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Cryoprotection in dampwood termites (Termopsidae, Isoptera)

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ABSTRACT

In contrast to the majority of the Order, the dampwood termites of the family Termopsidae found in colder regions can experience frost and snow, either in cool temperate areas at high latitudes (45°), or alpine areas at high elevations (>1000 m). This suggests that dampwood termites are adapted to cold climates. We investigated this hypothesis in two dampwood termites, *Porotermes adamsoni* Froggatt and *Stolotermes victoriensis* Hill. We measured nest temperatures and atmospheric temperatures of their alpine habitat during winter, and measured survival and recovery at subzero temperatures. We also determined the minimum temperature at which these species remain active and the LT50 values. We used a novel gas chromatographic strategy to examine eight metabolites from individuals of both species collected in winter and summer to identify possible cryoprotectants. Both *P. adamsoni* and *S. victoriensis* had significantly higher levels of trehalose, a known cryoprotectant, in winter than in summer; in addition *S. victoriensis* also had higher levels of unsaturated fatty acid ligands in winter than in summer, consistent with patterns observed for cold adaptation in other organisms. These results are the first to reveal that dampwood termites are adapted to cold climates and use trehalose and unsaturated lipids as cryoprotectants.

1. Introduction

Termites are generally known as social, wood-eating insects from the warmer climates in the world. The pattern of high species diversity in the tropics, decreasing with latitude is generally true (Emerson, 1955; Collins, 1989) except for one family in the order, the Termopsidae or dampwood termites (so called as they usually eat wet, rotted wood; Abe, 1987). Instead, the distribution of termopsid species is highly disjunct in two temperate zones around 25–45° north and south of the equator (Eggleton et al., 1994; Eggleton, 2000). The few tropical and subtropical locations where these termites are found are only at high altitude (700–2500 m; Imms, 1920; Basset, 1991; Eggleton and Tayasu, 2001). These regions experience frost and snow. As few or no other termites live in these cooler regions, wood-decaying fungi are the greatest competitors of termopsid termites. The distribution of these dampwood termites, and the lack of other termite species, suggests that they are capable of adapting to a much colder climate.

Termopsid termites may use either behavioural or physiological strategies, or both, to survive cold conditions (Downes, 1965). Behavioural adaptations can be grouped into either reduction to exposure or consumption of more calories, especially fats. Reduction to exposure can include simple behaviours such as basking or huddling, or more complex behaviours such as nesting. Basking requires exposure to sunlight, and consequently desiccation and predation; a strategy not favoured by termites. Huddling is more likely to work in endothermic animals and so is not available to poikilothermic termites. Nests can be made from or in insulating materials, such as wood and soil. Their nests are likely to provide some protection from the cold, as they are in wood—well known to provide insulation, but because termposid termites live in damp, rotted wood, the level of insulation will be less. The greater insulative properties of soil are beyond termopsids, because they have little, if any, capacity to tunnel in soil, unlike subterranean rhinotermitids (Cabrera and Kamble, 2001, 2004; Evans and Gleeson, 2001). Consequently, it is unlikely that dampwood termites use both behavioural and physiological strategies to survive cold temperatures.

Physiological adaptations are varied. Well known examples found in invertebrates include synthesis of simple metabolites and antifreeze proteins that serve as cryoprotectants, and also alteration of fatty acid ligand composition (Hoffmann, 1985; Storey and Storey, 1988; Lee, 1989; Duman et al., 1991; Danks, 2006). The most reported cryoprotectant metabolite in insects is glycerol, but low molecular-mass carbohydrates (such as glucose and trehalose) and polyhydric alcohols (such as sorbitol and mannitol) have also been found to accumulate in insects in response to cold induction (Crowe et al., 1984; Storey and Storey, 1991; Ishiguro et al., 2007; Overgaard et al., 2007). Cold induction changes the composition of fatty acid ligands in cell membranes of cold tolerant insects and other animals; in particular, it leads to an

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increase in their degree of unsaturation (Hazel, 1995; Bennett et al., 1997; Overgaard et al., 2006; Kayukawa et al., 2007).

The research hypothesis was to test whether termopsid termites use either behavioural or physiological strategies, or both, to survive subzero temperatures. We used two dampwood termites, *Porotermes adamsoni* Froggatt and *Stolotermes victoriensis* Hill, which are distributed in the cooler south east of Australia at higher elevations with regular frost and snow in winter. We measured nest temperatures to determine whether only a behavioural strategy was being used. We measured metabolites in individual termites in different seasons to identify possible physiological cryoprotectants by adapting our novel gas chromatographic strategy developed previously for milligram samples of nematodes (Pellerone et al., 2003).

