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ScienceDirect
Journal of Insect Physiology 54 (2008) 645–655
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*

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Received 15 June 2007; received in revised form 10 January 2008; accepted 16 January 2008

Abstract

The midge, *Belgica antarctica* Jacobs, is subjected to numerous environmental stressors during its 2-year life cycle on the Antarctic Peninsula, and in response it has evolved a suite of behavioral, physiological, and life-cycle modifications to counter these stressors, but thus far only a limited number of biochemical adaptations have been identified. In this study, we use a metabolomics approach to obtain a broad overview of changes in energy metabolism, amino acids, and polyols in response to three of the midge's major stresses: heat, freezing, and desiccation. Using GC-MS analysis, a total of 75 compounds were identified. Desiccation (50% water loss) elicited the greatest physiological response (as determined by principal components analysis) when compared to untreated controls, with many elevated metabolites from pathways of central carbohydrate metabolism and a decrease in free amino acids. When larvae were frozen (6 h at −10 °C), alanine and aspartate increased as well as urea. Freezing also increased three polyols (glycerol, mannitol, erythritol), while desiccation increased only two polyols (glycerol, erythritol). Heating the midges for 1 h at 30 °C elevated α-ketoglutarate and putrescine while suppressing glycerol, glucose, and serine levels. Freezing and desiccation elicited elevation of four shared metabolites, whereas no shared metabolites were elevated by heat. All three treatments resulted in a reduction in serine, potentially identifying this amino acid as a marker for stress in this species. A number of metabolic changes, especially those in the sugar and polyol pools, are adaptations that have potential to enhance survival during both cold and desiccation.

Keywords: Cold; Dehydration; Metabolism; Polar insect; Chironomidae

1. Introduction

The Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae), is the most southerly distributed insect and is found throughout the Antarctic Peninsula and its offshore islands (Sugg et al., 1983). This remarkable insect is active during the austral summer among pockets of primitive vegetation growing in nutrient-rich substrate near seal wallows and penguin rookeries (Usher and Edwards, 1984). During the 2-year life cycle, larvae overwinter as larvae (all four instars represented) in the frozen substrate (Sugg et al., 1983). Pupation occurs in early summer, and then the adults quickly eclose, mate, lay eggs, and die within a 2-week period.

Though air temperatures on the Antarctic Peninsula routinely drop below −15 °C, the temperature in the midge's hibernaculum, beneath the cover of ice and snow, remains fairly stable, in the range of 0 to −7 °C (Baust and Lee, 1981). Desiccation is an additional abiotic stress during the winter because all of the available water is tied up as biologically inactive ice, and during the summer high winds and periods of drought generate a highly desiccative environment. Furthermore, larvae are routinely exposed to heat stress during the summer; solar radiation generates substrate temperatures that far exceed the prevailing air

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0022-1910/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2008.01.003

temperatures (>20 °C). The Antarctic midge, not surprisingly, is quite intolerant of high temperature; larvae die within a week at 10 °C (Sugg et al., 1983; Baust and Lee, 1987; Lee et al., 2006) and survive only a few hours at 30 °C (Rinehart et al., 2006).

A few studies of *B. antarctica* have elucidated some of the physiological mechanisms involved in stress tolerance. In response to low temperature, this species accumulates three cryoprotective compounds: erythritol, glucose, and trehalose, in a manner dependent on temperature and geographic location (Baust and Edwards, 1979, Baust and Lee, 1983). Since this insect is freeze-tolerant, it is assumed that these three cryoprotective substances contribute to low-temperature survival by colligatively decreasing the amount of ice that forms internally (Baust and Edwards, 1979) and by hydrogen-bonding to proteins and membranes, rendering them less vulnerable to denaturation. In addition to these known cryoprotectants, larvae of *B. antarctica* constitutively produce a suite of heat-shock proteins (Hsps) known to enhance both high- and low-temperature tolerance by preventing or repairing thermal damage to proteins (Rinehart et al., 2006). This combination of low molecular weight cryoprotectants and constitutive Hsp expression presumably enables *B. antarctica* to survive the rapid temperature fluctuations that characterize the fringes of the austral summer and to tolerate freezing and/or cryoprotective dehydration during the long polar winter.

In contrast to what is known about low-temperature stress in the Antarctic midge, there is little information available on the physiological mechanisms used by this species to survive heat and desiccation. Even though desiccation tolerance is quite pronounced in this species (it can survive 70% water loss, Hayward et al., 2005), few of the physiological adjustments that accompany such a loss in body water have been identified. Using enzymatic assays, we previously determined that glycerol and trehalose accumulate in *B. antarctica* in response to desiccation, and a GC–MS analysis of surface hydrocarbons from desiccated larvae indicated that the hydrocarbon pool on the cuticle of this organism shifts to longer-chain hydrocarbons as it dehydrates (Benoit et al., 2007).

