were still reached within 60 min in most tissues.

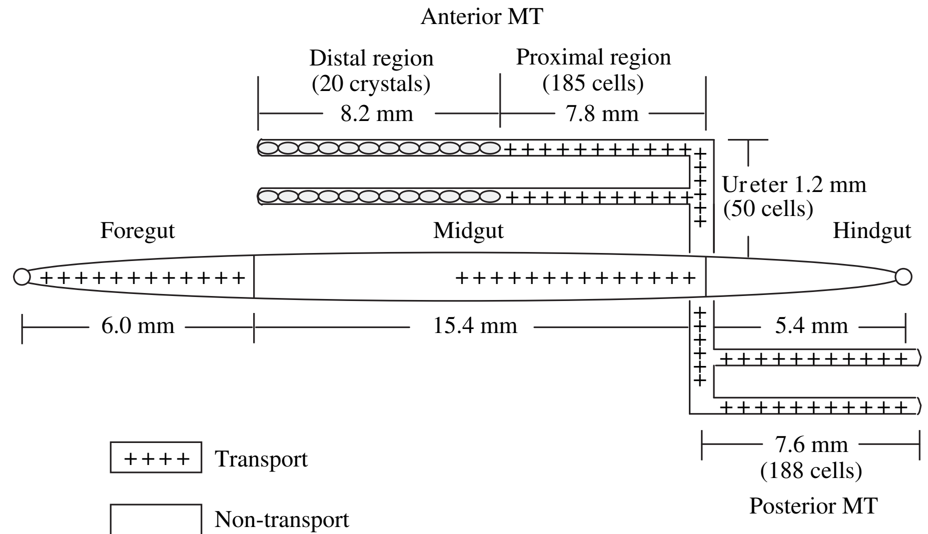

Fig. 3. Diagram summarizing the anatomical structure and transport function of the gut and Malpighian tubules. Regions of Chlorophenol Red transport are indicated by plus signs; calcium phosphate crystals by shaded ovals.

Seasonal decreases in transport function in MT

The rate of fluid secretion by the MT decreased markedly from September to December (Fig. 4). In September-collected larvae, isolated MT from both the anterior (MTA) and posterior pairs (MTP) secreted fluid at the highest rates (10.7±0.8 nl min^{-1} for the MTA; 12.7±1.0 nl min^{-1} for the MTP) when compared to the other collection times. During October and November, the rates declined sharply, and continued to decrease until secretion could no longer be detected in December-collected larvae (Fig. 4A). The rate of secretion from posterior tubules was greater than from the anterior pair in both October ($P<0.01$) and November ($P<0.05$), although no significant difference ($P>0.05$) was observed in September. For each monthly collection, the amount of fluid secreted by the MTA and MTP was linear for incubation periods between 30 and 120 min (Fig. 4B). Over a period of 120 min, each MTA tubule secreted 1.26±0.07 µl and each MTP tubule secreted 1.52±0.04 µl of fluid in September. In contrast, only 0.14±0.03 µl and 0.20±0.03 µl were secreted by the MTA and MTP, respectively in November over the same period.

Effect of freezing on transport in the gut and MT

The influence of freezing and larval survival on transport by the gut and MT was examined using larvae collected on Nov. 13 and held frozen for 40 days at –20°C (Table 3). Larvae were judged to have survived if they responded to touch after 5 h at 22°C. The gut and MT were then excised and dye transport measured as described previously. Overall, tissues from surviving larvae retained better ion transport capacity than from ones scored as dead (Table 3). However, significant levels of transport were evident in tissues removed from dead larvae. Compared to rates of dye transport from unfrozen larvae (see Table 2), freezing of the larvae decreased the rate of ion transport by the gut and MT epithelia. For example, in the posterior midgut of unfrozen larvae maximal transport rates were achieved within 10 min (Table 2), while 30 min were