2. Materials and methods

2.1. Field sites and temperature monitoring

The field site was in the Brindabella Range near Canberra, Australian Capital Territory (latitude 34.22°S, longitude 148.98°E, elevation 1298 m). The sites are heavily forested chiefly with *Eucalyptus viminalis* and *E. pauciflora*; the abundant standing and fallen dead trunks of these tree species are the food and nesting sites of the two termite species *P. adamsoni* and *S. victoriensis*. Four rotting logs were chosen for temperature monitoring in early August 2008. (The state of the logs was indicative that they could have contained termite colonies; however discovery of termites requires destruction of the logs.) Each log had four temperature probes (Hobo® data loggers, model H12-003, Onset Computer Corporation, Bourne, MA), which were placed: (1) suspended in the air ca. 1 m from the ground, (2) in the sapwood of the log, in a drill hole ca. 2 cm under the surface (the preferred habitat of *S. victoriensis*), (3) in the heartwood of the log, in a drill hole ca. 30 cm from the surface (the preferred habitat of *P. adamsoni*), (4) in the soil under the log, ca. 20 cm deep. Temperatures were recorded hourly for three weeks.

2.2. Field collection of termites

The two species of dampwood termites were collected from fallen and decaying *Eucalyptus* logs from the field site in the Brindabella Range in summer (March, mean daily air temperature range 6.6 to 22.1 °C) and winter (August, −1.8 to 10.9 °C). Termites used in bioassays were collected in August and returned to the laboratory in their log nests in insulated containers. The termites were kept in a cool-room (temperature 4 °C) to approximate natural field temperatures until they were used (from 2 to 20 days after collection).

2.3. Bioassays

Individuals of both species were removed from their log nests for laboratory bioassays to determine the lowest temperature at which they were active, speed of recovery after cooling and their LT50. The experimental unit consisted of a glass Petri dish (diameter 10 cm) with a circle of moistened filter paper (diameter 10 cm), on which a central circle (diameter 2 cm) was drawn. Termites were placed into a vertical PVC tube (diameter 2 cm) placed over the drawn circle, and then placed into a refrigerator set to the test temperature. After the 30 min equilibration period, the PVC tube was removed and the Petri dish was covered. The position of the termites was recorded 2 and 24 h later. The number of termites outside the drawn circle was recorded as the capacity for movement over these times. The Petri dishes were left for 2 h at room temperature (10 to 15 °C) and the number of termites walking was recorded as capacity to recover. All dead termites were recorded and removed. Each species had 10 replicate Petri dishes each containing 10 termites for each temperature setting examined. Actual temperatures in the refrigerator were measured using the temperature probes (Hobo® data loggers as above). Data were compared for actual temperatures, not temperature settings.

2.4. Measurement of metabolites

Termites used for measured metabolites were preserved over dry ice immediately upon collection in the field. The termites were dried *in vacuo* in the laboratory and stored in a vacuum desiccator over silica gel. The proportion of water in the termites was calculated by comparing wet and dry weights. The various metabolites in each individual termite were recovered by successive solvent extractions and their subsequent chemical derivatives were quantified by capillary gas chromatography (GC, helium carrier gas, cool on-column injection, flame ionisation detection). The individuals for metabolic analysis ($N = 6$ for each species and for each season) were selected at random.

All extractions and derivatisations were carried out in heated, screw-capped glass tubes (Alltech, Australia, 2 mL capacity, PTFE-faced silicone seal). The seals were replaced before each heating episode. Two glass beads (3 mm diameter) were included in the tubes to fracture the dried insect and to promote intimate contact with the termite residue during the solvent extractions. The internal standards used to quantify the metabolites selected in this study were ethyl n-heptadecanoate (for trans-esterified lipids), mannose (for hydrolysed glycogen), 1,2,4-butanetriol (for glycerol) and sucrose (for trehalose). The identities of the derivatised metabolites were validated by gas chromatography/mass spectrometry (GC/MS) (Hewlett Packard 5890 GC coupled to VG Trio 2000 mass spectrometer, 70 eV electron ionisation, MassLynx software) and the standard plots for quantification by GC were shown to be linear over the range of relative concentrations encountered in the present study. In those instances where the GC peak for the target derivative was too small for reliable quantification, enhanced signal-to-noise ratios were obtained by cumulative injections on to the retention gap (phase-free forecolumn) before initiating the temperature program (Lacey and Sanders, 1992).