Metabolomics, an emerging field concerned with the study of an organism's physiological state at the substrate level, offers a means to obtain a comprehensive view of the changes in abundance of numerous compounds simultaneously (Dunn and Ellis, 2005; Weckworth and Morganthal, 2005). Sampling is accomplished by extracting small molecules, called metabolites, with general solvents and derivatizing them to improve chromatographic behavior. Chromatographic analysis is accomplished either though gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry or by nuclear magnetic resonance (Dunn and Ellis, 2005). Lastly, data analysis for metabolomics is accomplished through normal peak-by-peak analysis between treatments, followed by a multivariate principal components analysis (PCA) to determine whether the treatments did indeed produce different physiological states and to determine which metabolites contribute most to that change in state (Weckworth and Morganthal, 2005). The disadvantages of metabolomics are the semi-quantitative nature of the data and the limitations of chromatographic peak identification, but advantages lie in the unbiased nature of the data gathering and statistics, the ability to easily compare these data with other omics data, and the vast amount of data and hypotheses generated from this avenue of research (Kopka, 2006). Even though metabolic flux through biochemical pathways cannot be confidently deduced from metabolomic data, a number of testable hypotheses concerning these pathways may be generated from metabolomics data, rendering this technique an invaluable tool for hypothesis generation. Metabolomics has been used for physiological studies of plants (Weckworth et al., 2004), clinical screening of humans (Zytkovicz et al., 2001), and the detailed study of metabolism in bacteria (Buchholz et al., 2002), but thus far the technique has not been applied extensively to insect studies (c.f. Malmendal et al., 2006; Michaud and Denlinger, 2007). In this study we use this approach to monitor changes in *B. antarctica* elicited by heat shock, freezing, and desiccation.

2. Materials and methods

2.1. Insect collection and storage

Larvae of *B. antarctica* were collected in January 2006 from islands near Palmer Station, Antarctica (64°46′S, 64°43′W) and maintained in the laboratory at 4 °C and 100% relative humidity in their natural substrate (soil with some vegetation). In early February, the larvae were shipped to our research laboratory (The Ohio State University) where they were held for 2 weeks prior to experimentation. Larvae were of mixed stages, but the majority was fourth (final) larval instar and the others were third instars. Larvae maintained in this manner readily survived over one year in our home laboratory.

2.2. Stress exposure

All stress exposures (plus the untreated controls) were performed using groups of 25 larvae held in Eppendorf 2.0 ml microcentrifuge tubes (VWR Scientific, West Chester, PA). Tap water (500 μl) was added to each tube, with the exception of those used in the desiccation experiment. Untreated controls were homogenized at 4 °C, 1 h after removal from the substrate. For the heat-shock treatment, tubes containing the larvae were submerged for 1 h in a 30 °C bath (Brinkmann Instruments, Westbury, NY) containing 50% ethylene glycol, and the larvae were homogenized immediately thereafter. For the freezing treatment, tubes were placed for 6 h at −10 °C in a 50% ethylene glycol bath, and the larvae were homogenized after removal. For the desiccation treatment, larvae were

washed and placed for 6 d at 4 °C in a ventilated tube within a plastic desiccator containing an environment of 98.5% RH that resulted in a 50% loss of total body water (Benoit et al., 2007). Following desiccation, the larvae were immediately homogenized at 4 °C.

2.3. Separation and derivatization

One milliliter of dH_2O containing 25 μg heptadecanoic acid (internal standard not normally detected in polar fractions derived from *B. antarctica*) and 1 ml chloroform were added to each sample, and the sample was vortexed for 30 s. The resultant two-phase mixture was allowed to sit for 30 min and then vacuum-filtered through a glass filter to remove particulate matter and precipitates. The filter was washed with 1 ml chloroform:methanol:water (2:1:0.8) to remove soluble trace metabolites from the filter matrix. After filtration, the two-phase mixture was separated into an aqueous phase containing polar metabolites (sugars, polyols, amino acids) and an organic phase containing non-polar metabolites (lipids). The organic phase was used in a different experiment. The aqueous phase was placed under a gentle stream of nitrogen and evaporated to dryness at room temperature. The dried sample was immediately methoxymated by adding 1.0 ml methoxyamine hydrochloride in pyridine, incubated for 1 h at 60 °C (turns clear as metabolites dissolve), followed by a 16 h incubation at room temperature. The sample was then trimethylsilyated by the addition of 300 μl MSTFA (*N*-methyl-*N*-trifluoroacetamide) and incubated at 50 °C for 30 min. One hundred and fifty micro liters of the trimethylsilyated sample was added to a glass insert within an auto-sampler vial and analyzed with GC–MS. Aqueous samples processed in this manner produced distinctive and repeatable chromatographic peaks that differed across samples by 5–60%, within the expected range for this type of experiment.

2.4. Chromatographic analysis

One microliter of each sample was auto-injected into a Finnigan-Trace GC–MS and subjected to chromatographic analysis (50–450 m z^{-1}). The injector temperature was held at 280 °C. The oven temperature ranged from 50 to 300 °C, and the temperature increment was 5 °C min^{-1}, which produced a chromatograph of sufficient resolution to separate ~175 chromatographic peaks per sample. The column was a Restek 30 m fused silica column (I.D. 25 mm, 95% dimethyl siloxane, 5% diphenyl) with helium gas used as a carrier at a rate of 50 ml min^{-1}. Samples were run using the splitless mode. After each sample was run, the oven remained at 300 °C for 10 min to clean impurities from the column.

