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RESEARCH ARTICLE

Upper lethal temperatures in three cold-tolerant insects are higher in winter than in summer

Henry M. Vu* and John G. Duman

ABSTRACT

Upper lethal temperatures (ULTs) of cold-adapted insect species in winter have not been previously examined. We anticipated that as the lower lethal temperatures (LLTs) decreased (by 20–30°C) with the onset of winter, the ULTs would also decrease accordingly. Consequently, given the recent increases in winter freeze–thaw cycles and warmer winters due to climate change, it became of interest to determine whether ambient temperatures during thaws were approaching ULTs during the cold seasons. However, beetle *Dendroides canadensis* (Coleoptera: Pyrochroidae) larvae had higher 24 and 48 h ULT_{50} (the temperature at which 50% mortality occurred) in winter than in summer. The 24 and 48 h ULT_{50} for *D. canadensis* in winter were 40.9 and 38.7°C, respectively. For *D. canadensis* in summer, the 24 and 48 h ULT_{50} were 36.7 and 36.4°C. During the transition periods of spring and autumn, the 24 h ULT_{50} was 37.3 and 38.5°C, respectively. While *D. canadensis* in winter had a 24 h LT_{50} range between LLT and ULT of 64°C, the summer range was only 41°C. Additionally, larvae of the beetle *Cucujus clavipes clavipes* (Coleoptera: Cucujidae) and the cranefly *Tipula trivittata* (Diptera: Tipulidae) also had higher ULTs in winter than in summer. This unexpected phenomenon of increased temperature survivorship at both lower and higher temperatures in the winter compared with that in the summer has not been previously documented. With the decreased high temperature tolerance as the season progresses from winter to summer, it was observed that environmental temperatures are closest to upper lethal temperatures in spring.

KEY WORDS: Insect temperature tolerance, Upper lethal temperature, Insect plasticity, Climate change, *Dendroides canadensis*

INTRODUCTION

The lethal temperatures of poikilotherms normally correlate directly with laboratory acclimation and natural seasonal acclimatization temperatures (Brett, 1971; Somero, 2010). Thus, upper and lower lethal temperatures for an organism tend to increase with higher environmental temperatures associated with the summer season. Conversely, colder environmental temperatures during the winter tend to induce a decrease in both upper and lower lethal temperatures. At least part of the reason for this is that some low temperature survival mechanisms used by poikilotherms are not compatible with high summer temperatures. For example, the increased percentage of unsaturated fatty acids in membrane phospholipids in the winter lowers the functional temperature range of the membranes (Hazel and Williams, 1990; Hazel, 1995; Koštál, et al., 2003).

Overwintering adaptations of larvae of the non-diapausing, freeze-avoiding beetle *Dendroides canadensis* Latreille 1810 have been investigated for over 40 years, mainly in the region around South Bend, IN, USA. Mechanisms to lower their supercooling points (SCPs) and thereby depress their lower lethal temperatures (LLTs) in winter include the production of antifreeze proteins (AFPs) and antifreeze glycolipids (AFGLs), clearance of ice-nucleating microorganisms from the gut, reduction of ice-nucleating proteins in the hemolymph, and accumulation of high concentrations of glycerol (Wu et al., 1991; Olsen and Duman, 1997a,b; Olsen et al., 1998; Duman, 1980, 2001, 2002, 2015; Duman et al., 1998; Duman and Serianni, 2002; Walters et al., 2011; Nickell et al., 2013). These overwintering adaptations assist *D. canadensis* in supercooling to temperatures below −20°C (Duman, 2002, 2015). Winter supercooling points in *D. canadensis* are also their winter LLTs. In the summer, *D. canadensis* supercooling points range from −2 to −7°C, but LLTs are often higher (Olsen and Duman, 1997a).

Cucujus clavipes clavipes Fabricius 1781 and *Tipula trivittata* Say 1823 are two other insect larvae that have been studied to examine their subzero winter adaptations, once again in the area around South Bend, IN, USA. *Cucujus clavipes clavipes* is a freeze-avoiding beetle that uses polyols, AFPs and AFGLs to lower its SCPs and increase its winter cold tolerance (Bennett et al., 2005; Walters et al., 2011; Duman, 2015). *Tipula trivittata* is a freeze-tolerant cranefly that employs sorbitol, protein and lipoprotein ice nucleators, and AFGLs to survive subzero temperatures as larvae in the winter (Duman et al., 1985; Neven et al., 1989; Walters et al., 2011; Duman, 2015).