2.4.1. Analysis of palmitate, palmitoleate, stearate, oleate and linoleate

A solution of sucrose (100–200 μg, depending on the mean weight of the termite species) in 20% aqueous ethanol (50 μL) was added to a screw-capped sample tube and evaporated to dryness under a nitrogen stream. The freeze-dried termite was weighed to the nearest 10 μg (using a five-figure electronic balance, Ohaus) in the sample tube and a solution of 1,2,4-butanetriol (Aldrich, Sydney, 100–200 μg) and ethyl n-heptadecanoate (150–300 μg) in absolute ethanol (100 μL) was added, followed by additional ethanol (200 μL). The tube was capped tightly and heated at 110 °C for 1 h with occasional vortex mixing. The mixture was centrifuged and a small aliquot of the warm supernatant solution (70–100 μL) was transferred to a fresh sample tube. Ethanol containing 2% H_2SO_4 (100 μL) was added and the sealed tube was heated at 110 °C for 1 h to trans-esterify the lipids. Hexane (300 μL) was added and the solution mixed with crushed ice (0.5 g). The aqueous layer was discarded and the hexane solution washed twice with water (0.3 mL) and dried ($MgSO_4$).

The resultant ethyl esters of the fatty acid ligands were separated and quantified by GC (Varian 3300 gas chromatograph, data acquisition and processing software) using a carbowax column (Alltech, Sydney, ECWax, 30 m length × 0.32 mm ID,

phase thickness 0.5 μm, helium flow 1 mL/min, cool on-column injection, 3 m retention gap). The temperature program used was 50 °C for 1 min; a gradient of 25 °C/min to 170 °C; a gradient of 3 °C/min to 210 °C; 210 °C isothermal for 20 min. For quantification, corrections were not applied to the profiles of the C16/C18 ethyl esters since the molecular masses of their progenitors (mainly glycerides but also some phospholipids) are not known.

2.4.2. Analysis of trehalose and glycerol

The remainder of the ethanol extract and the termite residue were dried under a nitrogen stream and 20% aqueous ethanol (200 μL) was added. The sealed tube was heated to 110 °C for 15 min with occasional vortex mixing. After cooling to room temperature and centrifuging, the clear supernatant solution and subsequent washing (100 μL) were combined and evaporated cumulatively within a conical reaction vial (Alltech, Sydney, screw-capped, thick walled, 100 μL capacity) under a nitrogen stream. Silylating reagent (Alltech, Sydney, Tri-Sil, 15-50 μL) was added to the dry residue and the sealed tube was heated at 80 °C for 1 h with occasional vortex mixing.

The resultant persilylated glycerol and trehalose, together with their respective internal standards, were separated and quantified by GC using a 5% phenyl methylpolysiloxane column (Alltech, Sydney, EC5, 30 m length × 0.32 mm ID, phase thickness 0.5 μm, helium flow 1 mL/min, 3 m retention gap). The temperature program used was 60 °C for 3 min; a gradient of 15 °C/min to 120 °C; 120 °C isothermal for 6 min; a gradient of 25 °C/min to 250 °C; a gradient of 10 °C/min to 300 °C; 300 °C isothermal for 20 min.

2.4.3. Analysis of glycogen

The remaining termite residue was dried under a nitrogen stream at 40 °C and 30% KOH solution in water (200 μL) was added. The sealed tube was heated to 110 °C for 1 h with occasional vortex mixing. After cooling to room temperature and centrifuging, the clear supernatant solution and subsequent washing (50 μL) were combined in a clean sample tube, which was then filled to capacity with ethanol (ca. 1.5 mL). The solution became cloudy after shaking as glycogen was precipitated. After 20 min, the tube was centrifuged (3000 rpm, 3 min) and the clear supernatant solution discarded. The precipitate was vortex-mixed with 20% aqueous ethanol (200 μL), centrifuged (3000 rpm, 1 min) and the washing discarded. The residue was dried at 40 °C whereupon internal standard (mannose, 100–200 μg) in water (100 μL) and dilute hydrochloric acid (5%, 100 μL) were added. The sealed tube was heated at 110 °C for 1 h with occasional vortex mixing to hydrolyse the glycogen to glucose. The resultant glucose/mannose mixture was evaporated to dryness under a nitrogen stream at 40 °C and the dried residue extracted with 10% aqueous ethanol (100 μL, vortex mixing). The extract was centrifuged and the supernatant solution was evaporated to dryness within a conical reaction vial (100 μL capacity). Silylating reagent (Tri-Sil, 15–50 μL) was added to the dry residue and the sealed tube was heated at 80 °C for 30 min with occasional vortex mixing.

The resultant persilylated carbohydrates were separated and quantified by GC using the 5% phenyl methylpolysiloxane column. The temperature program used was 60 °C for 3 min; a gradient of 20 °C/min to 140 °C; a gradient of 5 °C/min to 250 °C; 250 °C isothermal for 10 min. The peaks for the α- and β-pyranose anomers of each silylated carbohydrate were summated for quantification. Hydrolysis of glycogen to glucose increases the mass of the glucosyl units and therefore the quantity of glycogen was estimated as 90% of that of the derived glucose. The amount of covalently bound protein in glycogen is very small (Calder, 1991) and thus was ignored in the calculation.

