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Supercooling point plasticity during cold storage in the freeze-tolerant sugarbeet root maggot *Tetanops myopaeformis*

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Abstract. The sugarbeet root maggot *Tetanops myopaeformis* (Röder) overwinters as a freeze-tolerant third-instar larva. Although most larvae are considered to overwinter for only 1 year, some may exhibit prolonged diapause in the field. In the laboratory, they can live for over 5 years using a combination of diapause and post-diapause quiescence. In the present study, the cold survival strategies of these larvae during storage is investigated by measuring their supercooling points in combination with survival data. Supercooling points (SCPs) change significantly during storage, highlighted by a marked increase in the range of SCPs recorded, although the ability to tolerate freezing is not affected. Additionally, a freezing event 're-focuses' the SCPs of aged larvae to levels similar to those seen at diapause initiation. This change in SCPs is dependant not only on the initial freezing event, but also on the parameters of the incubation period between freezing events. Finally, the temperatures of larval overwintering microhabitats are monitored during the 2007–2008 boreal winter. The results indicate that, although overwintering larva are physiologically freeze-tolerant, they may essentially be freeze avoidant during overwintering via microhabitat selection.

Key words. Cold storage, diapause, overwintering, sugarbeet root maggot, supercooling, *Tetanops myopaeformis*.

Introduction

The sugarbeet root maggot *Tetanops myopaeformis* (Röder) is a major insect pest in the sugarbeet-producing areas of the western and northern plains of the U.S.A. This insect feeds on the roots of sugarbeet plants throughout its larval life, after which it enters an obligate diapause as a third-instar larva (Callenbach *et al.*, 1957; Harper, 1962; Klostermeyer, 1973). In the field, *T. myopaeformis* overwinters buried 5–35 cm deep in the soil, where it usually remains until favourable conditions return (Callenbach *et al.*, 1957; Harper, 1962; Whitfield, 1984; Anderson, 1986; Bechinski *et al.*, 1989; Bechinski *et al.*, 1990), although multiple year diapause may also occur (Chirumamilla *et al.*, 2008). In the laboratory, *T. myopaeformis* can survive for up to 5 years when stored at 5 °C, through a combination of diapause and post-diapause quiescence (Kruger, 1986; Chirumamilla *et al.*, 2008).

The overwintering state of diapause in insects is accompanied by a compilation of interesting physiological characteristics, including developmental arrest, decreased respiration, increased stress tolerance, and differential gene expression (Tauber *et al.*, 1986; Danks, 1987; Denlinger, 2002; Denlinger *et al.*, 2005). The present study focuses on the cold tolerance of diapausing *T. myopaeformis*. Many diapausing insects achieve increased cold tolerance through freeze avoidance, using a combination of microhabitat selection to ameliorate low temperature exposure and physiological alterations (including the synthesis of polyols and other cryoprotectants) to lower their supercooling point (SCP), which represents the temperature at which their body water freezes (Zachariassen, 1985; Duman, 2001). However, some insects, including *T. myopaeformis* (Whitfield, 1984; Whitfield & Grace, 1985), become freeze-tolerant when overwintering, surviving ice

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formation inside their bodies (Zachariassen, 1985). Although freeze avoidance usually involves a relatively low SCP, freeze-tolerant insects generally have relatively high SCPs (Turnock & Fields, 2005). This is considered to be a key component of freeze-tolerance, by allowing ice formation in extracellular locations where it can be better tolerated by the organism (Storey & Storey, 1988). These alternative strategies of cold tolerance are further complicated by repeated freezing of freeze-tolerant insects, which can have deleterious effects, including the loss of freeze-tolerance in subsequent freezing events (Brown *et al.*, 2004; Sinclair & Chown, 2005).

In the present study, the SCPs of *T. myopaeformis* during extended periods of storage and in response to a prior freezing event are reported. SCPs change significantly during storage, marked by an increase in the range of SCPs recorded. Interestingly, the larvae remain freeze-tolerant, even when their SCPs are well below the normal range for a freeze-tolerant insect. Additionally, the SCPs could be altered by a prior freezing event, greatly reducing the range of SCPs, and in effect re-focusing the SCPs to levels similar to those that are observed at the beginning of diapause. Further analysis of the mechanisms underlying this plasticity of SCPs will be important for understanding the diapause stress physiology of this economically important species.