2.5. Quantification and data analysis

Individual integrated peak areas were converted to response ratios in relation to the internal standard (heptadecanoic acid) by dividing the peak area of the metabolite by the peak area of the internal standard. An analysis of covariance (ANCOVA) with an alpha level of $p \leqslant 0.05$ was performed on each metabolite to determine which individual metabolites were altered with respect to the controls. Type II error was corrected using a step-wise false discovery rate analysis (Q-value Software, plug-in for the R statistical software v2.6.1) to determine which values were truly significant (Benjamini and Hochberg, 1995; Storey, 2002). Peak identities were determined where possible by comparison of retention times with amino acid, sugar and polyol standards prepared in the same manner as the samples. Matches of spectral peaks with the National Institute of Standards and Technologies (NIST) and Wiley Chemical Structural Libraries (Excalibur software, Thermo Inc.) further confirmed or established metabolite identities, provided the match carried a reverse similarity index (RSI) value $\geqslant 700$ (on a scale from 1 to 999). For PCA, raw peak areas of all 141 metabolites were converted into response ratios and entered into MetaGeneAlyse 1.7 (Max Planck Institute of Molecular Plant Physiology, Berlin, Germany) and separated by their principal components. In addition to PCA, hierarchical clustering was performed using the same statistical software to determine overall relatedness between the physiological responses of *B. antarctica* to heat, freezing, and desiccation. The output of the cluster analysis was expressed in terms of Euclidean distance.

3. Results

3.1. General observations

Extracted and derivatized samples isolated from *B. antarctica* produced chromatographic peaks with fairly consistent retention times (within a few hundredths of a minute) and excellent resolution (92% of peaks were singular). The first half of each chromatogram contained mostly amino acids, small fatty acids, and small molecules (e.g. free phosphate), and the latter half of the chromatograph featured sugars, polyols, and longer fatty acids and hydrocarbons, although some overlap occurred (e.g. glycerol was found on the first half of the chromatograph). Metabolites from the glycolytic pathway and the Krebs cycle were found throughout the chromatographs. Each peak within a treatment group varied 5–60% in area when compared to peak area of the internal standard (heptadecanoic acid), indicating a moderate level of total variation for this experiment. The treatment that produced the most variation was the heat-shock treatment of 1 h at 30 °C.

A total of 75 metabolites out of 141 total peaks could be identified across all samples in this experiment; this is a high proportion of identified peaks for a metabolomics experiment (Schauer et al., 2005). Some (~30) of the peaks in the "unidentified" category had RSI values (>700) but could not be assigned a biological function based on the

available literature, thus these compounds did not receive much attention, but may be revisited as the database of spectral libraries increases. The 75 identified compounds included 14 amino acids, 13 sugars and polyols, 8 metabolic intermediates, 4 small molecules, 8 fatty acids and hydrocarbons, and 28 metabolites that did not fall into the above categories (Table 1).

3.2. Response to heat shock

The response ratios of a small number of metabolites from *B. antarctica* larvae were significantly altered (ANCOVA) by heat shock (30 °C for 1 h) relative to larvae continuously maintained at 4 °C (Fig. 1). Concentrations of two metabolites were elevated by heat shock (Fig. 1A),

Table 1
Metabolites identified from the Antarctic midge, *Belgica antarctica*

Amino acids	*Metabolic intermediates*	*Other metabolites*
β-Alanine	Phosphoglycerate	2-Butanoic acid
Asparagine	Acetic acid	2-Methyl propanoic acid
Glutamate	Cystathionine	2-Propenoic acid
Glutamine	Fumaric acid	4-Amino butanoic acid
Glycine	Isocitric acid	5-Amino pentanoic acid
Leucine	Malic acid	8-Hydroxysclerodin
N-glycyl alanine	Pyruvate	Citrulline
Ornithine	Succinic acid	Dihydrouracil
Phenylalanine	Urea	Galactouronic acid
Proline		δ-Amino levulinic acid
Serine	*Small molecules*	δ-Amino butyric acid (GABA)
Threonine	Free amine	Glucopyranoside
Tyrosine	Free phosphate	Glutaric acid
Valine	Phosphonic acid	Hydroxybutane
	Free sulphate	Isovaleric acid
Sugars and polyols		Lactone pentonic acid
3-Ketoglucose	*Fatty acids and hydrocarbons*	Metanephrine
5-Ketoglucose	Dodecane	Myo-inositol-1-phosphate
Erythritol	Hexanoic acid (6:0)	*M*-hydroxymandelic acid
Erythrose	Linoleic acid (18:2)	*N*-butylamine
Fructose	Nonanoic acid (9:0)	Nonadecanoic acid
Galactose	Octadecanoic acid (18:0)	Norvaline
Glucose	Oleic acid (18:1)	Oxalic acid
Glucoson	Tetradecane	Putrescine
Glycerol	Tetradecanoic acid (14:0)	Prostaglandin F-2
Inositol		Trihydroxybutyric acid
Mannitol		Xylopyranose
Myo-inositol-1- phosphate		
Sorbitol		

