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## RESEARCH ARTICLE

# Antifreeze proteins in the primary urine of larvae of the beetle *Dendroides canadensis*

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## SUMMARY

**To avoid freezing while overwintering beneath the bark of fallen trees, *Dendroides canadensis* (Coleoptera: Pyrochroidae) larvae produce a family of antifreeze proteins (DAFPs) that are transcribed in specific tissues and have specific compartmental fates. DAFPs and associated thermal hysteresis activity (THA) have been shown previously in hemolymph and midgut fluid, but the presence of DAFPs has not been explored in primary urine, a potentially important site that can contain endogenous ice-nucleating compounds that could induce freezing. A maximum mean THA of 2.65±0.33°C was observed in primary urine of winter-collected *D. canadensis* larvae. THA in primary urine increased significantly through autumn, peaked in the winter and decreased through spring to levels of 0.2–0.3°C in summer, in a pattern similar to that of hemolymph and midgut fluid. THA was also found in hindgut fluid and excreted rectal fluid, suggesting that these larvae not only concentrate AFPs in the hindgut, but also excrete AFPs from the rectal cavity. Based on *dafp* transcripts isolated from Malpighian tubule epithelia, cDNAs were cloned and sequenced, identifying the presence of transcripts encoding 24 DAFP isoforms. Six of these Malpighian tubule DAFPs were known previously, but 18 are new. We also provide functional evidence that DAFPs can inhibit ice nucleators present in insect primary urine. This is potentially critical because *D. canadensis* larvae die if frozen, and therefore ice formation in any body fluid, including the urine, would be lethal.**

Key words: antifreeze protein, insect cold tolerance, Malpighian tubule antifreeze, urine antifreeze, *Dendroides canadensis*.

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## INTRODUCTION

Overwintering insects adopt one of two strategies to survive low temperatures: freeze tolerance or freeze avoidance. Freeze-tolerant species tolerate freezing in extracellular spaces, and many have high supercooling points (SCPs; the temperature at which spontaneous ice nucleation occurs, also known as the nucleation or crystallization temperature), primarily because they produce ice-nucleating proteins and lipoproteins in the hemolymph to encourage ice formation in extracellular water (Zachariassen and Hammel, 1976; Neven et al., 1989). In contrast, freeze-avoiding insects are unable to survive any freezing of their body fluids and therefore have evolved strategies to supercool and avoid freezing. Adaptations of freeze-avoiding organisms include production of low molecular mass antifreezes (such as glycerol) and antifreeze proteins, and elimination of ice-nucleating compounds either on a seasonal or evolutionary time scale (Salt, 1953; Sømme, 1982; Zachariassen, 1985; Neven et al., 1986; Olsen and Duman, 1997a; Olsen and Duman, 1997b; Duman, 2002; Duman et al., 2010).

Antifreeze proteins (AFPs) and antifreeze glycoproteins were first discovered in Antarctic marine teleost fishes (DeVries, 1971). Since then, AFPs have been described in diverse taxa including terrestrial arthropods (Duman, 1977: Duman, 2001; Tyshenko et al., 1997; Andorfer and Duman, 2000; Duman et al., 2004; Graham and Davies, 2005; Neelakanta et al., 2010), bacteria and fungi (Duman and Olsen, 1993; Sun et al., 1995; Yamashita et al., 2002; Gilbert et al., 2005) and plants (Griffith et al., 1992; Urrutia et al., 1992; Hon et al., 1994; Duman, 1994; Smallwood et al., 1999; Griffith and Yaish, 2004). Antifreeze proteins lower the non-colligative freezing point of water without depressing the melting point. This results in a difference between the hysteretic freezing point and the equilibrium melting point, termed thermal hysteresis activity (THA) (DeVries, 1986). The generally accepted mechanism of action, adsorption inhibition (Raymond and DeVries, 1977), suggests that AFPs bind to the surface of a growing ice crystal through hydrogen bonding and/or hydrophobic and van der Waals interactions (Sicheri and Yang, 1995; Jia and Davies, 2002), forcing the ice crystal to grow in a high curvature, high free energy front, which requires a decrease in temperature for further ice crystal growth. In a recent study using highly active AFPs from the beetle *Dendroides canadensis* (Latreille), the antifreeze activity was attributed to these short-range interactions of the AFPs with ice, but also to long-range changes in the protein–water hydration dynamics (up to 27 Å from the protein surface) that support the short-range interactions (Meister et al., 2013).

*Dendroides canadensis* is a non-diapausing, freeze-avoiding beetle that overwinters in multiple larval stages under the bark of fallen trees. The larvae produce a family of tissue- and compartment-specific AFPs, as well as glycerol, which allows the insect to supercool to temperatures below −20°C (Duman, 2002). *Dendroides canadensis* AFPs (DAFPs) consist of 12- to 13-mer repeats and contain cysteines every six residues that form disulfide bridges to stabilize the barrel conformation and allow the hydroxyl groups of the threonine residues in the characteristic Thr-Cys-Thr motif to bind the oxygens in the ice crystal lattice (Duman et al., 2002;

Graether and Sykes, 2004; Duman et al., 2010). The THA of AFPs depends on protein concentration and specific activity as well as the presence or absence of enhancers. Enhancers associate with AFPs, thus lowering the hysteretic freezing point. DAFP activity can be enhanced by other DAFPs (Wang and Duman, 2005), polyols (Li et al., 1998a; Amornwittawat et al., 2009) and polycarboxylates (Amornwittawat et al., 2008). *Dendroides canadensis* also produce a thaumatin-like protein, which on its own has no THA, but enhances the thermal hysteresis activity of certain DAFPs (Wang and Duman, 2006). The cumulative effect of specific AFP activity, glycerol and protein enhancers often provides a much greater cryoprotective effect than the measured THA would indicate (Zachariassen and Husby, 1982; Olsen et al., 1998; Duman, 2001; Duman, 2002; Duman et al., 2004). DAFPs promote supercooling by associating with the epidermis and preventing inoculative freezing, and by binding to ice nucleators and/or embryo ice crystals on the surface of ice nucleators (Olsen et al., 1998).