2.5. Statistical analyses

The field temperatures were compared using a two-way repeated measures ANOVA, with 24 h time and probe position for the two treatments and date the repeated measure, in order to determine whether temperatures differed significantly between habitats. In the laboratory experiment, the activity at 2 h chilled, 24 h chilled, 2 h recovery and mortality were compared between temperatures for each species. Regression equations were fitted to determine the temperatures at which 50% of walking activity occurred after 2 and 24 h chilled, for 50% recovery in walking activity after 2 h at room temperature, and for LT50. For the metabolite data, the weights of metabolites were converted into percentages of body weight to control for differences in body sizes between species. Differences in metabolite levels between seasons in *P. adamsoni* and *S. victoriensis* were explored using unpaired Student's *t*-tests for each metabolite. The relative importance of fatty acid ligands were considered by comparing ratios of C16 ligands (palmitoleate/palmitate) and C18 ligands (oleate/stearate and linoleate/oleate) and also compared with unpaired Student's *t*-tests.

3. Results

3.1. Field temperatures

The temperatures of (1) air, (2) sapwood, (3) heartwood and (4) soil did show differences (Fig. 1). Air was highly variable, as expected, with averages across probes and dates ranging from around −1.5 to +2.5 °C. The remaining microhabitat temperatures were all fairly constant, with soil the highest, around +2.0 °C, heartwood (the preferred habitat of *P. adamsoni*) around +1.5 °C, and sapwood (the preferred habitat of *S. victoriensis*) around +1.0 °C. Sapwood was cooler and more variable than heartwood, demonstrating the insulative properties of wood. These temperature differences were significant, with the repeated measures two-way ANOVA finding that time of day ($F_{23,288} = 3.107$, $p < 0.001$), probe position ($F_{3,288} = 155.778$, $p < 0.001$) and date ($F_{7,2016} = 1388.569$, $p < 0.001$; the air temperature dropped over the period of measurement) all significant. However, there was an interaction between time of day and probe position ($F_{3,288} = 3.107$, $p < 0.001$), which was not surprising given the daily variation in air temperature, so the repeated measures two-way ANOVA was re-run without air temperatures.

There were significant differences in temperature between soil, heartwood and sapwood. The repeated measures two-way ANOVA

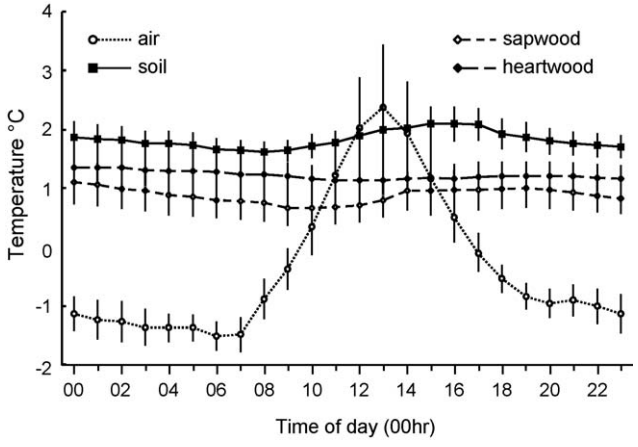

Fig. 1. The average (±standard error) daily range in temperatures over a three week period in winter (August 2008) observed in the field habitats of dampwood termites. The preferred habitat of *Porotermes adamsoni* is heartwood, and that for *Stolotermes victoriensis* is sapwood.

found that time of day was not significant ($F_{23,216} = 0.132$, $p = 1.000$), showing that temperatures were relatively constant over the 24 h day in these probe positions. However, there were significant differences in temperatures between probe positions ($F_{2,216} = 34.241$, $p < 0.001$), with soil warmer than heartwood, which was warmer than sapwood. Date was significant again ($F_{7,2016} = 1335.138$, $p < 0.001$), which demonstrated that the drop in air temperature also caused a drop in temperature in the other probe positions, indicating that the insulative properties of rotting wood were not perfect. There were no significant interactions.

The total number of below zero degrees Celsius temperature records differed for each location: there were 966 (34%) in air, 238 (13%) in sapwood, 243 (14%) in heartwood, and 0 in soil, of the 1920 temperature data records for each location. All of the 243 below zero degrees Celsius temperature records in heartwood were from one probe, whereas those for sapwood came from all probes. Re-calculating for averages across probes; the total number of below zero degrees Celsius temperature records were: 233 (49%) in air, 16 (3%) in sapwood, 0 (0%) in heartwood and soil, of the 480 averaged temperature data records for each location.

3.2. Bioassays

Termites collected from the field were active at the time of collection: individuals were walking around, and eggs and larvae were found, indicating that breeding took place right throughout winter. Only fifth or sixth instar nymphs (or pseudergates) were used for the laboratory experiments.