With recent general increases in the frequency of winter freeze–thaw cycles and overall warmer winters due to climate change, including locally in the state of Indiana (Sinha and Cherkauer, 2008), it became of interest to identify upper lethal temperatures (ULTs) for *D. canadensis*, *C. clavipes clavipes* and *T. trivittata* and determine whether *in situ* temperatures in their log microhabitats during winter warm spells approached their ULTs. It was expected that the insect larvae would have lower ULTs in winter than in summer. More specifically for *D. canadensis*, based on our previous knowledge of their LLTs throughout the year, we predicted their monthly ULTs using the following reasoning (Fig. 1). The mean ULT in a review of 119 insect species was 39.3°C (Hoffman et al., 2013). We felt that this value was a reasonable approximation for *D. canadensis* as most of the data used by Hoffman et al. (2013) were based on insects from temperate latitudes. Therefore, we set that temperature as our predicted summer ULT where 50% mortality would occur within 24 h (ULT_{50}). Based on this predicted summer ULT and our known summer LLTs (approximately −4°C based on past research), we predicted that *D. canadensis* would have a 24 h

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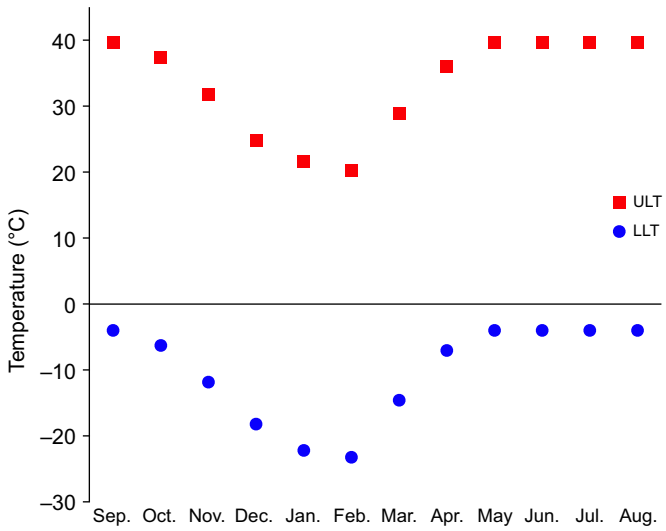

Fig. 1. Predicted upper lethal temperatures of *Dendroides canadensis* over the course of a year. Predictions of upper lethal temperatures (ULTs) were based on our previously determined lower lethal temperatures (LLTs), the mid-summer ULT of numerous insects of 39.3°C (Hoffman et al., 2013), and a predicted temperature range of 43°C. (See Materials and methods for details.)

lethal temperature range of about 43°C in mid-summer (ULT=39.3°C and LLT= −4°C). We then extrapolated the ability of the insect larvae to survive over the course of the year within this approximate temperature range of 43°C. Therefore, in the winter months, with a known LLT of approximately −23°C, we anticipated that they should have an ULT of approximately 20°C (Fig. 1). Because this predicted value is only slightly higher than occasional winter ambient high temperatures in recent years, we proceeded to determine the actual ULTs of these insects.

Here, we show that the 24 h ULTs for all three insects, *D. canadensis*, *C. clavipes clavipes* and *T. trivittata*, were actually higher in the winter than in the summer, contrary to previous thoughts about temperature tolerance and our predicted ULTs shown in Fig. 1. We are unaware of any other documented higher ULTs for organisms in winter compared with summer, with the exception of some anhydrobiotic organisms (Crowe et al., 1992; Watanabe et al., 2004). ULTs have not been previously measured in cold-adapted insects in the winter.

MATERIALS AND METHODS

Field collection of *D. canadensis*, *C. clavipes clavipes* and *T. trivittata* larvae

Larvae of *D. canadensis*, *C. clavipes clavipes* and *T. trivittata* were collected from underneath the bark of decaying trees, generally on the ground, multiple times a month over the course of 4 years from woodlots in the vicinity of South Bend, IN, USA (latitude ∼41.76°N, longitude ∼86.25°W) in northern Indiana and southwestern Michigan. The insect larvae were transported in an insulated container to maintain temperature close to that at the time of collection.

ULT_{50} testing

In the laboratory, the larvae were placed in containers lined with a moist paper towel and the containers were put into temperature-controlled chambers ranging from 33 to 41°C to determine their ULT. The chambers were set at a specific temperature prior to introduction of the larvae. The chamber temperature was not increased incrementally because the insects' heat tolerance may increase if they are given time to adapt to gradual increases in temperature. Insect larvae were scored for survival after 24 and 48 h. Between 10 and 42 larvae were tested at each temperature on a given date. The temperature at which 50% mortality occurred was identified as the ULT for that time period (24 and 48 h ULT_{50}).

The insects were grouped seasonally into four categories. The autumn months were considered to be October and November. The winter months were classified as the beginning of December until the time when we observed feeding by the larvae (generally sometime in March when they had food in their guts). Feeding is an indicator of a decrease in cold tolerance ability in *C. clavipes clavipes* and *D. canadensis* as SCPs rise (Nickell et al., 2013). The spring months were considered as the period between when we first observed food in the gut and May. The summer months were designated as June to September.