The presence of ice nucleators in *D. canadensis* hemolymph and gut fluid has been documented (Olsen and Duman, 1997a; Olsen and Duman, 1997b), but ice nucleators may also be present in the Malpighian tubule lumen. Some insects produce urine that contains crystals and spherules (Teigler and Arnott, 1972; Wigglesworth, 1974) with potential ice-nucleating activity. Spherules of calcium and phosphate have been found in the Malpighian tubules of the freeze-tolerant *Eurosta solidiganis*, and these showed ice-nucleating activity (Mugnano et al., 1996). Other homologues of crystals present in insect primary urine have ice-nucleating activity, including potassium phosphate, potassium urate, sodium urate and uric acid (Mugnano et al., 1996).

AFPs and/or THA have been described previously in the hemolymph and gut fluid of freeze-tolerant and freeze-avoiding arthropods including *D. canadensis*, but the presence of AFPs or THA has not been reported in primary urine (Ramsay, 1964; Duman, 2001; Duman et al., 2002; Duman et al., 2010). However, hexagonal crystal growth, which is indicative of the presence of low levels of AFP activity, was shown in Malpighian tubule homogenate in the New Zealand alpine cockroach (Wharton et al., 2009). The present study identifies transcripts encoding DAFPs in Malpighian tubule epithelia of *D. canadensis* larvae, demonstrates the presence of significant thermal hysteresis in the primary urine, explores the seasonal timing and relationship between THA in various body fluid compartments (hemolymph, midgut, hindgut, primary urine), and investigates the potential role of AFPs in the primary urine of *D. canadensis* by illustrating the ability of DAFPs to mask potential ice-nucleating crystals that might be present in the Malpighian tubules of overwintering arthropods.

## MATERIALS AND METHODS

### Air and microhabitat temperature measurement

Air temperature and temperature beneath the bark of a large, partially decomposed fallen log were monitored using a Hobo Pro series data logger (H08-031-08; Onset Computer Corporation, Bourne, MA, USA) and BoxCar Par 4 software (Onset Computer Corporation). The data logger housing was mounted 1 m above the ground to monitor air temperature, and the external temperature sensor was inserted beneath the bark on the top of the fallen tree. The area of the tree in which the sensor was inserted was ~1.5 m off the ground, and was only insulated through the winter by snow that settled on the top of the tree. The tree was similar in location and dimension to the trees from which *D. canadensis* were collected, and therefore gives a reliable representation of conditions experienced by animals collected for this study.

### Field collection of *D. canadensis* larvae

Larvae were collected from beneath the bark of decomposing logs in wood lots located in north central Indiana and southwestern Michigan. The larvae were transported (in a cooler) to the laboratory, where dissections and fluid collection took place. It is important to note that all animals used in the hemolymph, midgut fluid and primary urine comparison study were collected from the same woodlot, from late November through March. Most were collected from the same tree, thus decreasing population and habitat variability.

### Larval dissection and fluid collection

Hemolymph was obtained from each individual by piercing the cuticle of the insect with a 28-gauge needle, and drawing the hemolymph into a 10 µl capillary tube. The sample was then sealed, kept on ice and THA was determined as described below within 24 h of collection or kept sealed at −80°C for later analysis.

After hemolymph collection, each individual was pinned, dorsal side up, to a SYLGARD-184 (Dow Corning Corporation, Midland, MI, USA) elastomer-coated Petri dish. Care was taken not to pierce or damage the gut or Malpighian tubules. Incisions were made on the lateral sides of the cuticle with a pair of dissecting scissors, and the dorsal cuticle was reflected back. The cavity was rinsed with a small volume (~20 µl) of modified Hank's insect medium, and the two anterior Malpighian tubules were removed from the animal and placed on another SYLGARD-coated plate under mineral oil. The tubules were stretched slightly and the ends of the tubule were wrapped around two pins, and 20 µl of physiological saline were added under the oil near the distal blind end of the tubule. Fluid expressed from the proximal end of the tubule was collected in a pulled 10 µl capillary tube. Fluid was collected for 1–2 min in order to avoid dilution of the fluid that might otherwise take place. The sample was then held sealed on ice and THA was determined as described below within 24 h of collection or the sample was kept under oil at −80°C for later analysis.

The midgut was removed from the animal and rinsed with physiological saline to remove hemolymph from the gut surface. The outside of the gut was then dried by dabbing with a Kimwipe and placed on a clear area of the same SYLGARD-coated Petri dish. The midgut was pierced, and the gut fluid was collected in a pulled 10 µl capillary tube, and held under oil, on ice. THA was determined as described below within 24 h of collection, or the sample was kept sealed at −80°C for later analysis.

The hindgut was also removed from the animal and, like the midgut, rinsed with physiological saline, dabbed dry and placed on a clear area of the SYLGARD-coated Petri dish. The hindgut epithelium was pierced and the fluid was collected in a pulled 10 µl capillary tube and held under oil, on ice, and THA was determined as described below within 24 h of collection, or the sample was kept sealed at −80°C for later analysis.

The dissecting physiological saline contained NaCl (90 mmol $l^{-1}$), KCl (50 mmol $l^{-1}$), $MgCl_2$ (5 mmol $l^{-1}$), $CaCl_2$-$2H_2O$ (2 mmol $l^{-1}$), $NaHCO_3$ (6 mmol $l^{-1}$), $NaH_2PO_4$ (4 mmol $l^{-1}$), glycine (10 mmol $l^{-1}$), proline (10 mmol $l^{-1}$), serine (10 mmol $l^{-1}$), histidine (10 mmol $l^{-1}$), glutamine (10 mmol $l^{-1}$) and glucose (50 mmol $l^{-1}$) in ultrapure water. The osmotic concentration of the modified Hank's insect medium was 392 mOsm.

### Rectal excretion collection and analysis

In early October 2008, 80 *D. canadensis* larvae were collected from a woodlot on the University of Notre Dame campus. These insects, still actively feeding, were placed individually in Petri dishes lined

with Parafilm. The insect-containing Petri dishes were then placed in a high-humidity (saturated) environmental chamber at 4°C under short photoperiod (8 h:16 h light:dark) conditions to encourage the insects to void their guts. Insects were visually monitored at regular intervals in order to track when individuals cleared their guts. When it was observed that an individual had cleared its gut, the droplet of fluid (volume <<1 μl) on the Parafilm was drawn up into a pulled 10 μl pipette and the opposite end of the tube was sealed with a flame. The sample was then spun down into the closed end of the tube, the other end was sealed with oil, and the sample was placed on ice. At that time, the individual was dissected as described above, and the hemolymph, midgut fluid and urine were also collected and held under oil, on ice. THA was measured on a nanoliter osmometer as described below within 24 h.