Table 1. *Effect of inhibitors on transmembrane transport of Chlorophenol Red into the lumen of the gut and Malpigian tubules using larvae collected on Nov. 13, 2001*

	Transport (coloration)							
	Gut				Malpighian tubules			
	FG	MGA	MGP	HG	MTU	MTA (cell)	MTA (cry)	MTP
Control	+++	–	+++	–	+++	+++	–	+++
+KCN	++	–	++	–	+	±	–	±
+Ouabain	++	–	++	–	++	+	–	+

Observations were made for 20 minutes at 22°C (N=9)

All experiments were conducted in Coast's solution; the concentration of both KCN and ouabain was 0.01 mol l^{-1}. FG, foregut; MGA, midgut anterior portion; MGP, midgut posterior portion; HG, hindgut; MTU, Malpighian tubules ureter; MTA (cell), proximal region of anterior Malpighian tubules (cell); MTA (cry), distal region of anterior Malpighian tubules (crystal); MTP, posterior Malpighian tubules.

Red color increased in the lumen with time. Coloration was scored as: –, no color; ±, very slight color; +, positive color; ++, more color; +++, very strong color.

Table 2. *Effect of temperature on transmembrane transport of Chlorophenol Red into the lumen of the gut and Malpighian tubules*

Temperature (°C)	Incubation (min)	Transport (coloration)			
		Gut		Malpighian tubules	
		FG	MGP	MTA (cell)	MTP
22	5	+	+	+	+
	10	+++	+++	+++	+++
10	5	–	–	–	–
	10	+	+	±	±
	20	+	++	+	+
	30	++	+++	+++	+++
0	10	–	–	–	–
	20	+	+	–	–
	30	+	++	±	±
	40	++	++	++	++
	60	++	+++	+++	+++

Tissues and coloration scores were the same as Table 1 (*N*=9).

required to reach this level in previously frozen larvae (Table 3).

Cholesterol and cold tolerance

Since cholesterol has been implicated in processes related to seasonal cold-hardening, we monitored larvae for seasonal changes in cholesterol levels in the hemolymph and the MT membranes. Through the autumn, cholesterol concentrations in the hemolymph increased nearly fourfold (Fig. 5A). From September to October, hemolymph cholesterol level doubled, and doubled again from October to November. In contrast to the hemolymph, the ratio of cholesterol to protein content (nmol mg^{-1}) in the MT membrane of untreated (control) larvae remained relatively constant (22~24 nmol mg^{-1} protein) during the entire period (Fig. 5B).

Larval freezing caused a significant decrease in cholesterol levels in the hemolymph and the MT membranes. November-collected larvae that had been frozen for 20 days at either –20 or –80°C had significantly lower levels of cholesterol in their hemolymph compared to unfrozen control larvae (Fig. 5A, insert). Freezing of larvae at either subzero temperature decreased cholesterol concentrations to a level equivalent to that found in October-collected larvae (Fig. 5A). Similarly, freezing at –20 or –80°C generally lowered membrane cholesterol content in MT compared to that of their respective unfrozen controls (Fig. 5B). In all cases, –20°C decreased membrane cholesterol levels significantly more than did treatment at –80°C.