LLT_{50} testing

The autumn, winter and spring LLTs were determined by measuring the SCPs as the 24 h LLT of these insects is the same as the SCP in these seasons (note this is not the case in the summer). The average SCP of a month was determined to be the LLT of the insects. To determine the SCPs, the larvae were individually placed in 1.5 ml centrifuge tubes. A thermocouple was placed on the larva and foam was stuffed into the tube to hold the thermocouple and insect in contact with each other. The centrifuge tubes were individually placed into glass test tubes and submerged in an ethanol bath at −1°C. The thermocouple was attached to a multichannel thermometer (Iso-thermex, Columbus Instruments, Columbus, OH, USA). The temperature of the bath was lowered at 0.2°C min^{-1}. The computer recorded temperature readings every 5 s. A SCP was identified as the temperature when an exotherm representing the release of the latent heat of fusion from the insect freezing was observed. In summer, groups of larvae were placed at different temperatures from 4°C to −8°C for 24 h to determine LLTs. The LLT_{50} was calculated to be the temperature at which 50% of the population died.

Desiccation testing

To examine whether mortality of the insect larvae was due to temperature or to desiccation, we placed *D. canadensis* into individual containers at 40°C, after each insect was weighed. Some containers were lined with paper towels dampened with water while others had dry paper towels. These individual containers were then placed into a desiccator filled with desiccant, sealed with vacuum grease and placed at 40°C. The insects were quickly brought out of the 40°C chamber every hour and weighed at 1 h intervals for 12 h and again after 24 h to determine whether changes in mass occurred. Survivorship was also determined.

Microhabitat and air temperature measurement

A Hobo Pro series data logger (H08-031-08; Onset Computer Corporation, Bourne, MA, USA) was used to measure the microhabitat temperature of insects under the bark of logs comparable in size and location to those from which the insects were collected, as well as the air temperature around the logs over a 3 year period from September 2011 to July 2014. The external temperature sensor of the logger was placed beneath the bark of the tree while the data logger itself was placed above the log to record air temperature. BoxCar Par 4 software (Onset Computer Corporation) was used to display the different temperature readings from the data logger.

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Statistical analysis

Tukey's/Holm–Šídák multiple comparisons test was used to analyze the data for statistical significance. Statistical analysis was done by using Graphpad Prism and in R using RStudio with an alpha value of 0.05. To determine the ULT_{50}, a regression analysis was performed in R using RStudio.

RESULTS

Microhabitat and air temperature measurement

The temperatures recorded by the data logger from 2011 to 2014 revealed that the temperature underneath the bark in the winter months reached a high of 13°C (Fig. 2). The temperature of the logs reached the highest annual readings of 30°C in the spring months of March, April and May in 2012, 2013 and 2014 (Fig. 2). Leaves do not typically appear before to mid to late April. Therefore, the logs are not shaded prior to this time, and direct solar radiation warms the log, especially during warm and sunny days. After the leaves fall in the autumn, the logs are likewise exposed to solar radiation.

Winter and summer larvae 24 h survivorship at different temperatures

Dendroides canadensis larvae were tested for survival at numerous temperatures ranging from 30 to 43°C over the course of the first year of the study. Based on these data (not shown), subsequent, more directed, experiments were conducted. The following data are taken from these studies. When placed at 38, 39 and 40°C for 24 h, winter-collected *D. canadensis* had higher survivorship than summer-collected larvae (Fig. 3).

Survivorship at a constant temperature of 39°C for larvae collected over 13 months

The survivorship of *D. canadensis* collected over a period of 13 months and placed at a constant temperature of 39°C for 24 h is shown in Fig. 4. Larvae from the colder months of November–March had increased survivorship compared with those from the warmer months of May–September.

Autumn and spring 24 h survivorship at different temperatures

As seen in Fig. 4, but contrary to expectation, during the seasonal temperature transition periods of autumn and spring, *D. canadensis* showed increased heat tolerance for 24 h in the autumn as the days grew generally colder moving from October to November (Fig. 5A) and decreased heat tolerance in the spring as days generally warmed from March to April (Fig. 5B).