A cohort of winter-acclimatized larvae was also collected. Hemolymph, midgut, Malpighian tubule and rectal fluids were collected as described above, with one variation. Because these animals were not feeding and therefore would not clear the rectal space, rectal fluid was obtained by inserting a pulled 10 μl glass capillary tube through the anal opening and into the rectal space. A small volume of fluid was drawn into the capillary tube and held under oil, on ice, and THA was measured on a nanoliter osmometer as described below within 24 h.

### THA determination

THA of hemolymph, mid-gut fluid, hindgut fluid, rectal excretion and Malpighian tubule fluid was measured using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand). The sample wells of the nanoliter osmometer were filled with heavy mineral oil and ~50 nl of the respective body fluid was delivered *via* micrometer syringe into the sample well. The sample was frozen by rapidly decreasing the temperature to −40°C. The temperature was then slowly raised until a single, stable ice crystal remained in the sample. The temperature was then raised again until the crystal melted (melting point). After the melting point was obtained, the sample was re-frozen by rapidly decreasing the temperature to −40°C. The temperature was subsequently raised to just below the melting point temperature until only a single ice crystal remained in the sample, and the temperature was then slowly decreased until the crystal grew rapidly at the hysteretic freezing point. Thermal hysteresis is calculated as the difference between the melting point and the hysteretic freezing point. In addition to melting point and hysteretic freezing point temperatures, crystal morphology was also noted.

### Larval SCP determination

A cohort of insects collected at the same times and location as the individuals used for fluid and tissue collection was used to determine the SCPs for the respective population. The larvae were inspected to confirm that the cuticle was intact because many winter-collected individuals were in close contact with ice and sometimes required considerable effort to extract from the log. Those individuals whose cuticle had been compromised were excluded from SCP determination as a damaged cuticle could give an inaccurately high SCP reading. Individual larvae were placed in a 1.5 ml plastic microcentrifuge tube and a thermocouple was placed against the cuticle of the larva, held in place by small pieces of foam. Thermocouples were visually inspected to confirm contact with the animals. The thermocouples were attached to a multichannel thermometer (Iso-Thermex, Columbus Instruments, Columbus, OH, USA). The temperature of each larva was recorded by computer every 5 s. The tubes containing the larva and attached thermocouples were placed in 500 ml glass test tubes submerged in a circulating ethanol bath. After the larvae reached a temperature of −1°C, the temperature of the bath was reduced at a rate of 0.2°C $min^{-1}$. Temperature plots for each larva were observed, and the lowest temperature before the exotherm representing the latent heat of fusion as the larva froze was recorded as the SCP.

### Glycerol analysis

Hemolymph, midgut fluid and urine were pooled from winter-acclimatized insects. A spectrophotometric assay was employed to quantify glycerol concentrations in various compartmental fluids (Boehringer Mannheim, Marshall, MI, USA). The protocol was adjusted to accommodate the small volumes of midgut fluid and urine. The samples and dilutions used to calculate the standard curve were run in triplicate on a 96-well plate and read in a Spectramax M2 spectrometer multidetection microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Effects of DAFPs on ice nucleators

To determine whether DAFPs were able to inhibit the ice-nucleating activity of various crystals present in insect urine, SCPs of solutions containing the crystals were measured with and without DAFPs. SCP determinations were made on a TCP-6 thermoelectric cooling plate (Thermoelectrics Unlimited, Wilmington, DE, USA) (Vali and Stansbury, 1966; Vali, 1995). Aluminum foil was adhered to the copper plate and coated with a thin layer of petroleum jelly prior to placing the 1 μl droplets ($N$=30) onto the surface. New foil was used for each replicate. The cooling plate was covered by a piece of glass supported by a polystyrene block that served to insulate the supercooled droplets from the ambient air. In order to avoid condensation on the plate and potential nucleation of the droplets, centrifuge caps of $CaSO_4$ desiccant were placed on the cooling plate. Once the droplets were arranged on the foil, the temperature was decreased (~0.5°C $min^{-1}$) until freezing of the samples was observed visually.

Grace's insect medium with 0.5 mol $l^{-1}$ glycerol served as a control simulating the environment in the Malpighian tubule lumen in the absence of DAFPs. Supersaturated solutions of crystals potentially present in Malpighian tubule fluid were made up in Grace's insect medium containing 0.5 mol $l^{-1}$ glycerol. Solutions containing crystals of calcium phosphate, potassium phosphate, sodium urate and uric acid were tested. In order to determine whether DAFPs could inhibit the nucleating effects of these crystalline compounds, SCPs were also determined for identical solutions containing 0.5 mg $ml^{-1}$ DAFP-1 and -2. Recombinant DAFPs were produced as in Wang and Duman (Wang and Duman, 2005). A solution of Grace's insect medium with 0.5 mol $l^{-1}$ glycerol and 0.5 mg $ml^{-1}$ DAFP-1 and -2 resulted in a THA of 1.59±0.08°C, slightly lower than, but comparable to, the THA observed in primary urine collected from winter-acclimatized *D. canadensis* larvae. SCPs of the various fluids were compared using a non-parametric ANOVA and Tukey's multiple comparison tests.