Materials and methods

Insects

All experiments were carried out on field-collected third-instar larvae of *T. myopaeformis*. Larvae were collected over 4 years (2004–2007) from multiple field sites, all near St Thomas, North Dakota. In all cases, larvae were hand-collected from sugar beet fields, washed, and then stored in moist silica sand at 6 °C for up to 3 years. Larvae collected in June were considered to be in a pre-diapause feeding phase, whereas larvae collected in October were considered to be in diapause.

SCP determination

SCPs were determined using two different methods. When survival was not of interest, SCPs were determined using a Pyris-1 (Perkin Elmer, Waltham, Massachusetts) differential scanning calorimeter (DSC) with Cryofill attachment, programmed for a cooling rate of −1.0 °C min^{-1}. The SCP was defined as the last temperature recorded before the latent heat of fusion from body water freezing was recorded. Body mass was determined before freezing by use of a UMT-2 six place balance (Mettler Toledo, Columbus, Ohio), and the percentage of body water of an individual was calculated by using the area under the latent heat of fusion curve and the transition energy of water (333.88 J g^{-1}).

When survival or secondary SCPs needed to be determined, a different method was employed to protect the organism from the additional stresses associated with the DSC. Individual larvae were placed in thin-walled plastic 'PCR' tubes, to which a type-T 30-gauge copper-constantan thermocouple was taped. The thermocouple was attached to an Omega HH506R data logger (Omega Engineering, Stamford, Connecticut), which recorded the temperature of the probe at 1-s intervals. The tubes containing the larvae were placed in a Nalgene Cryo 1 °C Freezing Container (Thermo Fisher Scientific Inc, Rochester, New York), which was then placed in a −70 °C freezer, thereby attaining a cooling rate of −1 ± 0.1 °C min^{-1}. As before, the SCP was defined as the last temperature recorded before realization of the latent heat of fusion. After the heat of fusion had fully dissipated, the freezing containers were removed from the freezer and returned to ambient temperature. This allowed the larvae to warm at a rate of 1 ± 0.1 °C min^{-1} until thawed.

When the temperature of a second SCP was to be determined, larvae were treated in one of three ways. Twelve larvae were immediately taken through the entire process of freezing twice without a recovery period. Twelve more larvae were placed in 15-mL centrifuge tubes containing moist silica sand and returned to their storage temperature of approximately 6°C for 18 h before being subjected to a second round of freezing. A third group of twelve larvae were placed in 15-mL centrifuge tubes containing moist silica sand and incubated at 25 °C for 18 h before the second round of freezing.

When the effects of inoculative freezing were analysed, individual larvae were placed in tubes in contact with small pieces of filter paper saturated with water, and then treated as before. During the cooling process, the latent heat of fusion from the saturated filter paper was observed, as well as the subsequent latent heat of fusion from the larvae at a lower temperature. Two heat of fusion peaks were observed in all instances.

When survival was of interest, thawed larvae were warmed to room temperature, and then placed in a six-well microwell plate on moist silica sand and allowed to recover overnight. Survival was defined as the ability to exhibit coordinated movement during the course of the day after thawing. To further assess survival, these larvae were then placed in 15-mL centrifuge tubes containing 5 mL of moist silica sand. In this case, survival was defined as the ability to develop into a pupa. All SCP data were analysed by one-way analysis of variance (anova) followed by Bonferroni's post-hoc test where appropriate.

Microclimate temperature

Two Hobo U-12 4-channel outdoor data loggers (Onset Computers, Pocasset, Massachusetts) equipped with 1.8-m long thermocouple cables were deployed in a sugar beet field near St Thomas, North Dakota. For each data logger, one thermocouple was set at the soil surface, whereas three others were buried to a depth of 10 cm, 35 cm, and 70 cm, respectively. Loggers recorded the temperature at each depth every hour from 1 November 2007 until 31 May 2008. Data were retrieved in the field from the loggers using the

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Hoboware software package (Onset Computers) installed on a laptop system. Corresponding climatic data were obtained from the St Thomas station of the North Dakota Agricultural Weather Network (http://ndawn.ndsu.nodak.edu). This station is located approximately 2.5 miles from the field site.