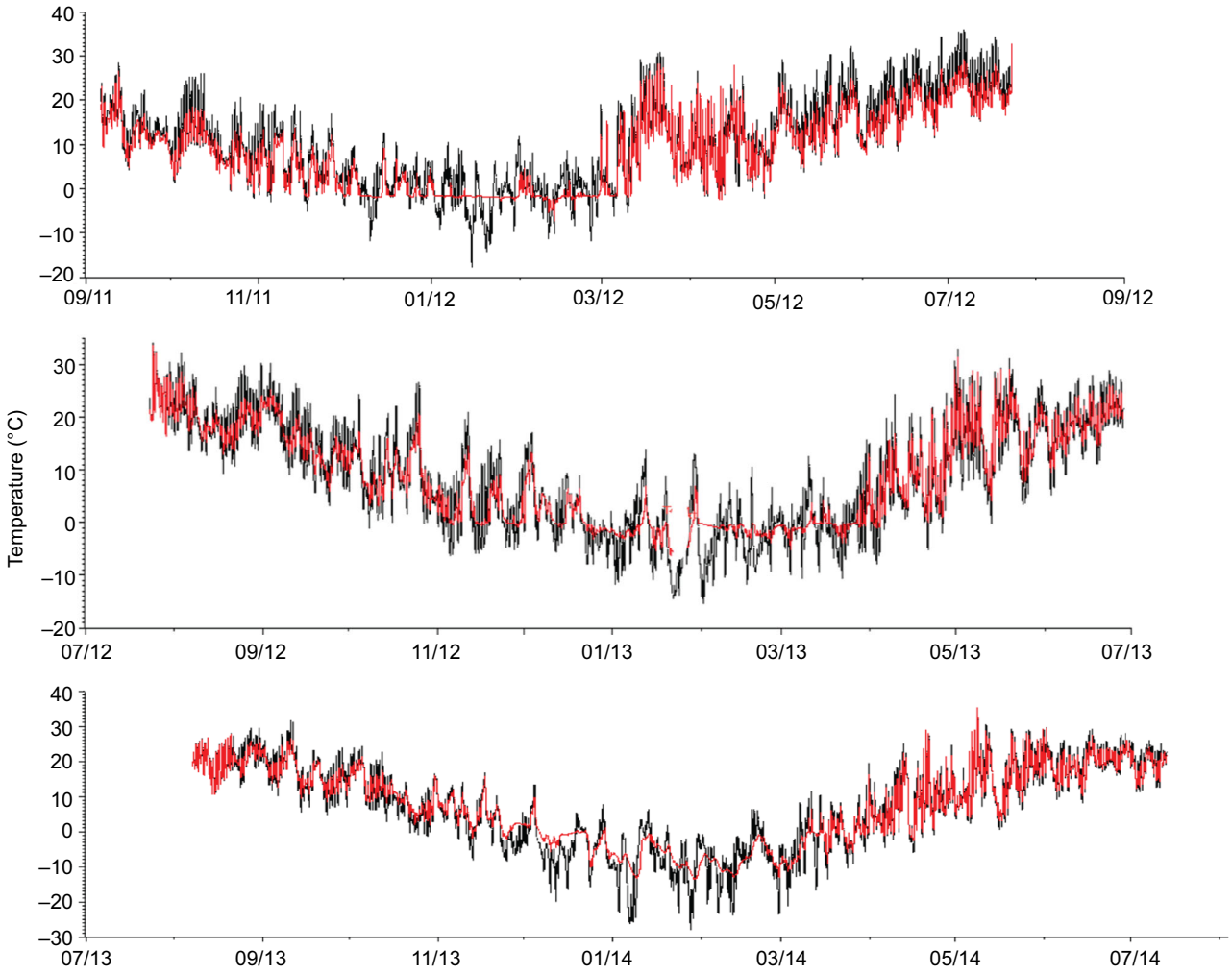

Fig. 2. Data logger temperature readings from September 2011 to July 2014 from a log in Michigan. The black line represents ambient temperature outside the tree. The red line represents the temperature underneath the tree bark.

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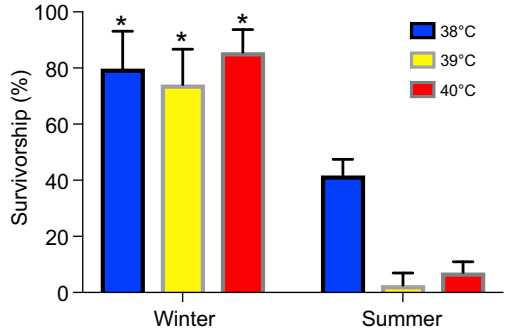

Fig. 3. *Dendroides canadensis* 24 h survivorship at different temperatures for larvae collected in winter and summer. Larvae were collected from 2011 to 2014 during January–February (winter) and June–August (summer) and immediately placed at various temperatures in the laboratory. The winter-collected larvae demonstrated higher survival than the summer-collected larvae (P<0.0001 at 38, 39 and 40°C with Tukey's multiple comparisons test). Values shown are means+1 s.d. Asterisks indicate a significant difference between the winter and summer values for a given temperature.

24 h ULT_{50} calculation for each season

We plotted the *D. canadensis* survivorship for each 24 h test conducted during the different seasons over 3 years. For each season, we performed a logistic regression via RStudio and calculated the 24 h ULT_{50} of the population. The calculated 24 h ULT_{50} was 40.9±0.3°C for winter larvae, 36.7±0.1°C for summer larvae, 37.3±0.3°C for spring larvae and 38.5±0.2°C for autumn larvae (Fig. 6 and Table 1).