### Isolation, cloning and sequencing of cDNA clones encoding AFPs from Malpighian tubule epithelia

Malpighian tubule tissue dissected from pools of 10 *D. canadensis* larvae collected periodically (in June, July, November and December) and tubule tissue dissected from individual larvae collected in January and August, were used to isolate RNA using the RNAqueous Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Using oligo dT primers and AMV reverse transcriptase (Promega, Madison, WI, USA), tubule tissue RNA was reverse

Table 1. Primers, based on previously known *dafp* genes, used to identify *dafp* transcripts present in *Dendroides canadensis* larvae Malpighian tubule epithelia

| Gene | Reverse primer (5′–3′) | Forward primer (5′–3′) |
|---|---|---|
| *AFP-1* | caatgtactggtggttccgattgtcgc | gcctgtaccgattccacgggatgtcca |
| *AFP-3* | caatgtactggtgctcccgttgtagt | ccatctgcatacatccaccagtcaac |
| *AFP-4* | caatgtactggaggttccgattgtcaa | aagacctgtaccgattctacgggatgtcca |
| *AFP-5* | gtatgtacaggggtccgaac | tcctgtgttaattccacgggatgtccaccaccg |
| *AFP-6* | caatgtactggaggttccgattgtagc | tgtaccgattctacgggatgtccatgatcttattc |
| *AFP-7* | gtatgtactgggggcggcaat | gcctgtgttaattctacgggatgtccacca |
| *AFP-8* | caatgcactggaggttccgattgtagc | actactgcctgtacagattcaactggatgtcca |
| *AFP-9* | caacaatgtactgggggttccgac | tcttctgcttcagtcaaacaaatcaag |
| *AFP-10* | caagaatgtactgggggttcc | tcatccgtttcggtcaaacatatcaag |
| *AFP-11* | gaatgcactggaggttccgattgtcgc | actggatgtccaggatctggtgct |
| *AFP-12* | gtatgtacaggggtccgaac | tcctgtgttaattccacgggagtccaccg |
| *AFP-14* | caacaatgtactgggggttccgac | tctgcttcagtcaaacaaatcaag |

transcribed into cDNA. PCR (50 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s with a final extension of 72°C for 10 min) was performed using primers designed by the Primer3 program (Rozen and Skaletsky, 2000) for the partial sequences. Primers for the full tubule sequences were designed to encode the amino and carboxy termini of each of the 12 previously known mature DAFPs (Table 1). Primers were prepared by Integrated DNA Technologies (Coralville, IA, USA). RT-PCR products were gel purified using the QiaQuick gel extraction kit (Qiagen, San Diego, CA, USA), cloned into the PCR2.1 vector (Life Technologies, Grand Island, NY, USA) and sequenced using an Applied Biosystems 3730xl DNA analyzer (Grand Island, NY, USA).

## RESULTS

### Microhabitat data

Many insects seek out microhabitats in winter that will provide thermal buffering from air temperatures, multiple freeze–thaw cycles, or both (Danks, 1991). Microhabitat selection is of particular importance to freeze-avoiding species in order to buffer against large temperature changes that may occur throughout the late autumn and winter. The actual temperatures to which overwintering *D. canadensis* larvae are exposed are often quite different than the ambient air temperature (Sformo et al., 2010). Therefore it is important to determine the temperature that overwintering organisms experience. Consequently, microhabitat temperature was monitored under the bark of a log similar to those from which larvae were collected for this study (Fig. 1).

From late September through mid-November 2008, the temperature beneath the bark of the log increased and decreased with changes in the air temperature; however, the magnitude of temperature fluctuation within the log was buffered by the thermal inertia of the log itself (Fig. 1). Only once before 16 November, for a period of 2 days, did the temperature beneath the bark reach 0°C or lower. The point at which sufficient snow accumulated on the log to provide an insulating effect is obvious because on this date (16 November) the temperature beneath the bark was considerably higher than the air temperature and remained steady between 0 and −1°C for 2 weeks. From 16 November through 31 December 2008, the temperature beneath the bark remained below zero except for one unseasonably warm 24 h period on 27 December. Because the insulating snow layer had melted during this record-setting warmth in late December, the temperature beneath the bark rose and fell with the air temperature until the next snowfall in January 2009. In early January a storm began 14 consecutive days of snowfall totaling 74.7 cm. During this period, in spite of insulating snow, the temperature beneath the bark reached the lowest recorded temperature of the season, −10.2°C, but was considerably higher than the corresponding air temperature of −27.5°C on that date.

A similar trend was observed during the autumn and winter of 2009–2010, although the temperatures were milder than those observed in 2008–2009. Snowfall in December 2009 was well below the historical average, resulting in greater fluctuation in log temperatures in December 2009 than in December 2008, including a 24 h temperature decrease of 7°C on 15 December (Fig. 1). Snowfall in January 2010 resulted in a moderation of log temperatures until a mid-winter thaw in February melted the snow, after which time log temperatures reached the seasonal low of −9.2°C. Log temperatures remained below 0°C until 9 March 2010, when log temperature rose, remaining above 0°C for the duration of the study.

### Whole-body SCPs

The SCP of every larva collected over the 2 years of the study was lower than the lowest log temperature recorded on the date of collection (Fig. 2). Mean winter 2008–2009 SCPs of *D. canadensis* were significantly lower ($P<0.05$) than those observed in early autumn from 19 November 2008 through 9 March 2009, excluding 15 December 2008 and 19 January 2009, when larvae had mean SCPs of −8.0±1 and −11.7±1.2°C, respectively. Mean summer and early autumn 2009 SCPs ranged from −5 to −6°C and decreased to

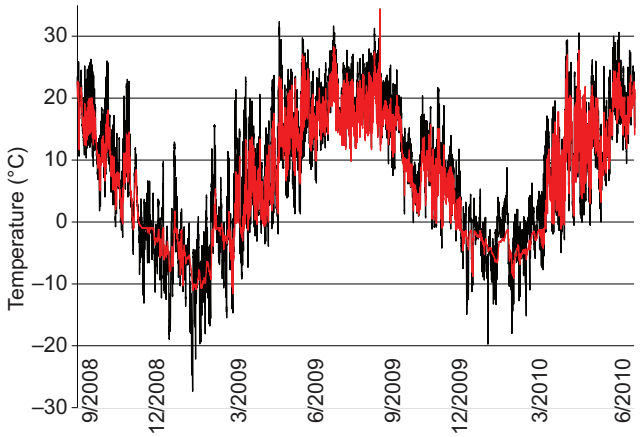


Fig. 1. Temperatures recorded beneath the bark of a fallen tree (red) and the corresponding ambient air temperatures (black) from autumn 2008 through spring 2010. In winter the daily minimum temperature beneath the bark is generally higher than that of the air because of the insulating effect of snow cover and the thermal inertia of the log.

