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Water balance in the sugarbeet root maggot *Tetanops myopaeformis*, during long-term low-temperature storage and after freezing

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Abstract. The sugarbeet root maggot *Tetanops myopaeformis* Röder (Diptera: Ulidiidae) can be stored in moist sand at 4–6 °C for up to 5 years and is freeze-tolerant. The majority of stored larvae survive in a state of post-diapause quiescence and the remainder are in a multi-year diapause. The present study aims to determine larval water content and water loss rates in diapausing and low-temperature stored larvae. Body water content ranges from 57% to 70.1%. Two distinct groupings of larvae are revealed based on dry weights. The first group consists of the diapausing larvae and larvae stored for 1 year. This group has significantly higher dry weights than the second grouping, which consists of the larvae stored for 2 and 3 years. There are no significant differences within each group. Larval water losses follow a first-order kinetic relationship with time. Larvae stored for 2 years lose water at a significantly higher rate than diapausing larvae. Larvae exhibit no active water uptake at storage temperatures. A freezing event does not induce a significant decrease in wet weights, nor does it increase larval water loss rates. These results indicate that metabolic water and the microclimate during storage are key factors enabling the long-term survival of *T. myopaeformis* larvae during low-temperature storage, and may provide insights for maintaining other insect species under similar conditions.

Key words. Diapause, low-temperature storage, overwintering, sugarbeet root maggot, *Tetanops myopaeformis*, water loss rates.

Introduction

There is an increasing need for high-quality insects for use in biocontrol, research, pollination and conservation programmes. A key component in meeting this need is the development of storage protocols. Currently, three general strategies are employed for the storage of insects: (i) subambient temperature storage of nondiapausing insects; (ii) storage of diapausing insects at overwintering temperatures; and (iii) cryopreservation in liquid nitrogen (Leopold, 1991, 1998, 2007; Van Lenteren & Woets, 1988). Of these three strategies, the use of diapause for the storage of insects has received the least amount of attention (Gilkeson, 1990; Tauber *et al*., 1993; Garcia *et al*., 2002; Foerster & Doetzer, 2006).

The sugar beet root maggot *Tetanops myopaeformis* Röder (Diptera: Ulidiidae) is currently being investigated as a model for enhancing low-temperature insect storage. It is a major pest of sugarbeet in the Red Valley of North Dakota and Minnesota, U.S.A. Adult flies emerge in May and June and the females deposit eggs near the sugarbeet seedlings during mid to late June. Larvae complete their development by late July to September and then migrate 5–35 cm down into the soil and initiate diapause. By late March and early April, the larvae move to within 10 cm of the soil surface and pupate (Callenbach *et al*., 1957; Harper, 1962; Whitfield & Grace, 1985; Anderson, 1986; Bechinski *et al*., 1989, 1990). The unique feature of *T. myopaeformis* that makes it a promising model system for exploring storage physiology is that the mature larvae can be stored in moist sand at 4–6 °C for up

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to 5 years. The majority of stored larvae survive storage in post-diapause quiescence and are able to resume development if exposed to favourable temperatures. The remaining larvae are in a state of prolonged diapause that can last at least 5 years (Chirumamilla *et al*., 2008). Sixty-two percent of larvae stored for 4 years pupate and, of those, 47% emerge as adults. Females that are maintained in cold storage for 4 years are fertile, and 81% of their eggs hatch (Chirumamilla *et al*., 2008).

Another remarkable feature of *T. myopaeformis* is that it is freeze-tolerant (Whitfield & Grace, 1985; Rinehart *et al*., 2009). Two cold-tolerance strategies are commonly employed within Insecta. Most insect species are killed if freezing of extracellular body water occurs (e.g. freeze-intolerant). These species use various cryoprotectants to decreases the temperature at which their body water will freeze. By contrast, freeze-tolerant species have mechanisms that promote the freezing of extracellular body water (Chown & Nicolson, 2004).