24 h ULT_{50} and LLT_{50} (and temperature range) for each month

We sorted the measured 24 h ULT and LLT survivorship by month (Fig. 7), and thereby determined the monthly 24 h lethal temperature range of *D. canadensis* larvae. The measured 24 h ULTs in winter (Fig. 7) were much higher than our predicted ULTs (Fig. 1). Consequently, the measured 24 h lethal temperature range was 64°C in the winter, rather than the predicted 43°C; however, the actual 40°C range in May to September (Fig. 7) was close to the 43°C predicted summer value.

Winter and summer 48 h ULT_{50}

When placed for 48 h at high temperatures, *D. canadensis* larvae collected in winter revealed an increased survivorship compared with those collected in summer as the winter 48 h ULT_{50} was 38.7°C compared with the summer ULT_{50} of 36.4°C (Table 1). While the summer 24 h and 48 h ULT_{50} were approximately equal (36.7 and

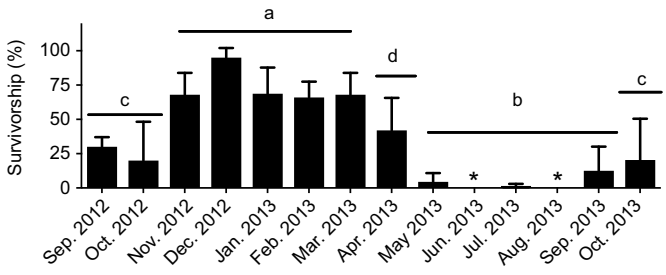

Fig. 4. *Dendroides canadensis* 24 h survivorship at 39°C for larvae collected over 13 months. n=40–100 larvae tested per month. Different letters above the error bars indicate statistical significance (P<0.05), using Tukey's multiple comparisons test. Data are means+s.d. *0% survivorship.

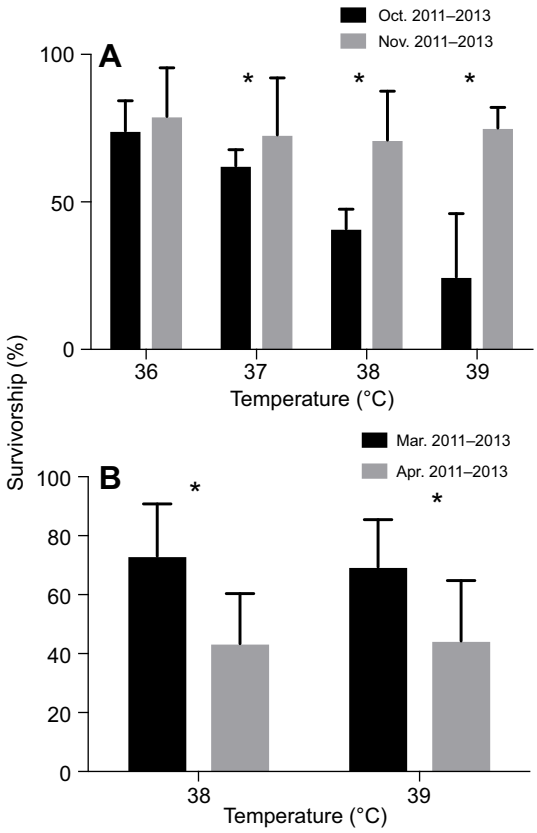

Fig. 5. *Dendroides canadensis* 24 h survivorship for larvae collected in the autumn and spring transition months. (A) Autumn transition: October and November. (B) Spring transition: March and April. n=40–142 larvae tested per month. Data are means+s.d. Comparison of data for larvae collected in the traditionally warmer months of October and April with those of larvae collected in the generally colder months of November and March, respectively, revealed the former had significantly decreased survivorship (*P<0.0001 from Holm–Šídák multiple comparisons test).

36.4°C, respectively), the winter 24 h ULT_{50} (40.9°C) was 2.2°C higher than the winter 48 h ULT_{50} (38.7°C).

Cucujus clavipes clavipes and *T. trivittata* high temperature survivorship over a year

To determine whether the ability to survive higher temperatures in winter relative to summer is unique to *D. canadensis*, we examined two other insects in which we had previously investigated cold tolerance ability and mechanisms. When tested at 39°C for 24 h, the freeze-avoiding larvae of the beetle *C. clavipes clavipes* had over 75% survivorship in the colder months from January to mid-March, but 0% survivorship from late March into May (Fig. 8). Likewise, when tested at 35°C for 24 h, the freeze-tolerant larvae

Table 1. The calculated seasonal 24 and 48 h ULT_{50} of *Dendroides canadensis*

Season	24 h ULT_{50} (°C)	48 h ULT_{50} (°C)
Winter	40.9±0.3	38.7±0.1
Summer	36.7±0.1	36.4±0.1
Autumn	38.5±0.2	36.9±0.1
Spring	37.3±0.3	35.9±0.2

Data are from Fig. 6. Each season's ULT_{50} (the temperature at which 50% mortality occurred) is significantly different from the ULT_{50} of every other season at both 24 h and 48 h (P<0.05).