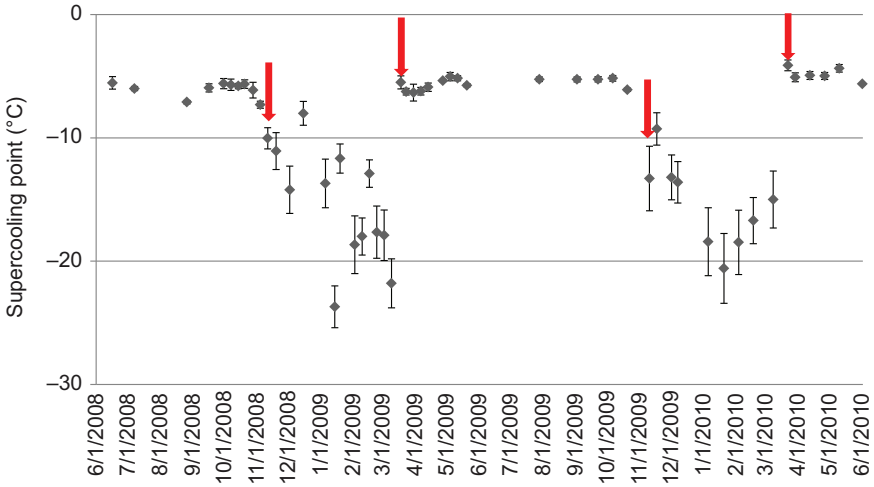


Fig. 2. Supercooling points (mean ± s.e.m.) of field-collected *Dendroides canadensis* larvae from June 2008–June 2010. Red arrows indicate the dates when 50% of the larvae collected had stopped feeding in the autumn or begun feeding in the spring. Cessation of feeding corresponded to a significant decrease in supercooling points.

−10°C in November. Winter 2009–2010 mean SCPs displayed a significant decrease from summer SCPs from 10 November 2009 through 8 March 2010, after which time SCPs rose abruptly, corresponding with resumed drinking and feeding on rotted wood, as confirmed by visual inspection of gut contents of field-collected larvae, and the concurrent consumption of exogenous ice nucleators.

### Compartmental fluid analysis

Measureable THA was present in *D. canadensis* primary urine throughout the study, including low levels in the summer. Mean primary urine THA of populations sampled throughout the year followed a general trend of increasing through the autumn until peaking in winter at mean values between 3.4 and 4.2°C, decreasing through spring, and reaching levels less than 0.5°C throughout the summer (Fig. 3). The lowest mean observed THA in primary urine over the course of the study was 0.20±0.03°C in July 2008.

Hemolymph and midgut fluid mean THA demonstrated similar patterns to that of primary urine: increasing through autumn, peaking during winter and decreasing through spring into summer (Fig. 3). However, mean THA in hemolymph and midgut fluid rose earlier in the autumn, reached greater magnitude in winter and remained elevated later in the spring than in primary urine. Mean THAs in midgut fluid and hemolymph were over 1°C in late September and remained greater than 1°C until late March, while mean THA in primary urine rose above 1°C 2 weeks later in the autumn and dropped below 1°C in early March, presumably when the larvae began drinking again and AFPs that may have been concentrated in the Malpighian tubules were flushed out into the hindgut. The maximum mean THAs observed in each fluid were 4.27±0.29°C in January 2009 in hemolymph, 4.88±0.26°C in January 2009 in midgut fluid and 2.65±0.33°C in November 2008 in primary urine. Mean midgut THA was occasionally higher than

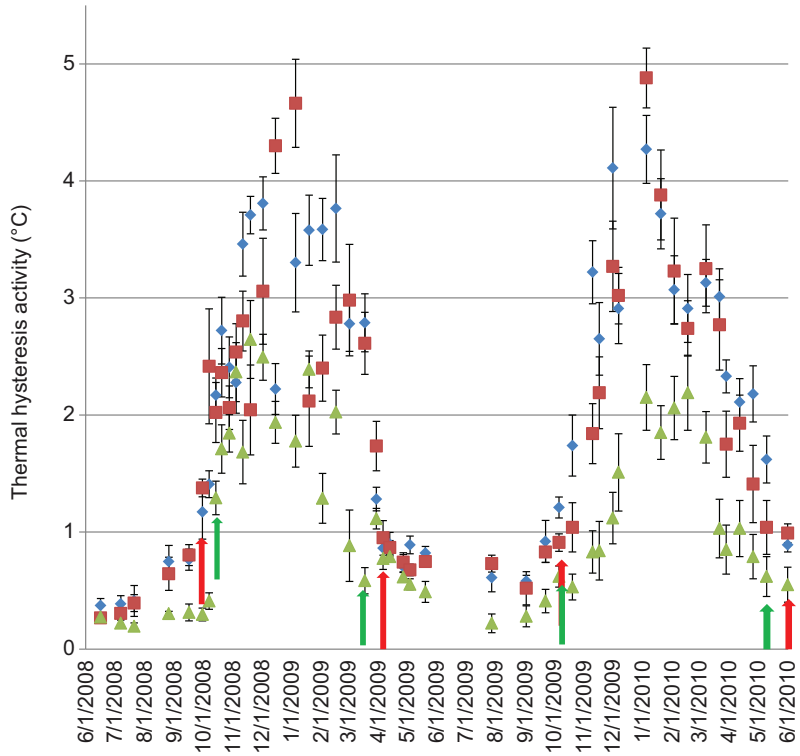


Fig. 3. Thermal hysteresis activity (THA; mean ± s.e.m.) of hemolymph (blue diamonds), midgut fluid (red squares) and primary urine (green triangles) harvested from field-collected *Dendroides canadensis*. Red arrows represent the date THA of hemolymph and midgut fluid increased significantly relative to summer levels in the autumn and returned to summer levels in the spring. Green arrows represent the date THA in primary urine increased significantly relative to summer levels in the autumn and returned to summer levels in the spring.