Results

SCPs as a function of age

The SCP of *T. myopaeformis* larvae changed significantly ($F_{7,88} = 19.89$; $P < 0.0001$) over time when measured by differential scanning calorimetry (Fig. 1a). Actively feeding larvae collected in June had a SCP of −0.56 °C ± 0.26 °C (mean ± SE), which fell to −6.88 °C ± 0.43 °C in larvae collected in October that were no longer feeding. SCP remained rather constant over 4 months of storage, before starting a downward trend. By 6 months of storage, the SCP was −8.94 °C ± 0.26 °C and, after 1 year of storage, it was −12.38 °C ± 1.09 °C. After 1 year, mean SCP values did not change significantly. Interestingly, a major factor contributing to these differences in mean SCP values appears to be the range of SCPs recorded (Fig. 1b). The range of SCPs recorded was only 3.7 °C in June, 4.7 °C in October and after 1 month of storage, and 4.8 °C after 4 months of storage. The range expanded to 5.1 °C after 6 months of storage, and subsequent values varied widely: 10.4 °C for larvae stored 1 year, 18.2 °C for larvae stored 2 years, and 15.4 °C for larvae stored for 3 years. Of those larvae stored at least 1 year, SCP values ranged from a high of −4.21 °C to a low of −25.8 °C, with several recordings being below −15 °C. This surprising range of SCPs could not be explained by the initial body weight (Fig. 2a) or the percentage of body water (Fig. 2b), which showed correlation coefficients of 0.35 and 0.13, respectively.

Inoculative freezing

To test the possibility of inoculative freezing as a component contributing to the range of SCP measurements, 12 larvae greater than 1 year old were frozen in dry conditions, whereas 12 additional larvae were frozen when in contact with moistened filter paper. The presence of external water had no effect

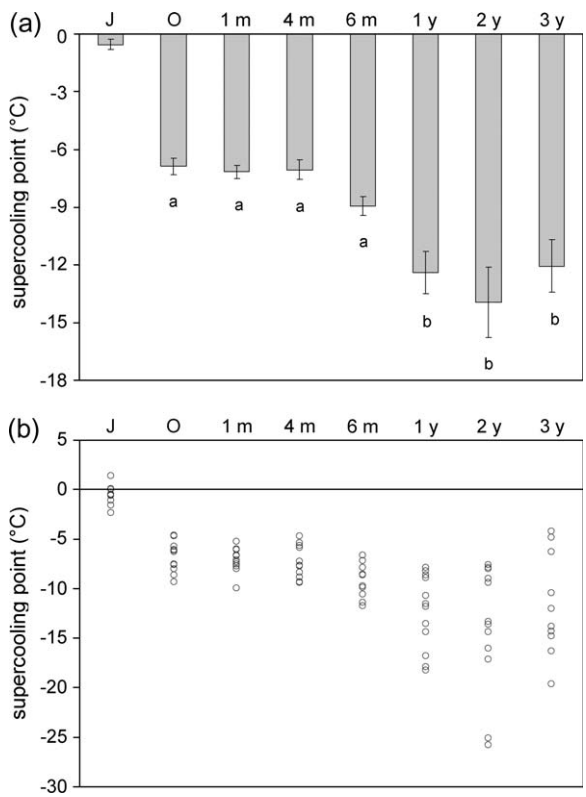

Fig. 1. Supercooling points as a function of larval age. (a) Mean ± SE of supercooling points (SCPs) by larval age. Letters denote means that are not significantly different when analysed by Bonfoerroni's post-hoc test. (b) Distribution of SCPs by larval age. J, July collected larva; O, October collected larva; 1–6 m, months in storage; 1–3 y, years in storage.