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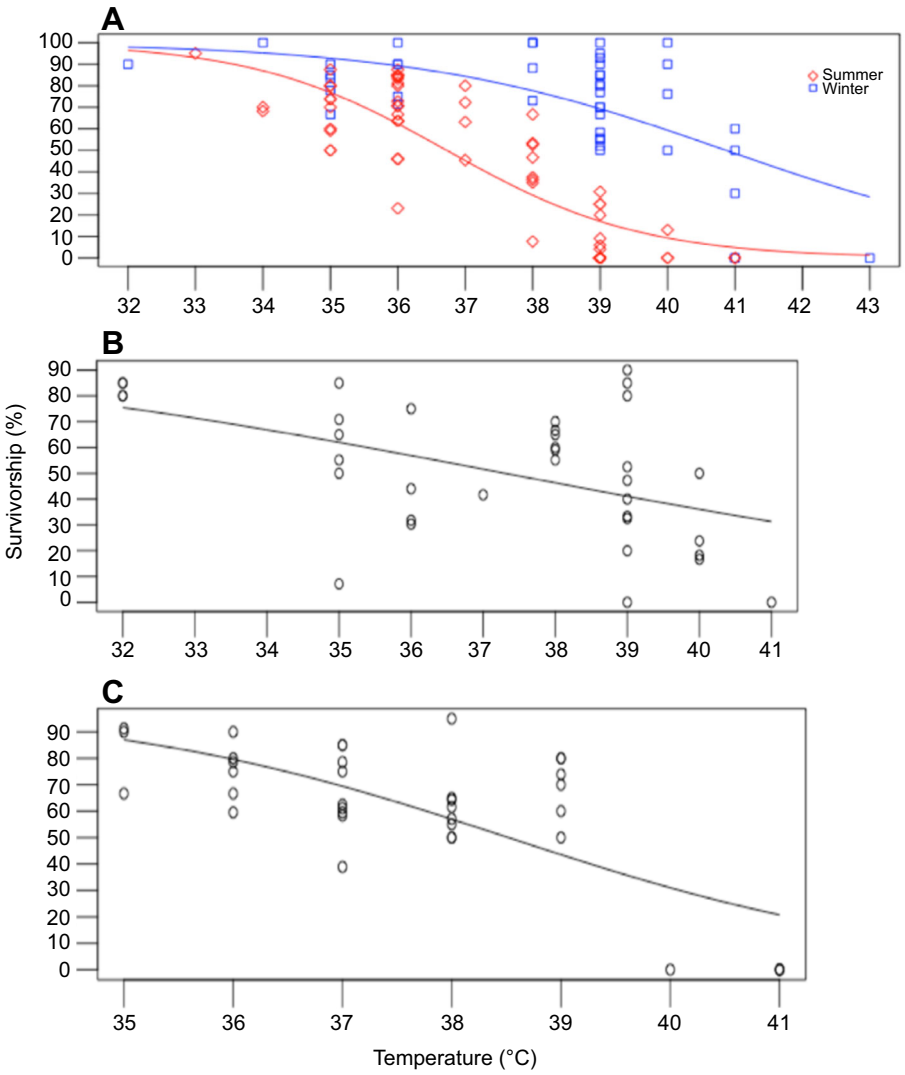

Fig. 6. *Dendroides canadensis* 24 h survivorship for larvae collected in different seasons over a 3 year period. (A) Summer and winter, (B) spring and (C) autumn. Data are means±s.e.m. The line represents the logistic curve fit for the data. The 24 h ULT_{50} (the temperature at which 50% mortality occurred) was calculated to be 36.7±0.1°C for summer, 40.9±0.3°C for winter, 37.3±0.3°C for spring and 38.5±0.2°C for autumn larvae. Each season's ULT_{50} is significantly different from that of every other season (P<0.05).

of the tipulid *T. trivittata* showed increased heat tolerance in the winter months (Fig. 9), and survivorship began to taper off as the seasons progressed from winter to spring. While the level of testing in these two species was not as exhaustive as that with *D. canadensis*, clearly *D. canadensis* larvae are not unique in their unexpected ability to better survive high temperatures in winter than in summer.