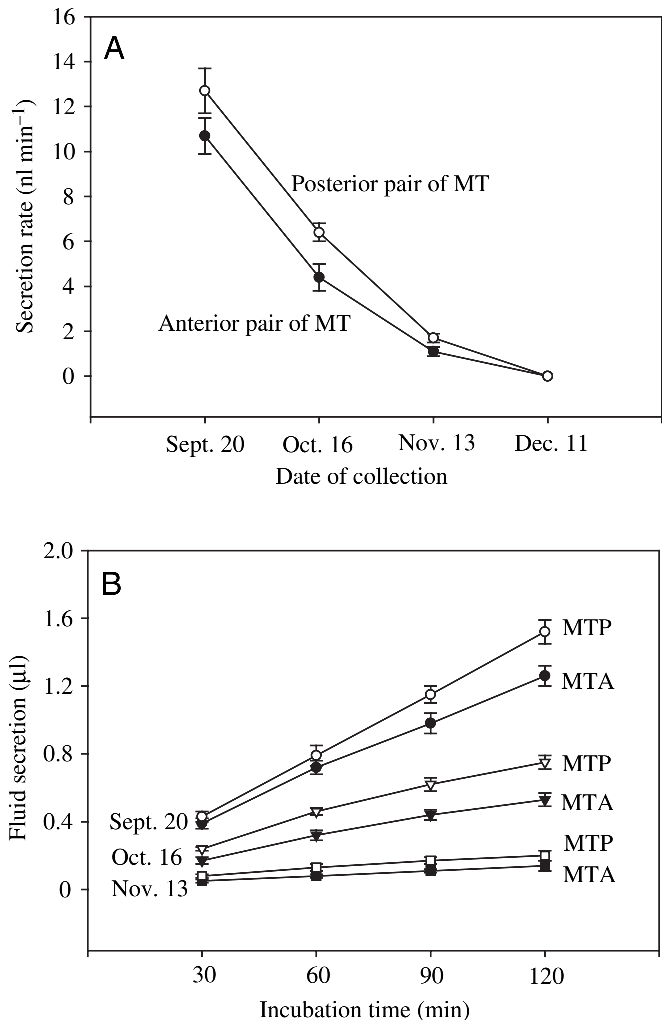

Fig. 4. *In vitro* fluid secretion by the isolated Malpighian tubules (MT) from larvae collected at different times. (A) Seasonal changes in secretion rate (nl min^{-1}) by anterior and posterior tubules. (B) Time course of fluid secretion during a period from 30 to 120 min after dissection. MTA, anterior pair of MT; MTP, posterior pair of MT.

Discussion

During September and early October larvae completed feeding and growing and reached their peak mass of ≈60 mg (Fig. 1). By late November, larval mass had decreased by approximately 10 mg, which is consistent with previous reports for this species (Lee et al., 1995; Rojas et al., 1986). The decrease in body mass is associated with the relatively higher environmental temperatures, and thus, higher metabolic and lipid utilization rates in late autumn compared to mid-winter (Lee et al., 1995; Irwin and Lee, 2003).

The seasonal acquisition of increased cold tolerance was evidenced by increases in hemolymph osmolality, elevated SCP values and survival rates after freezing (Lee and Hankison, 2003). From September through late December (Fig. 2A) hemolymph osmolality more than doubled, reaching 926 mOsmol kg l^{-1}. Since previous studies (c.f. Baust and Lee, 1981; Storey and Storey, 1986) have demonstrated that this increase is due to the accumulation of low molecular mass cryoprotective compounds, primarily glycerol, sorbitol and trehalose, monitoring hemolymph osmolality provides a convenient way to monitor cryoprotectant accumulation.

Table 3. *Transmembrane transport of Chlorophenol Red into the lumen of the gut and Malpighian tubules of larvae after freezing at –20°C for 40 days*

Time of incubation (min)	Coloration in the lumen							
	FG		MGP		MTA (cell)		MTP	
	S	D	S	D	S	D	S	D
5	–	–	–	–	–	–	–	–
10	+	±	+	+	+	–	+	–
20	++	+	++	++	++	±	++	±
30	++	+	+++	++	++	+	++	+
60	++	+	+++	++	++	+	++	+

Larvae were collected on Nov. 13, 2001 and frozen for 40 days at –20°C. Larvae were then thawed at 22°C for 5 h before tissues were removed and incubated in Chlorophenol Red–Coast's solution. S, survived; D, died.

Tissues and coloration scores were the same as Table 1 (*N*=9).