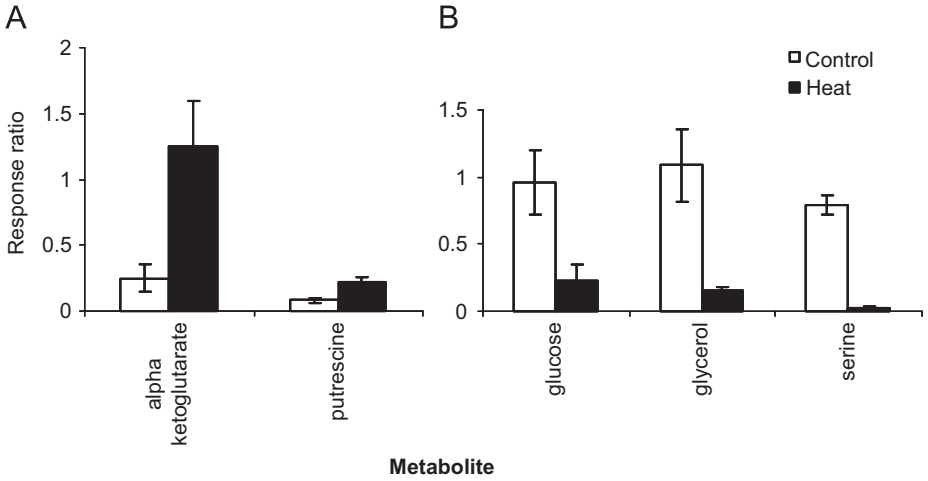

Fig. 1. Metabolites elevated (A) or reduced (B) in larvae of the Antarctic midge, *Belgica antarctica* by 1 h heat shock at 30 °C. GC–MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4 °C (white bars, untreated) were compared with peaks observed following heat shock (black bars). Response ratios are the ratio of the metabolite peak area to the peak area of an internal standard. Two metabolites accumulated in response to heat shock while 3 other metabolites were reduced in abundance. All bars are means ± SD of five determinations. Substances listed here were significantly different (ANCOVA with False Discovery Rate correction, df = 1.8).

and three were reduced (Fig. 1B). Serine ($p = 0.001$, $F_{1,8} = 33.39$) was reduced to 4% of its untreated level in response to heat shock, and the amino acid derivative, putrescine ($p = 0.01$, $F_{1,8} = 12.18$) was elevated by 75% when compared to untreated controls. Two known cryoprotectants, glucose ($p = 0.002$, $F_{1,8} = 21.17$) and glycerol ($p = 0.002$, $F_{1,8} = 22.35$), dramatically decreased to 30% or less of the control levels in response to heat shock. The intermediate of cellular respiration, α-ketoglutarate ($p = 0.003$, $F_{1,8} = 20.32$) increased in response to heat shock. A total of 5 "unknown" metabolites also were altered in heat-shocked larvae: 2 of these were elevated and 3 were reduced (data not shown).

3.3. Response to freezing

A comparison between the response ratios of chromatographic peaks from midges that had been frozen for 6 h at −10 °C and midges held continuously at 4 °C (ANCOVA) revealed a total of 10 metabolites that changed in concentration in response to freezing. Eight of these metabolites increased (Fig. 2A) and two decreased (Fig. 2B). Two constituents of the amino acid pool (alanine [$p = 0.000$, $F_{1,8} = 40.09$], aspartate [$p = 0.015$, $F_{1,8} = 10.36$]) were elevated 2–3 fold by freezing, while glycine ($p = 0.000$, $F_{1,8} = 106.03$) and serine ($p = 0.000$, $F_{1,8} = 51.17$) decreased to less than 50% of the concentrations found in untreated larvae. Among the cryoprotective polyols and sugars, there was a 2–5 fold elevation of three polyols: erythritol ($p = 0.006$, $F_{1,8} = -14.94$), glycerol ($p = 0.000$, $F_{1,8} = 136.15$) and mannitol ($p = 0.000$, $F_{1,8} = 61.56$). The volatile fatty acid, nonanoic acid (9:0, $p = 0.001$, $F_{1,8} = 26.28$), increased three-fold in response to freezing. The Krebs cycle intermediate, succinate ($p = 0.001$, $F_{1,8} = 27.18$), doubled in peak intensity, and the urea pool tripled ($p = 0.004$, $F_{1,8} = 17.34$). An additional 11 "unknown" metabolites changed in response to this treatment: 7 increased and 4 decreased (data not shown).

3.4. Response to desiccation

ANCOVA analysis applied to the response ratios of peaks isolated from midges held at 98.5% RH for 6 d (50%