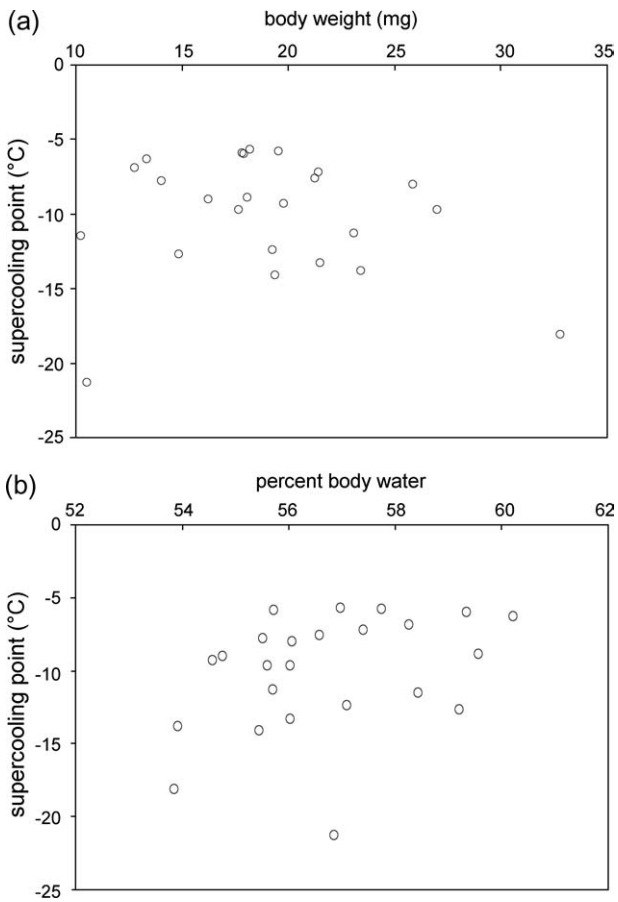

Fig. 2. Correlation of observed supercooling points: (a) as a function of initial body weight and (b) as a function of percent body water.

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(ANOVA: $F_{1,22} = 0.15$; $P < 0.7050$) on the mean SCP of these larvae (Fig. 3a). Similarly, there was no discernable difference on the range of SCPs in these two samples (Fig. 4a).

Freeze tolerance

In agreement with previous studies, the larvae in the present study exhibited freeze tolerance. When subjected to a single freezing event, 22 of 24 larvae (92%) exhibited coordinated movement after 1 day of recovery. Furthermore, 21 (87.5%) of these larvae were able to develop into pupae. There was no discernable correlation between mortality and SCP, with larvae with SCPs as low as −20 °C readily surviving (data not shown). Additionally, larvae from our inoculative freezing experiment (described above) and the effects of a second freezing (described below) were further observed to determine rates of survival. Freezing in the presence or absence of external ice had no detectable effect on survival rates because all 24 larvae in the inoculative freezing experiment survived the treatment. The addition of a second freezing event resulted in a slight reduction in survival. A total of 29 of the 36 larvae in these experiments (80.5%) exhibited coordinated movement 1 day after the second freezing event. There was no discernable difference in survival rates of the three treatment groups, with 83% survival for both groups allowed a recovery and 75% survival for the group without a recovery period between the two freezing events. Furthermore, the amount of change in SCP between the first and second freezing events had no discernable effect on survival (data not shown).

Effects of a second freezing event

Prior research (Brown *et al.*, 2004) has demonstrated that an initial freezing event can have substantial effects on the SCP of a second freezing event. Similar effects were tested in 1-year-old *T. myopaeformis*. When given a recovery period of 18 h at 25 °C, the mean of the second SCP changed significantly (ANOVA: $F_{1,22} = 7.17$; $P < 0.0137$) compared with the first SCPs recorded for the same animals (Fig. 3b). This effect can be further illustrated by looking at the individual SCPs for both treatments (Fig. 4b). Although SCPs ranged from −7.2 °C to −19.0 °C during the first SCP, they ranged from only −6.0 °C to −10.0 °C in the second SCP. However, the change in SCPs varied greatly when individual larvae were observed through the experiment. The mean change in SCPs was only 3.0 °C; however, the SCP of some larvae changed by as much as 11.1 °C, and others changed by as little as 0.3 °C. Additionally, not all SCPs moved in the same direction. Seven larvae experienced an increase in SCP temperature, whereas the SCP of five larvae decreased. Interestingly, this change in SCP was not solely dependant on the initial freezing event. When the 18-h recovery incubation was conducted at 4 °C, no significant change in SCP (ANOVA: $F_{1,22} = 1.84$; $P < 0.1885$) was observed (Figs 3c and 4c). Additionally, no significant change in SCPs (ANOVA: $F_{1,22} = 0.00$; $P < 0.9799$) was observed when the larvae were immediately refrozen (Figs 3d and 4d). As a control, 12 larvae were maintained at 25 °C for 18 h to determine the effect of the incubation period alone. This treatment had no observable effect on measured SCP (data not shown).