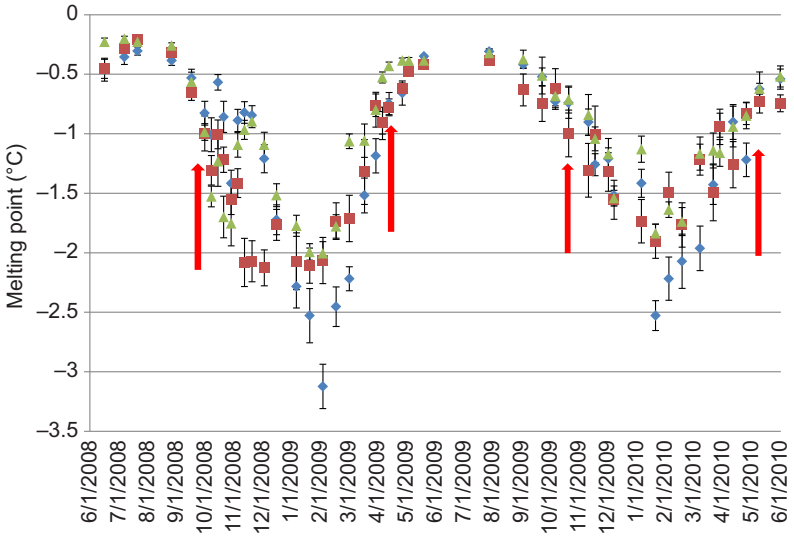


Fig. 4. Melting points (mean ± s.e.m.) of hemolymph (blue diamonds), midgut fluid (red squares) and primary urine (green triangles) harvested from field-collected *Dendroides canadensis*. Red arrows represent the dates that melting points of hemolymph, midgut fluid and primary urine decreased significantly relative to summer levels in the autumn and returned to summer levels in the spring.

mean hemolymph THA (i.e. on 15 December 2008 and 5 January 2009). Mean THA in hemolymph and primary urine generally plateaued through winter while mean THA in midgut fluid displayed much more variation, with low values coincident with warm spells, when the larvae presumably drank.

The melting points of all three fluids (Fig. 4) decreased from summer values through autumn and into the winter, probably reflecting accumulation of glycerol, the primary polyol accumulated by *D. canadensis* in winter (Duman and Serianni, 2002), and then rose in the spring. The mean melting point of the primary urine was generally lower than that of the hemolymph from late September through late October. Interestingly, the mean melting points of the hemolymph and midgut fluid were generally lower than those of the primary urine through late autumn and winter. The lower melting point of the hemolymph relative to the primary urine suggests that urine may not be produced at these times, a reasonable possibility as the larvae are not feeding or drinking for long periods.

The relationships of THAs of hemolymph, midgut fluid, primary urine, hindgut fluid, rectal fluid and excreted rectal fluid (in autumn) are shown in Table 2. All fluids, including rectal excretions, displayed THA, suggesting that in autumn *D. canadensis* excretes AFPs when it clears its gut. THA in hemolymph, hindgut and rectal excretions were significantly higher ($P<0.05$) than THA found in the midgut fluid and primary urine. Hindgut fluid and rectal excretions also exhibited melting points that were significantly lower ($P<0.05$) than those observed in hemolymph, midgut fluid and primary urine. Melting points of all the respective fluids were significantly lower ($P<0.05$) in winter (early February)-collected individuals than in those collected in autumn. THA was also significantly higher ($P<0.05$) in all fluids in winter-collected larvae than those collected in autumn.

Quantification of glycerol in fluids collected in winter 2008 demonstrated the presence of glycerol in the hemolymph, midgut and primary urine (Table 3). Hemolymph ($0.57\pm0.08\,mol\,l^{-1}$) showed the highest concentration of glycerol, followed by primary urine ($0.45\pm0.06\,mol\,l^{-1}$) and midgut fluid ($0.15\pm0.03\,mol\,l^{-1}$). The concentration of glycerol in the midgut was significantly lower than that in the hemolymph and primary urine. This indicates that glycerol and perhaps other colligative antifreezes are, at least in part, responsible for the melting point depression in primary urine in winter.

### Sequences of AFPs derived from Malpighian tubule epithelia

Fig. 5 shows the deduced amino acid sequences of the DAFPs encoded by *dafp* transcripts isolated from Malpighian tubule epithelia of *D. canadensis* larvae. Twenty-four different DAFPs were identified. Of these, six were known previously (DAFP-3, -5, -6, -8, -11 and -12) from other tissues in *D. Canadensis*; however, 18 are new and are currently known only from Malpighian tubules. Of the 24 Malpighian tubule *dafp*s, 12 were present only in winter (November and December; *dafp*s *1-1*, *5*, *5-2*, *5-3*, *5-6*, *8*, *8-1*, *10-1*, *11*, *11-4*, *12* and *12-1*), while 11 were present only in summer (June and July; *dafp*s *3*, *5-1*, *5-4*, *6*, *6-1*, *6-2*, *6-3*, *6-4*, *11-1*, *11-2* and *11-3*), and only one (*dafp 5-5*) was found in both summer and winter.

Table 2. Thermal hysteresis activity (THA) and melting points of autumn-collected *Dendroides canadensis* transported to the laboratory and held under conditions to induce gut clearance

| | Autumn | | Winter | |
|---|---|---|---|---|
| Fluid | THA (°C) | Melting point (°C) | THA (°C) | Melting point (°C) |
| Hemolymph | $1.17\pm0.09^{b}$ | $-0.51\pm0.06^{g}$ | $2.32\pm0.18^{d}$ | $-1.73\pm0.12^{j}$ |
| Midgut fluid | $0.78\pm0.08^{a}$ | $-0.85\pm0.08^{h}$ | $2.23\pm0.14^{d}$ | $-1.38\pm0.10^{i}$ |
| Primary urine | $0.57\pm0.10^{a}$ | $-0.56\pm0.07^{g}$ | $1.78\pm0.11^{c}$ | $-1.68\pm0.14^{j}$ |
| Hindgut fluid | $1.28\pm0.09^{b}$ | $-1.65\pm0.09^{j}$ | $3.18\pm0.20^{e}$ | $-2.37\pm0.12^{k}$ |
| Rectal excretion | $1.59\pm0.13^{c}$ | $-1.84\pm0.13^{j}$ | $3.77\pm0.26^{f}$ | $-2.93\pm0.17^{l}$ |

In the autumn THA was significantly higher in hemolymph, hindgut fluid and rectal excretion ($P<0.05$) than in midgut fluid and primary urine, and melting points were significantly lower ($P<0.05$) in hindgut and rectal excretion compared with hemolymph, midgut fluid and primary urine.

Data are means ± s.e.m.