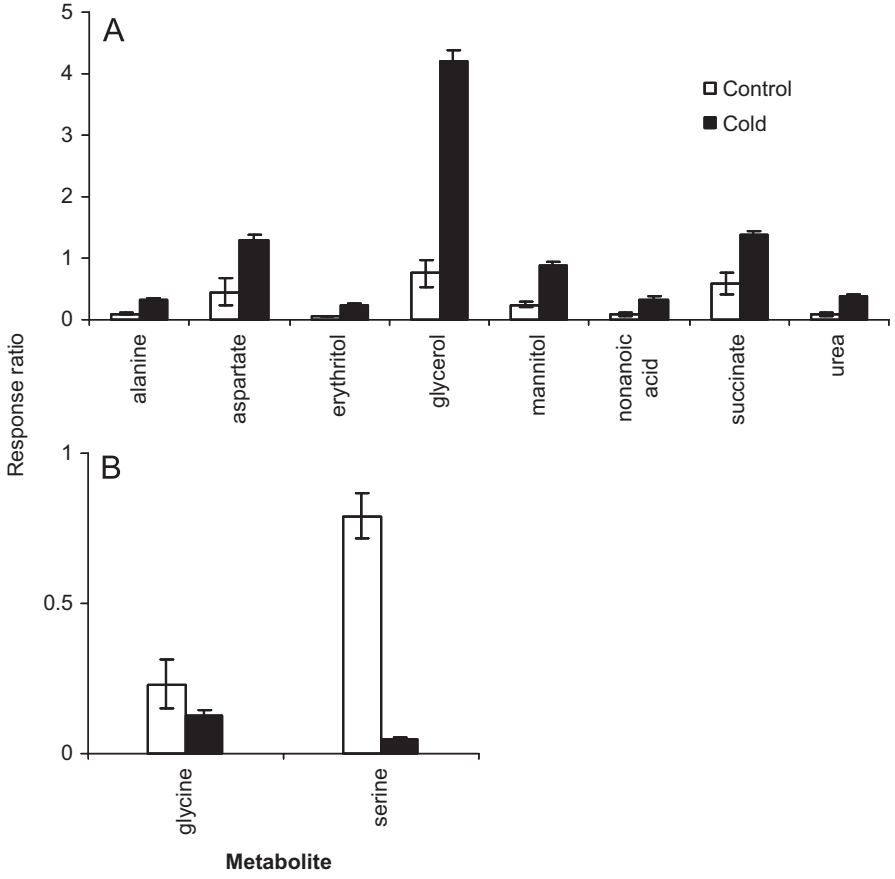

Fig. 2. Metabolites elevated (A) or reduced (B) in larvae of the Antarctic midge, *Belgica antarctica* by a 6 h freezing at −10 °C. GC–MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4 °C (white bars, untreated) were compared with peaks observed following freezing (black bars). Response ratios are the ratio of the metabolite peak area to the peak area of an internal standard. Eight metabolites accumulated in response to freezing, including 2 amino acids, 3 polyols, 1 Krebs cycle intermediates, and 2 other metabolites. Two metabolites decreased. Substances listed here were significantly different from untreated controls (ANCOVA with False Discovery Rate correction, df = 1.8). All bars are means ± SD of five determinations; some error bars are too small to be seen.

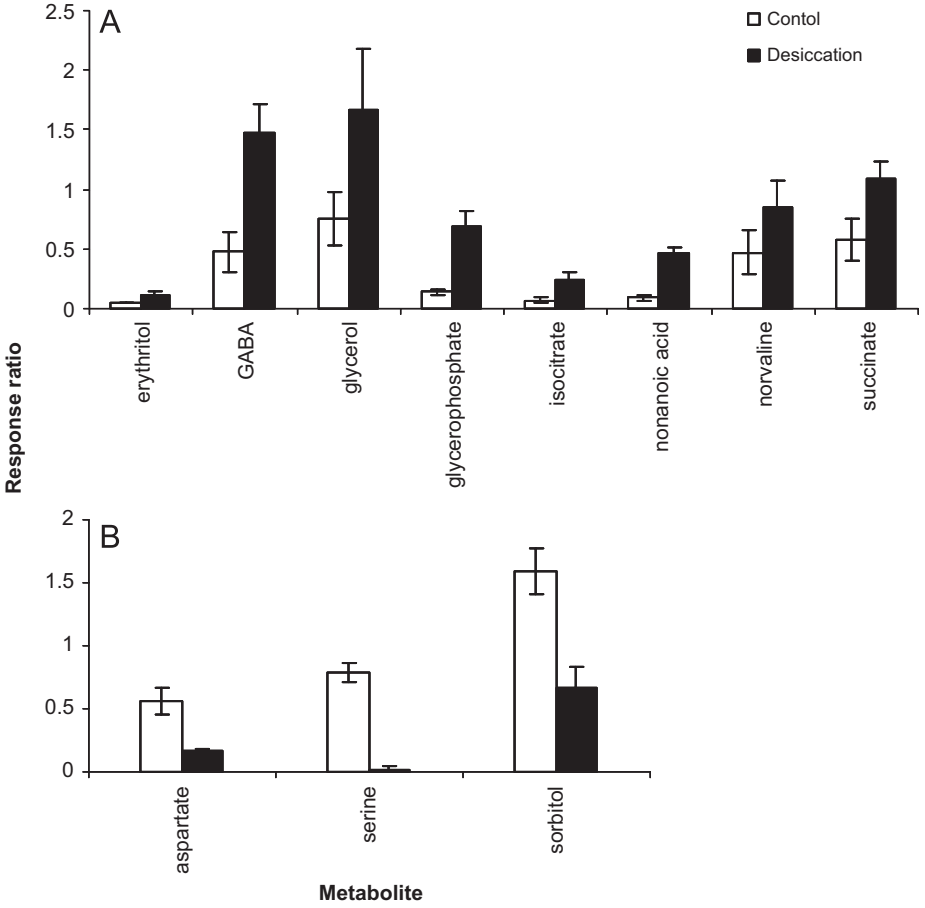

Fig. 3. Metabolites elevated (A) or reduced (B) by desiccation (5 d at 98% RH, resulting in a 50% water loss). GC–MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4 °C (white bars, untreated) were compared with peaks from larvae held for 5 days at 98% RH (black bars). Response ratios are the ratio of the metabolite peak area to the peak area of an internal standard. Eight metabolites increased in abundance, including 2 amino acid derivatives, 2 polyols, 3 intermediates of central metabolism, and 1 other metabolite. Three metabolites decreased in abundance. Substances listed here were significantly different from untreated controls (ANCOVA with False Discovery Rate correction, df = 1.8). All bars are means ± SD of five determinations; some error bars are too small to be seen.