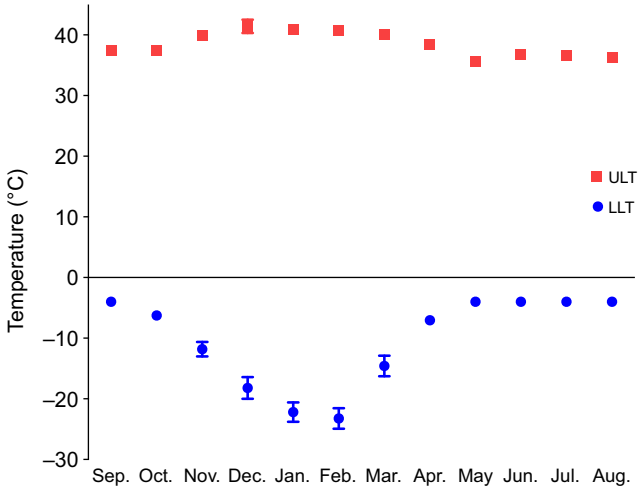

Fig. 7. Measured mean monthly 24 h ULTs and LLTs. Data are means±s.e.m.

Desiccation experiment

The following experiments were done to determine whether the mortality of *D. canadensis* larvae at high temperatures in the

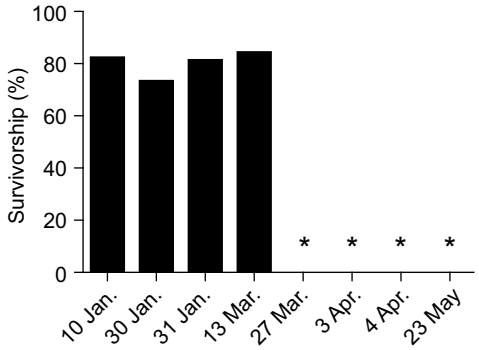

Fig. 8. *Cucujus clavipes clavipes* 24 h survivorship at 39°C for larvae collected periodically from January to May 2013. n=8–20 for each date. *0% survivorship.

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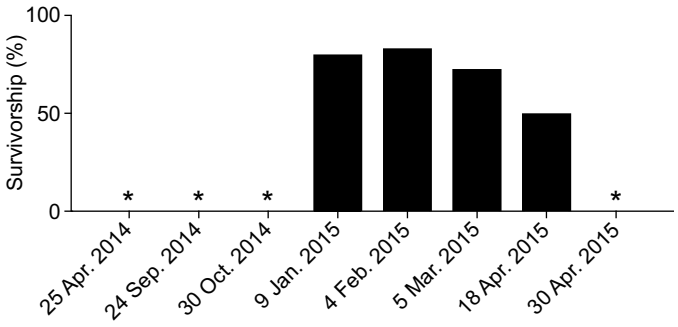

Fig. 9. *Tipula trivittata* 24 h survivorship at 35°C for larvae collected periodically between late April 2014 and late April 2015. *n*=8–20 for each date. *0% survivorship at 35°C.

previously described experiments was due to temperature or to desiccation. During our experiments at 40°C with dry, rather than wet, paper towels in the container with the larvae, the insects decreased in mass every hour and died after a few hours. In contrast, when a moist paper towel was placed with the larvae at various temperatures, *D. canadensis* did not change mass over 24 h. (Recall that the moist paper towel was part of the normal protocol in all of the previously described experiments.) The survivorship of the insects in this experiment at 40°C after 24 h with hourly mass checks was similar to the results of our other 24 h, 40°C experiments with no hourly mass checks. Consequently, temperature, not desiccation, was the cause of mortality in our normal ULT experiments.

DISCUSSION

This is the first study to document higher ULTs of insects in winter than in summer. Winter *D. canadensis* larvae showed increased survivorship compared with summer larvae when tested for 24 and 48 h at higher temperatures. We expected *D. canadensis* to have a temperature range of approximately 43°C between the insect's 24 h ULT and LLT through the four seasons. However, we found that winter *D. canadensis* both lower their LLT and raise their ULT, resulting in a 64°C range between their 24 h ULT and LLT, while this range shrinks in May through the summer to ∼40°C as the LLT increases and the ULT decreases. In addition, *C. clavipes clavipes* and *T. trivittata* had increased survivorship at higher temperatures in the colder months compared with the warmer months.

Underneath the bark of decaying logs where these three insects live, the microhabitat temperature was highest in the spring, with maximum temperatures of approximately 30°C over the 3 years of this study. Consequently, the closest the insects came to their ULT was in spring, when *D. canadensis* larvae were occasionally within 5–6°C of their ULT. The logs were at their warmest temperature in the early spring months as a result of the rising air temperatures combined with the absence of leaves to block the sunlight from reaching the logs prior to leaf development on the trees. Also, by late March, the larvae have begun to feed and have lost some of their winter adaptations, and their ULTs have begun to decrease. In addition, the microhabitat temperatures (Fig. 2) were measured in large logs that, because of their size, have significant thermal inertia. This can be seen in the difference between microhabitat and air temperatures (Fig. 2). This is noteworthy because larvae are sometimes found off the ground in the smaller branches of dead trees. These less thermally buffered sites more closely track air temperature and therefore on warm days they are often warmer than the main log temperatures presented in Fig. 2. Also, it is quite possible that during especially warm summers microhabitat temperatures may be higher than those measured in this study, thereby placing the insects at risk.