As a result of their large surface-to-volume ratio, insects are vulnerable to excessive body water loss. Therefore, insects have evolved a number of highly efficient mechanisms to manage and conserve body water (Wharton, 1985; Chown & Nicolson, 2004). The issue of water loss is even more critical for diapausing insects because of their limited access to water (Danks, 2000). To decrease their water loss during diapause, insects employ a number of different strategies, such as microenvironment selection, physical barriers (e.g. cocoons or increased cuticular lipid layers) and a decrease in metabolism aiming to reduce water loss as a result of respiration (Danks, 2000). There is evidence that some diapausing insects can gain water during diapause by absorption (Yoder & Denlinger, 1991, 1992; Yoder *et al*., 1994). Because of its central role in survival during diapause, water balance in *T. myopaeformis* is examined in the present study, aiming to further the understanding of the physiology responsible for the remarkable longevity of the species during low-temperature storage. The objectives of the present study are: to characterize how water loss rates of *T. myopaeformis* change over the course of storage; to investigate whether a stress event (e.g. freezing) affects the ability of *T. myopaeformis* to maintain water balance; and to determine whether larvae of *T. myopaeformis* absorb water from the atmosphere during storage.

Materials and methods

Insects

All experiments were conducted on *T. myopaeformis* larvae collected during 2006–2011 in the Red River Valley of North Dakota and Minnesota, U.S.A., from fields surrounding St Thomas, North Dakota. Larvae were transported to the laboratory, washed to remove any surface soil and transferred into plastic bags with moist silica sand (20 mL water per 500 g microcrystalline silicon dioxide CAS #14808-60-7; Unimin Corporation, New Canaan, Connecticut). The larvae were stored at 4–6 °C in darkness.

Water loss experiments

Initial body masses (wet weight) of larvae were determined using a six-place balance (model UMT2; Mettler Toledo, Columbus, Ohio). The larvae were then placed individually into wells of a 24-well culture plate and the plate was transferred to a desiccator containing anhydrous calcium sulphate ($CaSO_4$; Drierite, Fisher Scientific, Pittssburgh, Pennsylvania) held at room temperature. Larval water loss was determined by weighing the individual larvae at various time intervals during their exposure to $CaSO_4$. Upon completion of the water loss experiments, larval dry weights were determined by transferring the larvae to 65 °C and taking multiple readings until the weights remained constant (approximately 5 days). Water loss values were then calculated (Wharton, 1985):

$$\text{Initial milligrams of water} = \text{wet weight (mg)} - \text{dry weight (mg)} \quad (1)$$

$$\text{Water loss rate} = \ln(m_t/m_o) \quad (2)$$

where m_t is water mass at time t and m_o is the initial water mass.

In experiments examining the effect of a freezing event on water loss, the larvae were weighed before and after they were frozen, and the post-freezing weight was used to calculate water loss values.

Water loss and water uptake at storage temperatures

Water loss during storage was measured as above, except that the larvae were held at storage temperature (4–6 °C) during the experiment. To determine whether larvae were able to absorb water from the environment, the specimens from water loss experiments were transferred either to 100% relative humidity or placed on moistened filter paper at 100% relative humidity and the larval weights measured at 6 and 24 h after transfer. Water uptake experiments were conducted at the storage temperature.

Freezing and supercooling point determination

To verify that individual larvae froze, the larvae were placed individually in 0.2-mL thin-wall polymerase chain reaction (PCR) tubes to which 30-gauge copper-constantan thermocouples were taped. Temperatures were recorded at 1-s intervals using an Omega HH506R data logger (Omega Engineering, Stamford, Conneticut). Larvae were cooled at -1 ± 0.1 °C min^{-1} using a Nalgene Cryo 1 °C freezing container (Thermo Fisher Scientific Inc., Rochester, New York) placed in a −70 °C freezer. The tubes were removed from the −70 °C freezer as the temperature began to decrease again after the peak of the latent heat of fusion (i.e. heat released as a result of freezing), and were then immediately transferred to a −10 °C water bath. The larvae were maintained at −10 °C overnight.

Larvae that were frozen in direct contact with water (frozen wet) were treated as above, except that the PCR tubes contained a small piece of filter paper wetted with 10 μL of water. Two exothermal peaks were observed in this experiment. A small peak occurred when the water on the filter paper froze, and it was followed by a much larger peak when the larvae froze. The frozen larvae were immediately transferred to 0.5-mL microcentrifuge tubes containing moist sand and placed into the −10 °C water bath. All larvae were thawed before conducting the water loss experiments. Water loss rates were determined as above.