loss of body water) and midges held continuously at 4 °C, 100% RH, revealed a total of 11 metabolite peak alterations. Eight metabolites were elevated (Fig. 3A), and three were reduced (Fig. 3B). Within the amino acid pool, two amino acids (aspartate [$p = 0.003$, $F_{1,8} = 20.84$], serine [$p = 0.000$, $F_{1,8} = 85.77$]) declined. Gamma amino-isobutyric acid (GABA, $p = 0.001$, $F_{1,8} = 25.43$) was elevated more than two-fold. Among the sugars and polyols, glycerol ($p = 0.004$, $F_{1,8} = 17.44$) and erythritol ($p = 0.000$, $F_{1,8} = 61.57$) levels doubled in response to desiccation, and sorbitol ($p = 0.001$, $F_{1,8} = 27.12$) decreased to slightly less than half the levels seen in fully hydrated midges. Nonanoic acid (9:0, $p = 0.001$, $F_{1,8} = 33.33$) increased two-fold. Among intermediates of cellular respiration, desiccation resulted in the accumulation of 3-phosphoglycerate ($p = 0.022$, $F_{1,8} = 8.59$), isocitrate ($p = 0.008$, $F_{1,8} = 13.13$), and succinate ($p = 0.004$, $F_{1,8} = 17.22$), all of which were elevated two- to three fold. A total of 12 ‘‘unknown’’ metabolic peaks were altered in intensity in response to desiccation. Of these, 8 were elevated and 4 were reduced.

3.5. Principal components analysis and clustering

Multivariate PCA statistics of normalized peak areas from all samples in this metabolomic experiment gives a measure of the degree of separation of each treatment group (or metabolic state) based on the contribution of sets of metabolites, or principal components, to the overall variance within the data set. The metabolites comprising the first principal component, PC1, containing sorbitol, glycerol, serine and GABA, accounted for 38.1% of the total variation in this experiment. Metabolites that comprised the second principal component PC2 (31.7% of total variation) included GABA, serine, malate, and glycerol.

Plotting the principal components of each sample within a treatment group yields a cluster of points that can be circumscribed spatially, and the resultant area can be compared with areas generated by other treatment groups to determine if these treatments are physiologically distinct from one another (if they do not overlap). For this experiment, none of the areas generated by the four

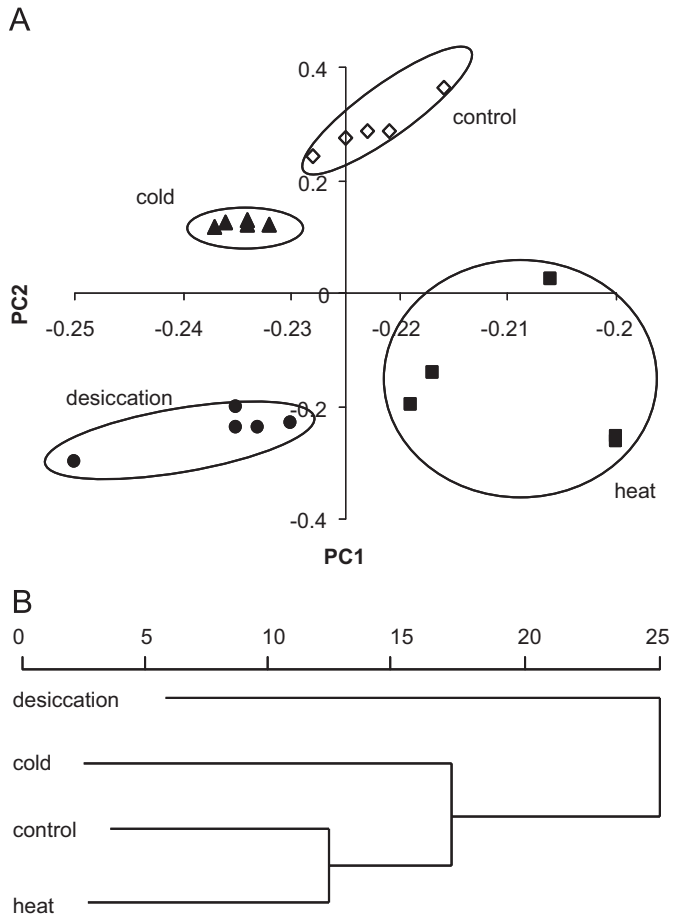

Fig. 4. Multivariate analysis of metabolomic data derived from larvae of the Antarctic midge *Belgica antarctica* subjected to heat shock, freezing and desiccation. Panel A illustrates that plotting the first two principal components of all samples of the stressed midges results in distinct treatment-dependent clustering along both principal components (MetGenAlyse v. 1.7). Hierarchical analysis (Panel B) demonstrates that the responses of the midge to heat (30 °C for 1 h) is most similar to that of the untreated control (held continuously in tap water at 4 °C) and that desiccation (50% loss of body water) produces a physiological response that is most distant from the untreated control. Numbers on the top bar indicate Euclidean distance. Freezing the midge for 6 h at −10 °C produced an effect that was an intermediate between the untreated control and desiccation.