Both species remained active below 0 °C. Around half of the *P. adamsoni* (Fig. 2) had walked outside the circle after 2 h at −2 °C and after 24 h at −3 °C, indicated that they were capable of walking slowly even at these low temperatures. A 24 h chilled temperature of around −5 °C seemed to be important as walking activity ceased at around −5 °C (2 and 24 h duration) and most termites recovered from −5 °C after 2 h at room temperature; mortality rose rapidly at temperatures below −5 °C. All *P. adamsoni* were dead at the coldest temperature, ca. −10 °C. The pattern for *S. victoriensis* was similar, although the temperatures were lower (Fig. 3). Around half of the *S. victoriensis* termites had walked outside the circle after 2 h at −2.5 °C and after 24 h at −3.5 °C. A 24 h chilled temperature of around −6 °C seemed to be important as walking activity ceased at around −6 °C (2 and 24 h duration) and most termites recovered from −6 °C after 2 h at room temperature; and mortality rose at temperatures below −6 °C, although not so much as *P. adamsoni*. Most *S. victoriensis* were dead at the lowest temperature, ca. −10 °C, but not all; around one termite per replicate did recover after 2 h at room temperature. The regression equations were of good fit, with high r^2 values—although the mortality equations were a little lower (Table 1). The 50% activity levels calculated from the regression equations matched observations; the LT50 for *P. adamsoni* was estimated at −5.66 °C and the LT50 for *S. victoriensis* was −7.32 °C.

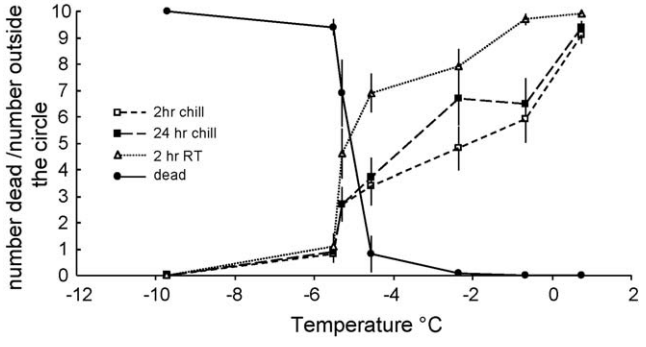

Fig. 2. The average (±standard error) movement and mortality data for *Porotermes adamsoni* from the laboratory experiment testing activity at different temperatures.

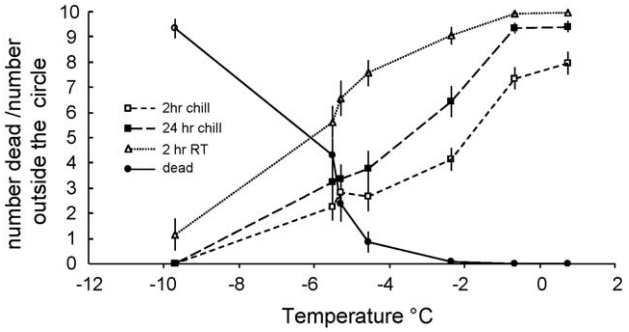

Fig. 3. The average (±standard error) movement and mortality data for *Stolotermes victoriensis* from the laboratory experiment testing activity at different temperatures.

3.3. Measurement of metabolites

The mean freeze-dried weight of termite workers collected in late winter (August) was: *P. adamsoni* (~8 mm long, 4.152 ± 0.201 mg), *S. victoriensis* (~6 mm long, 1.392 ± 0.075 mg). Considering the seasonal difference in the dampwood termites, *P. adamsoni* did not differ significantly in mass between March (4.120 ± 0.126 mg) and August (4.152 ± 0.201 mg) ($t_{10} = 0.134$, $p = 0.896$). *S. victoriensis* was significantly heavier in March (1.742 ± 0.054 mg) than in August (1.392 ± 0.075 mg) ($t_{10} = 3.792$, $p = 0.004$). Note that these differences were not due to a change in water in the termites: dry weight as a proportion of wet weight was not significantly different between seasons (*P. adamsoni* $t_{10} = 0.349$, $p = 0.734$; *S. victoriensis* $t_{10} = 1.307$, $p = 0.220$).

P. adamsoni did not change weight between seasons and the level of seven of the eight metabolites did not differ significantly over the same period: glycerol ($t_{10} = 1.549$, $p = 0.153$), glycogen ($t_{10} =$, 1.025, $p = 0.329$), palmitate ($t_{10} = 0.046$, $p = 0.964$), palmitoleate ($t_{10} = 0.390$, $p = 0.704$), stearate ($t_{10} = 0.148$, $p = 0.885$), oleate ($t_{10} = 0.774$, $p = 0.457$), linoleate ($t_{10} = 1.116$, $p = 0.290$). However, one metabolite, trehalose, was significantly higher in winter, showing a six-fold increase (4.3% dry body mass) compared with summer (0.7% dry body mass) ($t_{10} = 6.611$, $p < 0.001$) (Fig. 4).