Superscript letters (a–f for THA; g–l for melting points) indicate means that are statistically different from one another ($P<0.05$) using Tukey's multiple comparison test.

Table 3. Glycerol concentration of hemolymph, midgut fluid and primary urine from *Dendroides canadensis* collected in January

| Fluid | Glycerol (mol $l^{-1}$) |
|---|---|
| Hemolymph | 0.57±0.08 |
| Midgut fluid | 0.15±0.03* |
| Primary urine | 0.45±0.06 |

Data are means ± s.e.m.
Mean glycerol concentration was significantly lower (*$P$<0.05) in midgut fluid than in hemolymph or primary urine.

### Function of DAFPs in primary urine

Grace's insect medium containing ice-nucleating compounds common in insect primary urine had slightly, but significantly, higher SCPs than the medium alone (Table 4). However, the addition of DAFP-1 and -2 significantly lowered ($P$<0.05) the SCPs of all solutions containing ice nucleators such that they were then not significantly higher than the SCP of Grace's insect medium lacking ice nucleators, thereby indicating that the AFPs inhibited the ice nucleators. Note that because $KH_2PO_4$ contributed significantly to the osmotic concentration of the solution, the addition of DAFPs resulted in an SCP (−24.4±0.4°C) significantly lower than the control solution of Grace's insect medium with DAFPs (−22.1±0.4°C).

## DISCUSSION

In order for freeze-avoiding animals to survive at the lowest temperatures experienced, all fluids must remain in a liquid state, as ice formation in any fluid space is likely to spread throughout the animal. AFPs that prevent ice formation and maintain a supercooled liquid state have previously been described in the hemolymph, midgut fluid and epidermis of *D. canadensis* (Olsen et al., 1998; Duman, 2002). However, the present study provides the first definitive evidence of the presence of AFPs in the primary urine and hindgut of a winter-acclimatized insect. A previous study on a freeze-tolerant insect suggested the presence of ice-binding proteins in Malpighian tubule homogenate, but only showed hexagonal crystal growth, not THA (Wharton et al., 2009). Ramsay (Ramsay, 1964), in his classic study describing the cryptonephridial complex of *Tenebrio molitor*, was the first to mention a thermal hysteresis phenomenon; however, he did not report THA in fluid collected from Malpighian tubules, perhaps because the insects were not cold acclimated. THA measured in primary urine of *D. canadensis* was lower than that measured in corresponding hemolymph or midgut fluid. This is possibly because (1) the primary urine in the Malpighian tubule lumen is not at risk of inoculative freezing as the Malpighian tubules are isolated within the hemocoel, and (2) the ice nucleators present in the primary urine are not as potent as those present in the hemolymph or gut fluid. DAFPs in the primary urine of *D. canadensis* may play a considerable

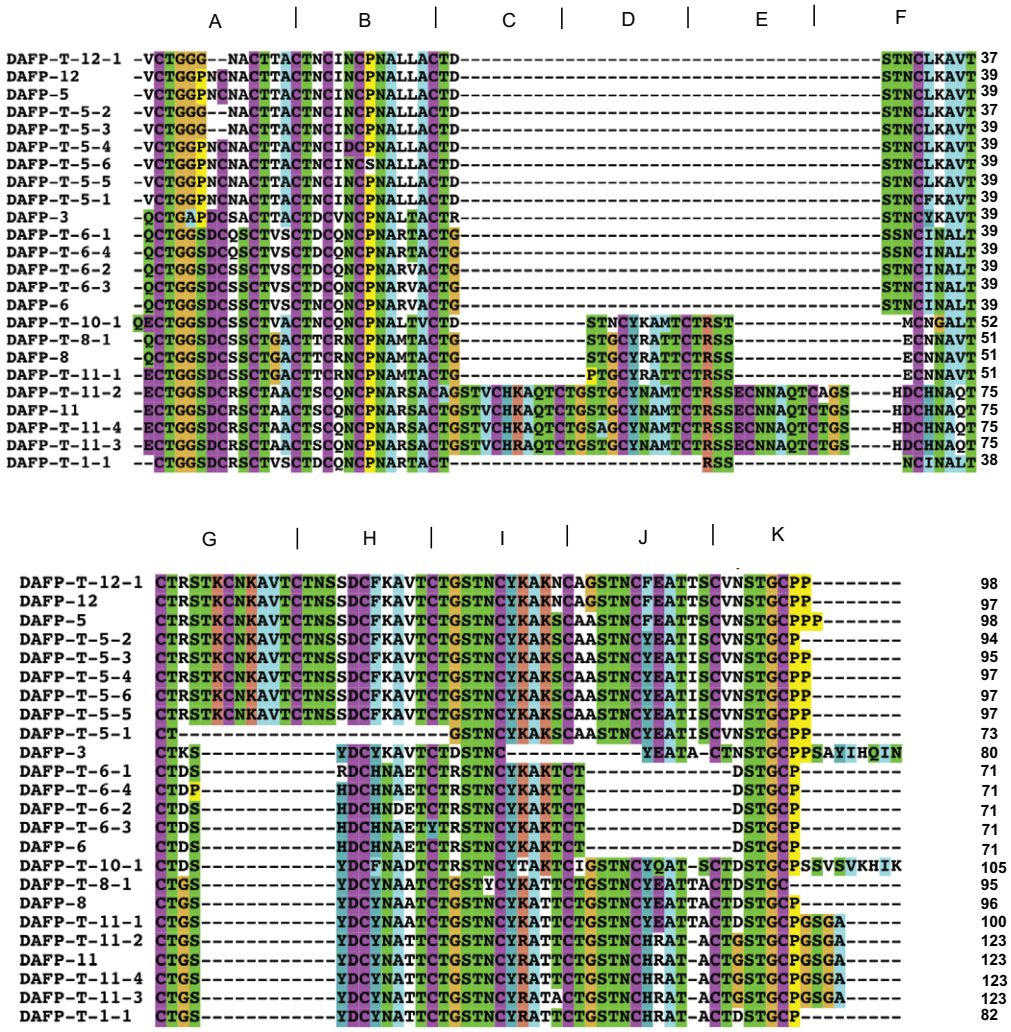


Fig. 5. Deduced amino acid sequences of the 24 antifreeze proteins from Malpighian tubule epithelia in *Dendroides canadensis*. The six *D. canadensis* antifreeze proteins (DAFPs) previously identified from other tissues are DAFP-3, -5, -6, -8, -11 and -12. The other 18 newly identified DAFPs are labeled as DAFP-T (for Malpighian tubule) followed by numbers that indicate similarity to previously known DAFPs. For example, DAFP-T-12-1 signifies that the sequence of this Malpighian tubule DAFP is most similar to DAFP-12. The letters A–K along the top of the sequences identify the various repeat units. Numbers to the right of sequences indicate the residue number of the last amino acid in each line.