treatment clusters (untreated, heat-shock, freezing, desiccation) overlapped (Fig. 4A). Variation along PC1 accounted for the separation of the heat treatments and the untreated controls from the cold and desiccation treatments, and variation along PC2 accounted for the separation of the heat and desiccation treatments from the untreated controls and the cold treatments. Two of the five samples produced by heat shock (1 h at 30 °C) deviated considerably from the bulk of their cluster, but this variation did not preclude physiological distinctiveness for this stressor. Deviation in these two samples is likely due to the inability of *B. antarctica* to survive a severe heat shock of 30 °C (Rinehart et al., 2006). Although the heat shock used in the current experiment does not immediately kill most larvae, the presence of some moribund individuals may have caused the large variation in metabolite concentrations observed for this treatment group.

Hierarchical clustering based on response ratios of peak areas across all samples (Fig. 4B) demonstrated that the metabolic response of *B. antarctica* to heat was most similar to the untreated control (one heat-shocked sample clustered with the untreated controls). The midges responded to desiccation in a manner that is considered to be an out-group with respect to the untreated control and the responses to freezing and heat, indicating that the greatest physiological adjustment for *B. antarctica* is in response to the loss of body water, followed by the response to freezing.

4. Discussion

4.1. Metabolic response to heat shock

B. antarctica constitutively expresses a suite of HSPs throughout its larval life (Rinehart et al., 2006). The heat-shock regime used in our current study (1 h at 30 °C) does not affect HSP expression nor is it lethal, but a 2 h exposure to this temperature shuts down heat-shock protein transcript expression, and a 3 h exposure to this temperature kills >50% of the larvae (Rinehart et al., 2006). Although we anticipated that our 1 h heat shock at 30 °C would be relatively mild, the varied response we observed in this metabolomic experiment suggests that this treatment may have ultimately been lethal to a significant portion of the larvae in two samples.

In response to heat shock, *B. antarctica* increased concentrations of α-ketoglutarate more than 4-fold. α-Ketoglutarate serves as both a Krebs cycle intermediate and a precursor to the amino acid biosynthesis of glutamine and glutamate. In addition, adding α-ketoglutarate to glucose-starved mouse cells leads to increased thermotolerance (Gomes et al., 1985). The use of whole-body samples for a metabolomics experiment requires that caution be exercised in designating a function to any upregulated or downregulated metabolite, but an increase in α-ketoglutarate does suggest that the Krebs cycle is perturbed by heat stress in the Antarctic midge.

Another notable metabolite elevated by heat shock in *B. antarctica* was a threefold increase in putrescine, a polyamine derived from the amino acid arginine. An elevation of putrescine due to stress is also seen in plants (Renaut et al., 2005), earthworms, nematodes, leeches, and planatrians (Hamana et al., 1995). Putrescine is used as a marker for stress in plants (Renaut et al., 2005) because stressed plants activate an enzyme, arginine decarboxylase, leading to putrescine conversion from arginine (Galston and Sawhney, 1990; Borrell et al., 1996). In animals, the conversion of arginine to putrescine is initially accomplished through the action of arginase. Arginase shows activation plasticity in response to stress for humans (Lange et al., 2004) and crabs (Reddy and Bhagyalakshmi, 1994). The increase in putrescine seen in this experiment,

however, was not coupled with a reduction in arginine, but such change is not necessary if the throughput for this pathway is high. Putrescine also can regulate the response of HSPs (Basra et al., 1997; Koenigshofer and Lechner, 2002) and change the topology of DNA in a manner that promotes survival (Tkachenko et al., 1998). Further investigation is necessary to determine which, if any, of these roles for putrescine are involved in the response to heat shock.

B. antarctica exhibited an overall pattern of moderate metabolite reduction in response to heat shock. We observed a reduction in serine, glycerol, and one sugar (glucose). Serine is synthesized from a pyruvate or phosphoglycerate precursor in glycolysis, indicating that the activity of one or both of these pathways is affected by heat shock. The reduction of glycerol levels points to temperature-dependent inhibition of the aldose reductase enzyme. Glucose reduction seen in this study could be driving all of these glycolysis-related changes in amino acids and polyols by reducing the abundance of substrates available for glycolysis, thus reducing the rate of all downstream processes. Conversely, the reduction of all of these compounds could be the result of rapid utilization of energy caused by a temperature-dependent increase in metabolism. More detailed study is needed to delineate which of these processes are responsible for the observed metabolite decreases due to heat shock.

4.2. Metabolic response to freezing

Seasonal studies tracking whole-body concentrations of cryoprotectants in *B. antarctica* previously reported that glycerol, erythritol, sorbitol, glucose, and trehalose concentrations are higher in the spring and fall than in the summer (Baust and Edwards, 1979; Lee and Baust, 1981). These concentrations are thought to be reliant upon diet and temperature, but not photoperiod (Baust and Edwards, 1979; Baust and Lee, 1983).