In contrast, *S. victoriensis* lost weight from summer to winter and the level of only three of the eight metabolites did not differ significantly over the same period: glycerol ($t_{10} = 1.992$, $p = 0.074$), glycogen ($t_{10} =$, 1.900, $p = 0.087$), and palmitoleate ($t_{10} = 1.327$, $p = 0.214$). Four metabolites, all fatty acid ligands, decreased significantly in winter: palmitate ($t_{10} = 2.306$, $p = 0.044$), stearate ($t_{10} = 3.301$, $p = 0.008$), oleate ($t_{10} = 3.280$, $p = 0.008$), and linoleate ($t_{10} = 3.031$, $p = 0.013$). As had been observed for *P. adamsoni*,

Table 1
Regression equations used to determine the temperatures (x) at which 50% of the activity of interest occurred ($y = 5$). LT50 is represented by mortality.

Metric	Regression equation	r^2	50% Temperature (°C)
Porotermes adamsoni			
2 h Chill	$y = 0.0598x^2 + 1.3638x + 7.61$	0.947	−2.1
24 h Chill	$y = 0.0484x^2 + 1.3458x + 7.3323$	0.929	−2.7
2 h Recovery	$y = -0.0017x^2 - 1.0393x + 9.8391$	0.858	−4.6
Mortality	$y = 0.0192x^2 - 0.9678x - 0.3961$	0.723	−5.1
Stolotermes victoriensis			
2 h Chill	$y = 0.0424x^2 + 1.1618x + 7.335$	0.971	−2.2
24 h Chill	$y = 0.027x^2 + 1.2153x + 9.1438$	0.977	−3.7
2 h Recovery	$y = -0.0606x^2 + 0.3282x + 9.9633$	0.993	−6.7
Mortality	$y = 0.106x^2 + 0.0379x - 0.1597$	0.953	−7.2

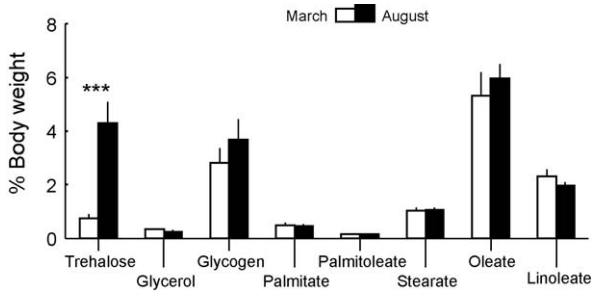

Fig. 4. Metabolite data for the dampwood termite *Porotermes adamsoni* for March (late summer, mean minimum temperature 6.6 °C, mean maximum temperature 22.1 °C) and August (late winter, mean minimum temperature −1.8 °C, mean maximum temperature 10.9 °C). Significance indicated as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

trehalose was significantly higher in winter, a fourfold increase (4.4% dry body mass) compared with summer (1.0% dry body mass) ($t_{10} = 6.317$, $p < 0.001$) (Fig. 5).

Since glycogen was not measured directly in the present study, it is possible that the relative quantities for glycogen noted in Figs. 4 and 5 are maximum levels. Cellulose is a potential contaminant in the termites but it does not contribute significantly to the glycogen analysis because it is insoluble in the alkaline extraction solvent. However, it is possible that partly digested cellulose is more soluble in alkali and therefore could contribute to the measured glucose levels after subsequent acid hydrolysis.

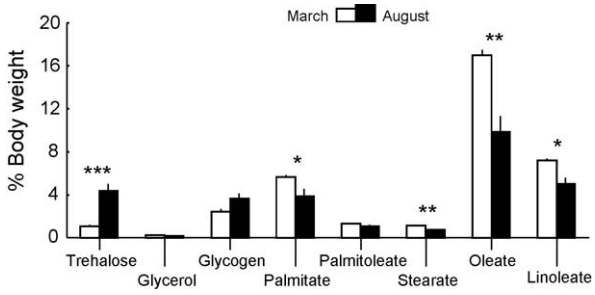

Fig. 5. Metabolite data (average + standard error) for the dampwood termite *Stolotermes victoriensis* for March (late summer) and August (late winter). Significance indicated as for Fig. 4.