Furthermore, since a primary mode of action for these compounds is colligatively based, it also provides a direct measure of this property (Lee, 1991). From the time of our first sampling date in September until mid December, SCP values were already elevated and they remained constant at temperatures near –10°C (Bennett and Lee, 1997). Elevation of the SCP promotes freezing tolerance and winter survival by slowing the formation of extracellular ice, reducing the metabolic rate and energy utilization, and decreasing the rate at which water is lost to surrounding frozen microhabitats (Zachariassen and Hammel, 1976; Storey et al., 1981; Lundheim and Zachariassen, 1993). Large crystalloid spheres of calcium phosphate within the Malpighian tubules serve as endogenous ice nucleators that elevate the SCP (Mugnano et al., 1996).

Between September and December, larvae progressively increased their tolerance of freezing, consistent with previous reports (Bennett and Lee, 1997; Lee and Hankison, 2003). Fully cold-hardened larvae are freeze-tolerant and survive freezing at –25°C with 75% reaching adulthood (Lee et al., 1993). In the present study, we used larval responsiveness to tactile stimuli as the criterion of survival. We found that all November-collected larvae survived freezing for 20 days at –20°C but only 83% survived –80°C, although we did not follow their late development to either pupariation or emergence (Fig. 2). Lee and Hankison (2003) reported that larvae collected in early autumn can survive freezing treatments at –4°C. Consequently, if September-collected larvae had been subjected to a milder freezing treatment, it is probable they would have survived.

Our observations of the gut and MT transport in *E. solidaginis* larvae are consistent with the general understanding of their function in other insects (Maddrell and O'Donnell, 1992). Active transport of a wide range of organic metabolites, including certain synthetic dyes is known (Maddrell and Gardiner, 1974). In this study, we used a pH-sensitive dye, Chlorophenol Red as an indicator to assess the transport function of the epithelial membrane from different regions of the gut and the MT. The dye is carried in the water that follows the active transport of potassium into the tubule lumen against a concentration gradient (Pritchard and Miller, 1993). As summarized in Fig. 3, the dye was readily transported into the lumen of the foregut, posterior region of the midgut, ureter, proximal region of anterior MT, and posterior MT, but not of the anterior region of the midgut, the entire hindgut and the spherule-containing distal region of anterior MT. This regional specificity in ion and water transport reflects different physiological functions of the gut

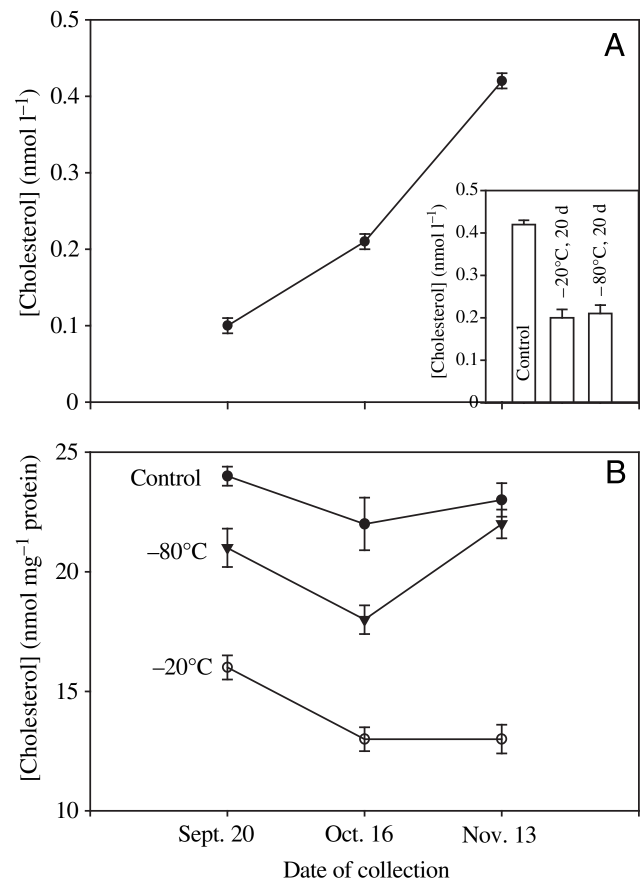

Fig. 5. Effects of season and freezing (inset) on cholesterol levels in the hemolymph (A) and Malpighian tubule membrane (B).