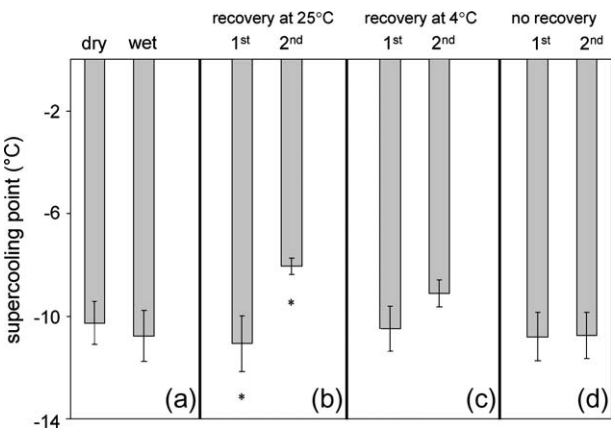

Fig. 3. Effects of (a) innoculative freezing and (b–d) repeated freezing on supercooling points (mean ± SE). (b) Effect of an 18-h incubation at 25 °C between freezing events. (c) Effect of an 18-h incubation at 4 °C between freezing events. (d) Effect on immediate re-freezing. *Means that are significantly different when analysed by analysis of variance.

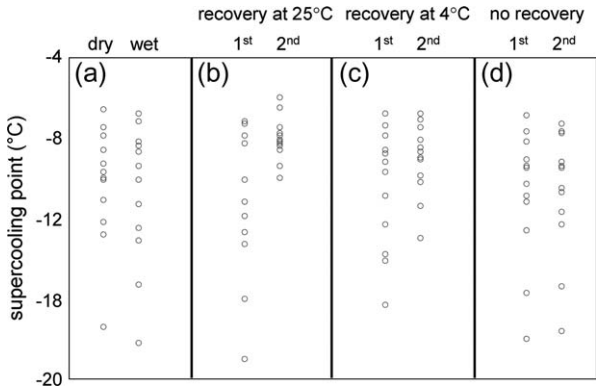

Fig. 4. Effects of (a) innoculative freezing and (b–d) repeated freezing on the range supercooling points. (b) Effect of an 18-h incubation at 25 °C between freezing events. (c) Effect of an 18-h incubation at 4 °C between freezing events. (d) Effect on immediate re-freezing.

Microclimate temperatures

During the recording period (1 November 2007 to 31 May 2008), the maximum ambient temperature recorded at the St Thomas weather station was 25 °C and the minimum temperature was −34 °C. During this period, the St Thomas station recorded 4 days with temperatures below −30 °C, 48 days with temperatures below −20 °C, and 108 days with temperatures below −10 °C. Included in these data was a 104-day period (26 November to 9 March) during which the

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temperature rose above 0 °C on only two dates. Additional summary data for air temperatures during this period are provided in Table 1.

The soil surface thermocouple first recorded a temperature below freezing on 3 November, and last recorded a freezing temperature on 11 May. This period included 113 days (25 November to 17 March) during which the temperature at the soil surface never rose above freezing. The maximum surface temperature recorded during the recording period (November 2007 to May 2008) was 24.7 °C and the lowest recorded surface temperature was −13.4 °C. The date of freezing varied directly with soil depth: the 10-cm deep probe first recorded freezing on 27 November, the 35-cm probe on 4 January, and the 70-cm probe on 14 Feburary. At 10 cm, the soil first thawed on 8 April, indicating a 133-day period during which the soil remained frozen at this depth. Interestingly, the 10-cm probe then recorded a series of six freeze–thaw cycles until the soil remained thawed on 27 April. During these cycles, the soil remained frozen for intervals ranging from hours to days. At this depth, the lowest recorded temperature was −8.0 °C. After the aforementioned freezing on 4 January, the probe at the 35-cm level remained frozen until 1 May, demonstrating a 118-day duration during which the soil was frozen at this depth. Unlike the 10-cm depth, no additional freeze–thaw cycles were recorded. The lowest temperature recorded at this depth was −3.7 °C. At the 70-cm depth, thawing was not observed until 21 May, indicating a 97-day interval during which the soil was frozen. Similar to our observations at 35 cm, no additional freeze–thaw events were recorded at 70 cm. The lowest temperature recorded at the 70-cm depth was −0.6 °C. Additional summary data for this time period are provided in Table 1. However, the summary data provide an incomplete representation of soil temperatures experienced at our field location. Although soil at the 10-cm depth remained frozen for an extended period of time, substantial variability in soil temperature was recorded at this depth (Fig. 5). Although small fluctuations were observed at this depth on a daily interval, substantial variation was repeatedly observed over the course of several days as a function of weather patterns. These cycles can cover a temperature change of several degrees, and can involve rapid temperature changes. At this depth, the largest recorded increase in temperature in a 1-h period was 0.7 °C, with the largest recorded hourly decrease being −0.4 °C. Temperature cycles were also observed at 35 cm, although with a lower frequency and less intensity (Fig. 5). The largest recorded increase in temperature in a 1-h period at this depth was 0.15 °C, and the largest recorded hourly decrease was −0.08 °C. At 70 cm, these fluctuations were largely absent (Fig. 5). The largest hourly increase at this depth was 0.10 °C and the largest hourly decrease was −0.03 °C.