Statistical analysis

Statistical analysis was conducted using SIGMAPLOT, version 11 (Systat Software, Inc., Chicago, Illinois). The initial water mass, dry weight and water loss data were analyzed by one-way analysis of variance and, if appropriate, post-hoc mean comparisons were carried out with Tukey's multiple comparison procedure ($\alpha = 0.05$).

Results

Initial water mass of stored larvae

The mean initial water mass of the larvae varied significantly in the range 11.5–14.8 mg ($F = 6.193$; d.f. = 3; $P < 0.001$) (Fig. 1A). Larvae stored for 1 and 2 years had a body water content equivalent to that of diapausing larvae but significantly less water by weight than larvae that were stored for 3 years ($P < 0.05$). The mean of the dry weights of the larvae varied significantly in the range 6.1–9.4 mg ($F = 13.937$; d.f. = 3; $P < 0.001$) (Fig. 1B). Analysis of the dry weights identified two statistically significant groupings of larvae ($P < 0.05$). The first group included the diapause larvae and larvae stored for 1 year and the second group consisted of the larvae stored for 2 and 3 years. The percentage body water of the larvae was in the range 57–70.1%.

Rate of water loss in stored larvae

Graphing the natural log of m_t/m_o produces a straight line, indicating that water loss in *T. myopaeformis* exposed to dry air involves a first-order kinetic relationship between water loss and time (Fig. 2) Water loss rates of diapausing and stored larvae varied significantly in the range 1.4–2.2% h^{-1} ($F = 5.856$; d.f. = 3; $P = 0.001$). The only group that lost water at a significantly higher rate ($P < 0.05$) than the diapausing larvae was the larvae stored for 2 years (Fig. 2A).

Water loss and water uptake of larvae exposed to normal storage temperature

Larvae stored for 2 years, when exposed to dry air at storage temperatures, lost water at a significantly ($F = 24.073$, d.f. = 1, $P < 0.001$) higher rate (0.68% h^{-1}) than diapausing larvae (0.48% h^{-1}) (Fig. 2B). Once larvae lost water, exposing them to either 100% relative humidity or placing them onto moistened filter paper failed to rehydrate them, which indicates that *T. myopaeformis* larvae are unable to take up water actively at low temperatures (data not shown).

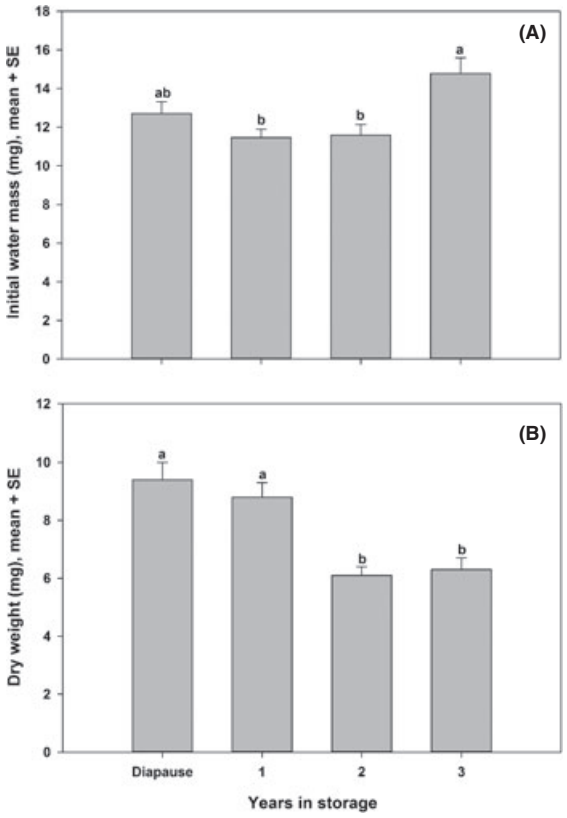

Fig. 1. (A) Initial water mass and (B) dry weights of diapausing and stored *Tetanops myopaeformis* larvae (mean ± SEM). Larvae were maintained at 4–6 °C for less than 3 months (diapausing), and 1, 2 or 3 years. Each bar is based on 20 larvae. Bars sharing the same lowercase letter are not significantly different ($P < 0.05$, Tukey's multiple comparison procedure).