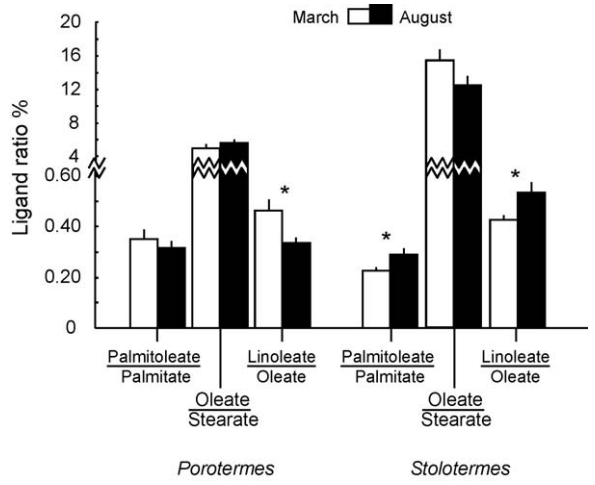

Fig. 6. Ratios (average + standard error) of unsaturated fatty acid ligands (palmitoleate, oleate and linoleate) to their more saturated equivalents (palmitate, stearate and oleate) from the dampwood termites *Porotermes adamsoni* and *Stolotermes victoriensis* for March (late summer) and August (late winter). Significance indicated as for Fig. 4.

The ratios of unsaturated fatty acid ligands to more saturated ligands varied between the two dampwood species and between seasons (Fig. 6). For *P. adamsoni*, no significant differences were found for the ratios of the C16 fatty acid ligands (palmitoleate/palmitate, $t_{10} = 0.781$, $p = 0.453$), or one of paired C18 ligands (oleate/stearate; $t_{10} = 1.339$, $p = 0.210$), but a significant decrease was found for the other paired C18 ligands (linoleate/oleate, $t_{10} = 2.989$, $p = 0.014$). For *S. victoriensis* significant increases from summer to winter were found for the ratios of the C16 fatty acid ligands (palmitoleate/palmitate, $t_{10} = 2.856$, $p = 0.017$), and one of the paired C18 ligands (linoleate/oleate, $t_{10} = 2.739$, $p = 0.021$), but not the other (oleate/stearate; $t_{10} = 1.711$, $p = 0.118$). Thus, there was no observed change to more unsaturated fatty acid ligands in *P. adamsoni*, less in fact, whereas there was a change to more unsaturated ligands in *S. victoriensis*.

4. Discussion

Glycerol did not change significantly between seasons for either dampwood species, demonstrating that this most common insect cryoprotectant (Chino, 1957; Sømme, 1982; Duman et al., 1991) was not used in termites. Instead, trehalose was the only metabolite of the eight measured in this study that varied in the same pattern between seasons for both dampwood species. This strongly suggests that the two dampwood termite species are using trehalose as a cryoprotectant in their cold adaptation. Trehalose is well known as a cryoprotectant in insects, plants and microorganisms (Duman et al., 1991; Storey and Storey, 1991; Chen and Haddad, 2004), including butterflies (Kukal et al., 1991) and beetles (Fields et al., 1998). Trehalose was initially found to be important in dry situations because of its ability to interact with phospholipids using hydrogen bonding, which replace analogous bonds that occur with water (Crowe et al., 1984). Similar adaptations have been found in insects to both cold and dry conditions (Danks et al., 1994).

Other metabolites may also be important for functioning at lower temperatures. *S. victoriensis* showed an increase in unsaturated fatty acid ligands relative to more saturated fatty acid ligands in winter. This increased degree of unsaturation has been documented previously for cell membranes in cold adapted organisms, e.g. in flies (Bennett et al., 1997; Kostal et al., 2003; Overgaard et al., 2005, 2006; Michaud and Denlinger, 2006) and bugs (Hodková et al., 1999; Slachta et al., 2002). An additional change in lipid composition has been observed from phosphatidylcholines to phosphatidylethanolamines (Slachta et al., 2002; Kostal et al., 2003; Overgaard et al., 2006; Tomcala et al., 2006); however, the phospholipids were not monitored separately in the present study. The alteration of cell membrane lipids in response to ambient temperatures has been termed homeoviscous adaptation (Hazel, 1995). It is notable that the greater proportion of linoleate relative to oleate during winter for the combined lipids as seen in *S. victoriensis* has previously been observed for the phosphatidylethanolamines of *Pyrrhocoris apterus* after cold acclimatisation (Hodková et al., 1999; Slachta et al., 2002) and the polar phospholipids of *Drosophila melanogaster* after rapid cold hardening (Overgaard et al., 2005).