Discussion

Similar to previous studies (Whitfield, 1984; Whitfield & Grace, 1985), the present study demonstrates that *T. myopaeformis* larvae are freeze-tolerant when overwintering. Throughout 4

Table 1. Summary of microclimatic data obtained during the 2007–2008 boreal winter near St Thomas, North Dakota.

				Freeze-thaw cycles		Recorded temperature	
	First freeze	Last freeze	Longest interval	Fall	Spring	Minimum	Maximum
Air	NA	NA	104	NA	NA	−34	25
Soil surface	3 November	11 May	113	13	37	−13.4	24.7
10 cm	27 November	27 April	133	0	6	−8.1	8.1
35 cm	4 January	1 May	118	0	0	−3.7	6.9
70 cm	14 February	21 May	97	0	0	−0.6	8.5
Average maximum temperature	November	December	January	February	March	April	May
Air	1.6	−9.5	−10.6	−10.0	−2.1	9.9	17.1
Soil surface	3.9	−3.8	−6.8	−6.8	0.4	14.7	29.4
10 cm	3.0	−1.4	−3.5	−4.7	−1.6	2.0	7.1
35 cm	4.3	0.4	−0.8	−2.6	−1.2	−0.2	2.9
70 cm	6.1	2.3	1.2	−0.2	−0.5	−0.2	0.3
Average minimum temperature	November	December	January	February	March	April	May
Air	−5.9	−18.1	−20.4	−20.7	−10.8	−0.7	3.0
Soil surface	−4.2	−6.1	−11.2	−10.7	−4.7	−1.4	0.4
10 cm	2.2	−1.8	−4.3	−5.6	−2.1	0.4	4.2
35 cm	4.0	0.3	−0.9	−2.9	−1.4	−0.2	2.4
70 cm	6.0	2.2	1.1	−0.3	−0.5	−0.2	0.1
Maximum daily range	November	December	January	February	March	April	May
Air	16.0	23.0	17.0	22.0	20.0	18.0	21.0
Soil surface	15.2	6.0	8.5	10.7	15.1	27.6	45.8
10 cm	1.6	0.8	2.6	2.0	1.8	3.9	4.9
35 cm	0.6	0.2	0.7	0.8	0.4	0.1	0.9
70 cm	0.3	0.2	0.1	0.1	0.1	0.0	1.4

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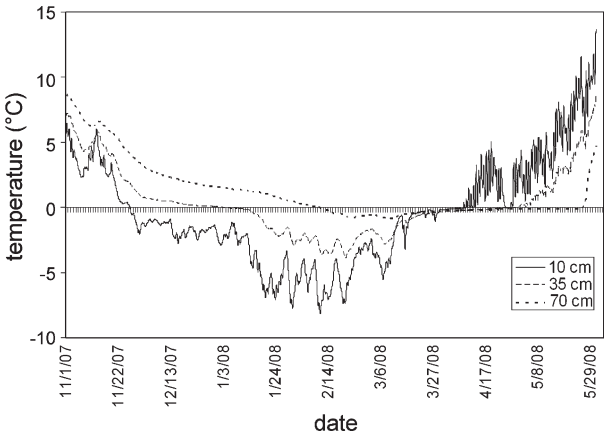

Fig. 5. Microclimate data at different soil depths in a sugarbeet field near St Thomas, North Dakota. Temperatures were recorded at hourly intervals between 1 November 2007 and 31 May 2008. Temperatures for 10 cm below the surface (blue), 35 cm below the surface (green), and 70 cm below the surface (red) are shown.