Water loss after a freezing event

The mean larval wet weights before (23.1 ± 1.1 mg) and after freezing (23.0 ± 1.1 mg) were not significantly different between wet- or dry-frozen larvae ($P > 0.05$) (Fig. 3). The water loss rates for larvae under these conditions were 1.9% h^{-1} (frozen dry), 2.8% h^{-1} (frozen wet) and 2.1% h^{-1} (nonfrozen controls), and were not significantly different (Kruskal–Wallis rank test: $H = 5.118$; d.f. = 2; $P = 0.077$).

Discussion

Surviving prolonged cold storage requires the sugarbeet maggot *T. myopaeformis* to maintain homeostasis under very

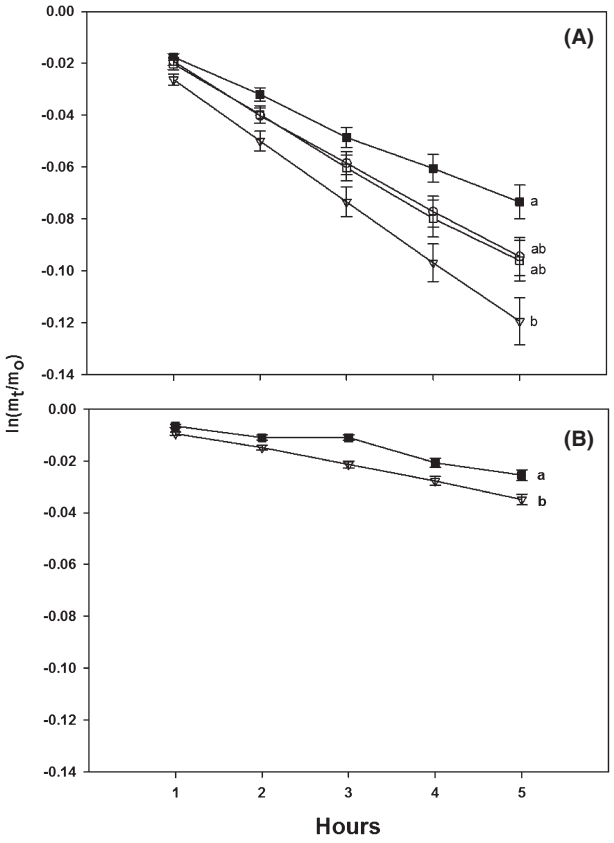

Fig. 2. Water loss of diapausing and stored *Tetanops myopaeformis* larvae exposed to (A) dry air at room temperature or (B) normal storage temperature (mean ± SEM). Larvae were maintained at 4–6 °C for less than 3 months diapausing (solid squares), and 1 year (open squares), 2 years (triangles) or years 3 (circles). Means are based on 12 larvae in (A) and 20–29 larvae in (B). Curves with the same lowercase letter are not significantly different ($P < 0.05$, Tukey's multiple comparison procedure).

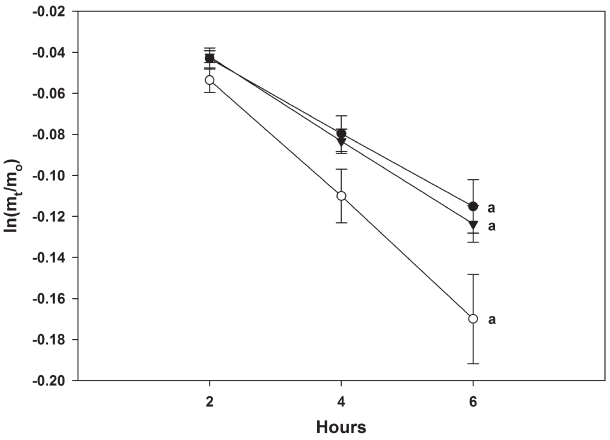

Fig. 3. Water loss in *Tetanops myopaeformis* larvae after a freezing event (mean ± SEM). Larvae were maintained at 4–6 °C for 1 year. Larvae were frozen either dry (solid circles) or in direct contact with water (open circles). Control larvae (solid triangles) were not frozen before the water loss experiment. Means are based on 12– 25 larvae. Curves sharing the same lowercase letter are not significantly different ($P < 0.05$, Tukey's multiple comparison procedure).