The two dampwood species did not have identical quantities of metabolites, nor was the seasonal change in metabolites always similar in pattern. Clearly, *P. adamsoni* and *S. victoriensis* have other differences, most notably in the amount of fat they accumulate over summer and lose during winter. This may be related to their differing food and nest habits. Although both species are found in fallen and decaying *Eucalyptus* logs, and indeed both species can be found in the same log, the location within the log is quite distinct for the two species. This may be due to food preferences (which allow co-existence): *S. victoriensis* is generally found in the more

heavily decayed outer sections of logs, attacking from the bark to the centre, perhaps indicating a preference for decayed sapwood, whereas *P. adamsoni* occupies the central section of a log, attacking outwards, eating decayed (but also sound) hardwood (Lenz and Barrett, 1982). These divergent feeding habits create differences in "nest" (these termites live in the voids made from their feeding) site selection. Field data revealed that the two termite species inhabit distinct temperature regimes. The nests of *P. adamsoni* in the centre of a log are more protected from exposure to low, and variation in, temperature compared with the nests of *S. victoriensis* on the outside of the tree. Consequently, perhaps, the central nesting habit may allow *P. adamsoni* to rely on trehalose as its only cryoprotectant of low molecular mass; indeed, trehalose levels in *P. adamsoni* during winter are 40% higher than those in *S. victoriensis*. On the other hand, *S. victoriensis*, within its colder outer nests in decayed wood, evidently requires additional metabolites to trehalose such as more unsaturated lipids as cryoprotectants.

This is one of the first biochemical studies of cold adaptation in termites and is the first demonstration that termites use trehalose as a cryoprotectant. Termites can also increase the proportion of unsaturated fatty acid ligands as a further protection of function under cold conditions. Termites are very closely related to cockroaches (Inward et al., 2007) and a cold adapted cockroach has been found in the mountains of New Zealand (Block et al., 1998; Wharton et al., 2009). Although the New Zealand cockroach is unlikely to be ancestral to dampwood termites (Legendre et al., 2008), the presence of a cold adapted termite and cockroach supports the contention that these adaptations are widespread in insects and increases the phylogenetic range of insects studied to the more ancestral types.

Although the accumulation of trehalose during winter is illustrated here for only two Australian species, there are several reasons why it is likely that this is a general protective strategy for other cold adapted dampwood termite species, such as the other *Porotermes* and *Stolotermes* species in Chile, New Zealand and South Africa (Gay, 1969; Gay and Calaby, 1970; Villance, 1972; Coaton and Sheasby, 1976, 1978), and the *Archotermopsis*, *Hodotermopsis* and *Zootermopsis* species in the northern hemisphere (Harris, 1970; Collins, 1989; Beljaeva, 2004a,b; Sornnuwat et al., 2004). It may also be true for subterranean species found in cool temperate regions, including *Reticulitermes* (Rhinotermitidae) in the northern hemisphere (Davis and Kamble, 1994; Cabrera and Kamble, 2004). Firstly, trehalose is ubiquitous in insects as the characteristic 'blood sugar' and is synthesised by glycogenolysis (Thompson, 2003). Secondly, it is the most effective of the carbohydrates for stabilising cellular membranes by replacing water and thereby maintaining the membrane in a liquid crystalline state during stress (Behm, 1997; Thompson, 2003). It also exercises a remarkably long-range perturbation of the solvation dynamics of surrounding water molecules (Heyden et al., 2008) and is especially effective in preserving the native conformations of proteins at low temperatures (Strambini et al., 2008). Thirdly, other species of insects in completely separate families have been shown to use trehalose for surviving low temperatures (see above).

Although more challenging than the analysis of amalgamated specimens, the metabolic profile of single insects has an advantage that any variation in the level of a metabolite in an individual responding to environmental conditions can be demonstrated to be a consistent change within the selected population. The present study demonstrates clearly that the biosynthesis of cryoprotectants of low molecular masses during cold adaptation of small animals such as insects can be quantified by the techniques of chemical derivatisation and gas chromatography (GC) and this strategy for gauging profiles of individual organisms may serve as an exemplary contribution to metabolomics. Moreover, the strategy can be applied to a wider evaluation of metabolic perturbations arising from exogenous or endogenous stresses within populations of insects and other arthropods because additional metabolites of low molecular masses (e.g., other polyhydric alcohols) can be accommodated within the chromatographic observation windows. GC/MS would be required for quantification in those cases where improved selectivity and sensitivity are necessary, particularly where the dry weight of the individual is significantly less than 1 mg.

A future area of research suggested by this study would be to use the techniques described above to evaluate biochemical changes in laboratory insects subjected to differing stresses. If the individual insects are of sufficient weight, the various glycerides and phospholipids in their ethanol extracts could be partitioned on the basis of polarity by solid-phase extraction before transesterification (Overgaard et al., 2005, 2006), assuming that appropriate internal standards were incorporated to allow quantification of the chromatographic fractions. Furthermore, the progenitor molecules containing the fatty acid ligands could be analysed qualitatively by liquid chromatography/mass spectrometry (Storey and Storey, 1988, 1991; Slachta et al., 2002; Kostal et al., 2003; Tomcala et al., 2006); or the regulation of the genes or enzymes responsible could be monitored (Pfister and Storey, 2006; Kayukawa et al., 2007).

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