The mechanism(s) whereby the insects' tolerance for high temperature increases in the winter relative to summer is unknown at this time. It is probable that multiple physiological and biochemical processes suffer at the extreme upper end of the temperature range, any one of which could result in mortality if it was to malfunction. Also, it is likely that the 'weak link' varies with time of year and with temperature. Consequently, it is likely that multiple factors are required to overcome these potentially lethal problems. However, at least a partial answer may involve the cold tolerance mechanisms of the insects: AFPs in *D. canadensis* and *C. clavipes clavipes*, AFGLs and polyols in all three species. Also, heat shock proteins are typically produced constitutively by insects in winter (Denlinger, 2002; Rinehart et al., 2007). *Dendroides canadensis* produces HSP-70 throughout the winter (J.G.D., unpublished).

One obvious problem resulting from the expansion of the temperature range of these insects in winter concerns membranes. It is well established that the functional ranges of membranes of poikilotherms change seasonally, shifting to lower temperatures in winter, generally by increasing the percentage of unsaturated fatty acids and shorter fatty acids incorporated into membrane phospholipids and increasing the level of membrane cholesterol (Hazel, 1995; Hazel and Williams, 1990; Somero et al., 2017). Likewise, seasonal lipid restructuring is well established in insects (Koštál et al., 2004; Tomcala et al., 2006), without which disruption of inorganic ion homeostasis can result in mortality (Koštál et al., 2003, 2006). While these membrane changes lower the lower temperature at which the membranes are functional in winter, they should also lower the upper end of the functional temperature range as well. Yet, in the case of *D. canadensis* larvae, the temperature range increases from 41°C in summer to 64°C in winter. How might this be explained? The answer is unknown, but certain fish AFPs have been shown to stabilize cell membranes at low temperature (Tomczak et al., 2002). Is it possible that some AFPs may also stabilize membranes at high temperature? In the desert beetle *Microdera punctipennis*, two AFP isomers, slightly different from the winter isomers, are naturally expressed in the rigorous desert summer (Qiu et al., 2013). Expression of one of the summer isomers as a fusion protein in yeast and bacteria increased the heat tolerance of these organisms, and the protein stabilized lactate dehydrogenase at high temperatures. AFP transcripts were upregulated by high temperature exposure in the laboratory in both *M. punctipennis* and the darkling beetle, *Tenebrio molitor* (Li et al., 2016). The AFPs of both of these beetles are structurally similar to those of *D. canadensis*. Although *D. canadensis* hemolymph thermal hysteresis activity decreases from ∼4–6°C in winter to ∼0.3°C in mid-summer, a few of the 30+ *D. canadensis* AFP (DAFP) isomers continue to be synthesized during the summer months, albeit at lower levels (Andorfer and Duman, 2000). Also, AFGLs are present mainly in the cellular membrane, but only in winter in all three of the insect species included in this study (Walters et al., 2009; Walters et al., 2011). Might the AFGLs function to stabilize the cell membrane from temperature stresses and contribute to the increased heat tolerance, along with cold tolerance, in winter? In addition, polyols such as sorbitol and glycerol are known to stabilize proteins and membranes at both high and low temperature extremes (Back et al., 1979; Gekko and Morikawa, 1981; Gekko, 1983). Sorbitol accumulates in silverleaf whiteflies during heat stress (Salvucci et al., 2000). Hemolymph glycerol concentrations in *D. canadensis* are typically 0.5–1.0 mol l^{-1} in winter, but are extremely low in summer (Duman, 1980; Duman and Serianni, 2002).

This study reveals the paradoxical and previously undescribed phenomenon of increased heat tolerance of insects in the winter months. For biomedical and research applications, understanding the mechanisms that permit increased survivorship at both high and low temperatures may be beneficial to the freeze–thaw processes of cryopreservation of human cells, tissues and organs for transplantation purposes, for example. With the drastic changes in temperature from 37°C to −135°C and back to 37°C in the process of cryopreservation, the cells, tissues and organs undergo extreme temperature stress. If the mechanisms underlying the insects' winter phenomenon of increased heat tolerance can be understood, the compounds driving the insects' winter survivorship could be used to increase the yield and success in cryopreservation and mitigate the damage resulting from temperature stress.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.M.V., J.G.D.; Methodology: H.M.V., J.G.D.; Validation: H.M.V., J.G.D.; Formal analysis: H.M.V., J.G.D.; Investigation: H.M.V., J.G.D.; Resources: H.M.V., J.G.D.; Data curation: H.M.V., J.G.D.; Writing - original draft: H.M.V., J.G.D.; Writing - review & editing: H.M.V., J.G.D.; Visualization: H.M.V., J.G.D.; Supervision: J.G.D.

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