months of storage, larvae exhibit a classic freeze tolerance, maintaining a relatively high supercooling point. However, after 6 months, the mean SCP begins to fall, concurrent with an increase in the range of SCPs recorded. The plasticity of SCPs during storage and the range of SCPs recorded in the later stages are unique observations. Traditionally, SCPs are considered to be relatively stable physiological parameters with respect to a given life stage (Bale, 2002) and only recent studies demonstrate that ecological factors can alter SCPs (Bale *et al.*, 2001; Brown *et al.*, 2004). In the present study, fluctuating SCPs are reported in a very stable environment over the course of several months. Additionally, unlike other studies, the range of SCPs cannot be interpreted as an indicator of cold survival strategy because the larvae with low SCPs in the present study survive freezing as readily as those with higher SCPs. Although freeze tolerance at a low SCP is reported (Ring & Tesar, 1981; Ring, 1982), it is the exception rather than the rule. Finally, it is important to note that, although the observed changes in SCP occur only after extended periods in storage, this does not necessarily mean that these observations are not ecologically relevant. Recent studies have raised the possibility of multiple-year diapause in the field in this species (Chirumamilla *et al.*, 2008).

What is the mechanism underlying these observed changes in SCP? Previous studies suggest that the long-term storage of *T. myopaeformis* larvae involves a combination of diapause and post-diapause quiescence, with the former being predominant early and the latter being predominant for larvae stored for 1 year or longer (Chirumamilla *et al.*, 2008). Hence, one possibility is that the observed changes in SCP are a function of the diapause state of the individual. Studies documenting changes in cold tolerance mechanisms with respect to post-diapause quiescence are available. For example, post-diapausing pupae of the flesh fly *Sarcophaga crassipalpis* undergo a decrease in glycerol concentrations, although the expression of heat shock proteins remains unchanged (Hayward *et al.*, 2005). Characterization of the mechanisms underlying these observations of SCP plasticity, and the ecological implications associated with them, will prove intriguing.

The plasticity of SCPs in *T. myopaeformis* is not specific to the age of the insect. A single freezing event, followed by a recovery period, restores the SCP to the original level. Changes in SCPs in freeze-tolerant insects in response to freezing are reported for the hoverfly *Syrphus ribessi* (Brown *et al.*, 2004) and the beetle *Hydromedion sparsutum* (Bale *et al.*, 2001). However, the changes observed in *T. myopaeformis* are fundamentally different. In previous studies, a freezing event leads to an increase in the range of SCPs, which corresponds with a shift in cold tolerance mechanisms. Insects with relatively high SCPs in such studies remain freeze-tolerant, whereas those with relatively low SCPs become freeze intolerant. The present study finds different responses, with the initial freezing event 're-focusing' the SCPs to levels normally associated with freeze tolerance, and no discernable effect on the cold survival mechanism employed by the insect.

Although the specific mechanism behind the observed changes in SCP cannot be determined from the present study, some inferences can be made. The observed changes cannot be solely the result of the freezing event itself because SCPs are not altered when larvae are refrozen immediately. Furthermore, changes in the SCP probably involve metabolic processes because SCPs are altered at a higher inter-freezing interval temperature but not with a lower temperature.

Another important component of the overwintering strategy of the sugarbeet root maggot is microhabitat selection. Previous overwintering studies indicate that this species is buried 5–35 cm below the soil surface (Callenbach *et al.*, 1957; Harper, 1962; Whitfield, 1984; Anderson, 1986; Bechinski *et al.*, 1989; Bechinski *et al.*, 1990). The microclimate recordings indicate that, although there is temperature fluctuation at these depths, there is considerable protection from the rigours of winter in the northern plains of North America. At 10 cm, the lowest recorded temperature is −8.1 °C (Table 1). Although overwintering *T. myopaeformis* larvae are protected from the extremes occurring above ground, and the temperatures are still more rigorous than those for overwintering of the Antarctic midge *Belgica antarctica* (Elnitsky *et al.*, 2008). Hence, at this depth, freeze tolerance is ecologically relevant to *T. myopaeformis*. Using differential scanning calorimeter-derived SCPs, the present data indicate that, at −8.1 °C, 92% of the 1-month-old larvae, 67% of the 4-month-old larvae, 33% of the 6-month-old larvae and 8% of the 1-year-old larvae would undergo freezing. However, the observation of −3.7 °C as the lowest recorded temperature at 35 cm indicates there is greater protection for the larvae at this depth because this temperature is well above the lowest recorded SCP. Hence, although overwintering larvae maintain a freeze-tolerant physiology, they would become essentially freeze-avoidant insects at these depths solely as a result of microhabitat selection.

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