and MT epithelial membranes in *E. solidaginis* (Table 1). After feeding, blood-sucking insects can secrete fluid across cells of the MT at phenomenally high rates (Maddrell, 1991), however the fluid secretion in the MT of *E. solidaginis* occurred more slowly (10.7–12.7 nl min^{-1}; Fig. 4), which is typical for herbivorous insects (Phillips, 1981; Neufeld and Leader, 1998c).

Effects of the metabolic poison (KCN) and the specific Na^+/K^+-ATPase inhibitor (ouabain) on dye uptake by gut and MT are consistent with species-specific differences reported by other investigators. In *E. solidaginis*, both inhibitors slowed the uptake of dye, indicating reduced rates of active transport of potassium (Table 1). However, KCN inhibited transport to a greater extent than ouabain. Previous studies on *Locusta* tubule cells showed that the trans-cellular gradients for Na^+, K^+ and Cl^- were altered by ouabain and *n*-ethyl maletimide (Pivovarova et al., 1994). In the New Zealand alpine weta *Hemideina maori*, however, fluid secretion was unaffected by ouabain or bumetanide, but the transport inhibitors Ba^{2+} and amiloride reduced secretion by 79 and 57%, respectively (Neufeld and Leader, 1998c). The high sensitivity of the apical cation pump, maintaining the cell interior as a K^+-rich, Na^+-poor environment (see Maddrell and O'Donnell, 1992), may explain why the function of most MT are not affected by treatment with ouabain.

Temperature had a significant effect on gut and MT transport. Predictably, transport occurred more rapidly at higher temperatures (Table 2). Nicolson and Isaacson (1996) reported that warming the MT from 20 to 30°C increased the MT secretion rate in the tsetse fly. Of special interest in our study was the fact that transport occurred at significant rates even in larvae held at 0°C, although 60 min were required before transport reached the same level as occurred after 30 min at 10°C (Table 2).

Seasonal acclimatization of larvae to overwinter also had a major effect on MT secretion. During the course of this study, larvae underwent a major transition from actively feeding larvae with negligible cold tolerance to diapausing larvae with well-developed freezing tolerance (Lee and Hankison, 2003; Bennett and Lee, 1997). In field-collected larvae, the rate of secretion by both the anterior and posterior MT pairs decreased markedly from Septemeber 20 until December 11 when secretion was no longer detectable (Fig. 4). This decrease matches larval entry into diapause, when metabolic rates decrease two to threefold (Irwin et al., 2001). To our knowledge these data are the first to show a seasonal decrease in MT transport rates associated with diapause.

Freezing and thawing subjects cells and organs to myriad chemical, mechanical and physiological stresses including cellular dehydration, anoxia, extracellular solute concentration and extreme osmotic flux across organelles and membranes (Pegg, 1987; Storey and Storey, 1996). Injury is frequently manifest at the level of the cell membrane. As pointed out by Neufeld and Leader (1998a) the single-cell-thick tubules constituting the MT are an especially good model for studying the effects of freezing in a relatively simple organ. They found that the MT of the freeze-tolerant weta (*H. maori*) readily tolerated *in vitro* freezing if high concentrations of trehalose or glucose were present in the bathing saline. However, membrane potential and secretion rate decreased markedly if MT were frozen in a saline solution lacking sugar (Neufeld and Leader, 1998a). The MT of this species is also highly tolerant of hyperosmotic exposure comparable to the osmotic shock experienced during freezing to –4°C in nature (Neufeld and Leader, 1998b).