unnatural conditions. As measured by initial water mass with up to 3 years in storage, larvae of *T. myopaeformis* show a remarkable ability to maintain this critical resource within a narrow range. The percentage body water values for larvae (57.0–70.1%) are within the normal range recorded in other insects (Wharton, 1985; Danks, 2000). This is also consistent with the findings of Chirumamilla *et al*. (2010). Specifically, wet weights of *T. myopaeformis* larvae remain stable over a course of 5 years in storage; however, after 2 years, there is a significant decrease in the dry weight of the stored larvae compared with diapausing controls. Similar results are found in the present study, except that the dry weights of the larvae stored for 2 years are significantly different from the diapausing larvae and larvae stored for 1 year. Insects use a number of different means to acquire water (e.g. feeding, drinking, active and passive absorption of atmospheric water and water generated by metabolism) (Chown & Nicolson, 2004). Larvae at the normal storage temperature of 4–6 °C are immobile and therefore cannot gain water from food or drinking. The present study demonstrates that these larvae are unable to uptake water actively or passively from the atmosphere during storage. Metabolic water appears to be the primary means by which larvae of *T. myopaeformis* maintain water balance during storage.

It is one thing for the larvae to be able to maintain their water balance under the high humidity conditions of the normal storage method. However, increasing the stress on the larvae by exposing them to 0% relative humidity could provide insights into how larval vigour changes during storage as they respond to this potentially lethal stress. At room temperature, larval water loss rates vary in the range 1.4–2.2% h^{-1}. The only group of larvae that lose water at a significantly higher rate than the diapausing controls are the larvae stored for 2 years. Water loss in the larvae stored for 3 years is not significantly different from that of the diapausing larvae. Lowering the temperature to the normal storage temperature decreases the water loss rate, although the larvae stored for 2 years lose water at a significantly high rate (0.68% h^{-1}) compared with diapausing controls (0.48% h^{-1}). The larvae used in these two experiments have different collection years, suggesting that the increased water loss rate seen in the 2-year stored larvae is a result of some other environmental factor that the larvae are exposed to before entering storage.

An examination of water loss rates after a freezing event identifies no significant differences between control larvae and those exposed to freezing. According to Rinehart *et al*. (2009), stored *T. myopaeformis* are able not only to maintain water balance after they have been frozen, but also to adjust other key physiological components to survive future stresses. The supercooling points of larvae stored for 1 year vary by 11.8 °C from −7.2 to −19.0 °C. After a freezing event and subsequent recovery at 25 °C, the range of supercooling points of larvae stored for 1 year decreases to only 4 °C (−6.0 to −10.0 °C), with both magnitude and absolute values at a range seen in diapausing nonstored larvae (Rinehart *et al*., 2009). These results

indicate that, even after 1 year in storage, *T. myopaeformis* larvae are still sufficiently healthy to respond appropriately to serious environmental stressors such as freezing.

In conclusion, storage even for 3 years does not affect the ability of *T. myopaeformis* larvae to maintain water balance. These results reinforce the findings of earlier studies indicating that storage for 3 years has very little discernible direct fitness cost to *T. myopaeformis* larvae (Chirumamilla *et al*., 2008). Three key factors are considered to be involved in the ability of *T. myopaeformis* larvae to survive multi-year laboratory storage: (i) the larvae are either in a state of prolonged diapause or post-diapause quiescence, which are naturally occurring physiological stages optimized for surviving prolonged or recurrent low-temperature stress (Chirumamilla *et al*., 2008); (ii) the overwintering larvae possess uncommonly large metabolic reserves (Chirumamilla *et al*., 2010); and (iii) exposing larvae to 4–6 °C in non-moistened sand results in 100% mortality within approximately 7 days (M. A. Boetel, unpublished observations). The rates of water loss observed in this investigation explain these observations and reinforce the importance of the high-moisture microenvironment needed by this organism. Understanding the relative contributions and interactions between each of these factors (as well as other possible undiscovered factors) affecting the larval tolerance of *T. myopaeformis* to prolonged storage could provide valuable insights into how to optimize storage protocols for other insect species.

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