Table 4. Supercooling points of primary urine ice nucleator homologues with and without addition of DAFPs

| Solution (with 0.5 mol $l^{-1}$ glycerol) | Supercooling point (°C) | |
|---|---|---|
| | Without DAFP-1 or -2 | With DAFP-1 and -2 |
| Grace's insect medium | –22.6±0.3[a] | –22.1±0.4[a] |
| Calcium phosphate, tribasic | –16.2±0.4[b] | –22.5±0.4[a] |
| Potassium phosphate, monobasic | –20.4±0.5[c] | –24.4±0.4[d] |
| Sodium urate | –20.8±0.6[c] | –22.3±0.5[a] |
| Uric acid | –20.1±0.5[c] | –23.1±0.4[a] |

Superscript letters indicate means that are statistically different from one another ($P$<0.05) using Tukey's multiple comparison test.

role in the ability of these organisms to supercool. The digestive/excretory anatomy of *D. canadensis* is quite basic. The gut lacks diverticula, and the midgut and hindgut are connected by a slight constriction where the Malpighian tubules intersect and empty into the hindgut and then into the rectal lumen, the primary site of reabsorption of water and ions in most insects. This allows for the intermingling of all gut and Malpighian tubule AFPs in the hindgut and rectal space and creates the potential for concentration of the DAFPs as well as enhancement of THA by other DAFPs or protein enhancers (Wang and Duman, 2005; Wang and Duman, 2006). Concentration of DAFPs in the rectal space could theoretically inhibit ice growing directly across the rectal opening in winter, while excretion of DAFPs would decrease the likelihood of the same situation if ice were present when the insect purged its gut.

In response to decreasing temperature and reduced photoperiod in the autumn, *D. canadensis* cease feeding and drinking, clear gut contents and synthesize AFPs, as well as antifreeze glycolipids and glycerol (Olsen and Duman, 1997; Duman et al., 2010; Walters et al., 2011). During warm periods in the winter and in early spring when snowmelt occurs, the larvae appear to drink, but this has not been conclusively demonstrated. As the insects drink they may consume bacterial ice nucleators that must then be masked or eliminated from the digestive tract before temperatures again decrease sufficiently to freeze the individual.

The influences of thermal inertia from the log microhabitat and insulation from snowfall in combination with antifreezes appear to be sufficient to protect *D. canadensis* larvae from rapid temperature decreases in late autumn/early winter and from temperature extremes throughout the winter. Every cohort of *D. canadensis* collected for this study supercooled sufficiently to survive the corresponding log temperature on a given date (Figs 1, 2). Prior to the first snowfall, temperatures beneath the bark of the log remained at or above 0°C even as corresponding air temperatures were several degrees below 0°C. Mean winter SCPs of all groups of *D. canadensis* excluding larvae collected 15 December 2008 and 17 November 2009 were sufficiently low for those individuals to survive the lowest log temperature recorded over two winters (−10.2°C) only because the layer of snow on top of the log provided an ~10°C temperature differential between the ambient air and the larval microhabitat temperature in the log.

This work illustrates, for the first time, the presence of AFPs, including several new DAFPs, and glycerol in the primary urine of a freeze-avoiding arthropod. Of the 24 DAFPs encoded by transcripts from the Malpighian tubule epithelium, 18 were DAFPs that had not previously been described. Most of the new DAFPs conform to the basic 12- to 13-mer repeat structure identified previously for DAFPs, where every sixth residue is a cysteine and certain other residues are highly conserved (Duman et al., 1998; Andorfer and Duman, 2000); however, three of these (DAFP-T-12-1, -T-5-2 and -T-5-3) are missing N and C residues at positions 7 and 8 in the first repeat. The absent C residue is potentially of consequence because this renders these DAFPs unable to form disulfide bridges between the C residues at positions 8 and 18, as is the case in all previously sequenced DAFPs (Li et al., 1998b). Tissue-specific expression of DAFPs has been reported (Duman et al., 2002), but it is interesting that although 13 DAFPs were previously sequenced from various tissues, nearly twice as many (24) were identified from Malpighian tubule epithelia. All 13 of the previously known DAFPs are produced in the fat body, eight are produced in the midgut epithelia and four to six are produced in the epidermal tissue. Only four DAFPs are present in the hemolymph. Ice formation in any body fluid is likely to be lethal in the freeze-avoiding *D. canadensis* larvae, especially as the ice is almost certain to spread quickly to other compartments. Consequently, prevention of freezing in the primary urine is essential. However, it is not obvious why so many more DAFPs are produced in the Malpighian tubules as compared with other tissues. Also, the presence of 12 of the 24 Malpighian tubule *dafp*s in the summer, and 11 of these only in summer, raises the question of the function of the DAFPs at this time of the year.

In addition to showing the presence of AFPs in primary urine, this study also demonstrates the presence of thermal hysteresis in the hindgut and rectal fluids and provides a summary of the freeze-avoiding response of *D. canadensis* over multiple years.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFP | antifreeze protein |
| DAFP | *Dendroides canadensis* antifreeze protein |
| SCP | supercooling point |
| THA | thermal hysteresis activity |

## AUTHOR CONTRIBUTIONS

P.K.N. was responsible for all aspects of this study (designed and performed experiments, analyzed data, writing), except for DAFP sequencing. S.S. assisted with insect collecting, measuring supercooling points and thermal hysteresis. D.V. determined DAFP sequences. E.M.B. collected the initial Malpighian tubule fluid and trained others in this methodology, and assisted with writing. J.G.D. oversaw the research, secured funding and assisted with manuscript writing.

## COMPETING INTERESTS

No competing interests declared.

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