In the present study, we examined gut and MT that were removed from *E. solidaginis* larvae after 40 days of freezing at –20°C (Table 3). For surviving larvae, so judged for their capacity to move in response to touch, gut and MT function returned within 30 min, although more slowly than for unfrozen larvae (Table 2). Even gut and MT from larvae that were scored as dead exhibited transport function, albeit at lower rates than surviving larvae (Table 3). Death at cellular levels does not always correlate with organismal mortality (Yi and Lee, 2003). Previously, we reported that fat body survived freezing at significantly lower temperatures than did the intact *E. solidaginis* larvae (Lee et al., 1993). Using fluorescent vital dyes to assess tissue viability in these larvae, Yi and Lee (2003) found that integumentary muscle, hemocytes, trachea, MT, fat body and gut were more tolerant of freezing than the whole animal. Ultrastructural manifestations of lethal freezing in these larvae suggest that the brain exhibits greater structural perturbations than muscle or MT (Collins et al., 1996, 1997).

Membranes are highly complex structures, characterized by domains of non-randomly distributed protein and lipid components; only recently has the important functional role of these domains begun to be appreciated (Brown and London, 1998; Hazel, 1995; Williams, 1998). Mounting evidence makes it clear that cholesterol plays a multiplicity of roles in membrane function including regulation of membrane fluidity and maintenance of sphingolipid rafts (Hochachka and Somero, 2002; van Meer, 2002; Simons and Ikonen, 2000). Evidence from diverse sources suggests that cholesterol plays special roles in membrane function at low temperature. Crockett (1998) reviewed the diversity of roles that membrane cholesterol plays in temperature adaptation. It is commonly reported that membrane cholesterol increases with acclimation to higher temperatures, consistent with the homeoviscous adaptation model, in which cholesterol functions as a membrane stabilizer (Hazel, 1995). However, in some tissues cold acclimation increases membrane cholesterol, as found in brush border membranes from intestinal epithelia of trout (Crockett and Hazel, 1995). Furthermore, mammalian sperm membranes with naturally higher or artificially elevated levels of cholesterol are more resistant to cold shock (Drobnis et al., 1993; Zahn et al., 2002).

Concomitantly with increases in hemolymph osmolality and cold tolerance, cholesterol levels in the hemolymph increased fourfold in the autumn (Fig. 5A). This result suggests the possibility that cholesterol plays a role in the seasonal acquisition of freeze tolerance in insects. We have evidence that increased membrane cholesterol also enhances chilling

tolerance and rapid cold-hardening in *Drosophila melanogaster* (S.-X.Y. and R.E.L., unpublished data). Although the membrane ratio of cholesterol to protein in the MT epithelium remained relatively constant during the course of this study, we did not measure membrane levels in other types of cells. Insects cannot synthesize cholesterol and must obtain it in their diet. Since larvae of *E. solidaginis* cease feeding in September, the increase in hemolymph cholesterol, observed late in the autumn, must have come from other stores.

Freezing is known to cause changes in the composition and thermotropic properties of cell membranes and the composition of membrane components (McKersie et al., 1989; Crowe et al., 1989). In Dunning prostate tumor cells, freezing increased the phase transition temperature, elevated membrane fatty acids and caused membrane protein denaturation (Bischof et al., 2002). In this study freezing of intact larvae decreased cholesterol levels in the hemolymph and in MT membranes (Fig. 5A,B). Freezing at –20°C had the greatest effect on MT membrane levels, however both freezing treatments caused a similar reduction in cholesterol concentrations in hemolymph. These results suggest that future investigations of the role of cholesterol in insect cold-hardiness will prove fruitful.

In summary, this study demonstrated changes in the functional responses of gut and Malpighian tubules to seasonal acclimatization, chilling and freezing in a freeze-tolerant insect. These results indicate that cold acclimatization occurs, not only at the cellular level, but also at the organ level in insects. Future studies of organ-level function should improve our understanding of fundamental mechanisms underlying freezing injury and cryoprotection.

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