In the present study, metabolomics identified a number of known and previously unknown metabolic changes in response to short-term freezing at −10 °C. Confirming previous results, erythritol and glycerol increased in response to freezing. Another polyol previously found in *B. antarctica*, mannitol (Block, 1982), increased in response to freezing, but this is the first time that this polyol has been found to change in response to a stressor in this species. Overall, three polyols increased. The multi-polyol system seen in the Antarctic midge appears to have complex control in a manner similar to the spruce bark beetle (Koštál et al., 2007). All of the polyol increases in this experiment likely contribute to cold survival by decreasing the amount of ice that forms in the larvae. In addition, polyols can contribute non-colligatively to survival by hydrogen-binding to proteins to prevent low-temperature denaturation (Tang and Pikal, 2005) and by prevention of membrane damage (Tsvetkova and Quinn, 1994). Glucose and trehalose did not change in response to our freezing regime, although both were previously reported to change in response to seasonal acclimation in *B. antarctica* (Lee and Baust, 1981; Baust and Lee, 1983).

The free amino acid pool was considerably altered by freezing. Alanine and aspartate increased while glycine and serine levels decreased. Glycine and serine are linked in the same biosynthetic pathway, thus a decrease of glycine coupled with a marked reduction in serine indicates that one or more of the enzymes involved in serine biosynthesis may be inhibited by freezing or one of the pathways using these amino acids as a substrate is activated. Serine levels also were reduced in overwintering larvae of *Ostrinia furnacalis* (Goto et al., 2001).

Another free amino acid, alanine, increased due to freezing. Alanine upregulation has been correlated with a number of insect cold-hardy states (e.g. Kukal et al., 1991; Fields et al., 1998; Rivers et al., 2000; Goto et al., 2001). This amino acid also protects proteins from cold inactivation (Carpenter and Crowe. 1988) and may contribute to cold survival by providing a less toxic end-product than lactic acid in an environment where the Kreb's cycle is slowed due to cold inactivation (as indicated by the accumulation of succinate in this experiment). Studies in plants suggest that a shift to alanine synthesis from pyruvate is preferable to the neurotoxic alternative, production of lactic acid (Touchette and Burkholder, 2000; Xiang and Bergold, 2000). Indeed, lactic acid did not accumulate due to freezing in this study, indicating that lactic acidosis is not a component of the winter biology of *B. antarctica*. Aspartate is the final amino acid upregulated due to freezing in the Antarctic midge, although the function of this metabolite in freezing stress is unclear. It may be that this increase is an adaptive response to freezing, but also, this increase could be a secondary response to a disruption in glycolysis, as evidenced by the accumulation of glycerol and alanine. Aspartate also increased due to cold acclimation in the rusty grain beetle, *Cryptolestes ferrugineus* (Fields et al., 1998).

Since GC–MS metabolomics also determined that urea levels increased due to freezing in the midge, an overall nitrogen cycle perturbation is likely. Perturbation of the nitrogen cycle was also observed when the flesh fly, *Sarcophaga crassipalpis*, was subjected to low temperatures for the induction of rapid cold-hardening (Michaud and Denlinger, 2007). However, urea apparently also functions as an osmoprotectant and cryoprotectant in freeze-tolerant ectotherms (Costanzo and Lee 2005).

Subjecting larvae of *B. antarctica* to freezing temperatures also caused succinic acid, a Krebs cycle intermediate, to accumulate. Elevation of this compound suggests that the enzyme immediately downstream (succinate dehydrogenase) has been cold inactivated, a condition similar to that seen in anoxic goldfish (Van Den Thillart and Smit, 1984; Lahnsteiner et al., 1996). Previous research showed that *B. antarctica* does not have an aerobic compensatory mechanism for low temperatures (Lee and Baust, 1982), as

seen in some other species (Sommer and Poertner, 2002; Siddiqui and Cavicchioli, 2006), therefore a loss of Krebs cycle activity is not surprising. Under these physiological circumstances, the frozen midge either has greatly reduced cellular energy requirements or is deriving its energy from a source other than aerobic respiration, most likely through glycolysis. It is assumed that the energy demands of the Antarctic midge are low during winter because *B. antarctica* does not appreciably develop during this period (Sugg et al., 1983), therefore energy derived from glycolysis may be all that is needed for survival.

Freezing the Antarctic midge caused nonanoic acid to increase. This volatile short chain fatty acid has phytotoxic (Lederer et al., 2004), nematocidal (Davis et al., 1997), and fungicidal (Aneja et al., 2005) activity. Upregulation of this compound in response to freezing may allow the Antarctic midge to repel infection while in a non-motile, frozen state. Although the midge is not known to have vertebrate predators, it is interesting to note that nonanoic acid can also function as a mosquito repellant (Schultz et al., 1983).

4.3. Metabolic response to desiccation

The loss of 50% of the total body water in larvae changed the chromatographic profiles of a number of metabolites, but many of these changes were seen in the freezing treatments, as well (Fig. 5). Like freezing, desiccation caused the accumulation of nonanoic acid, possibly for the same defensive purposes discussed above. Accumulation of free fatty acids is correlated with desiccation survival in *Aedes egyptii* (Sawabe and Mogi, 1999) but also may be a marker for cells that have endured membrane stress (Hoekstra et al., 2001).

The amino acid pool of *B. antarctica* changed in response to a 50% loss of body water, but most of these changes were decreases. Serine and aspartate decreased in response to desiccation while the amino acid derivative, norvaline, increased. All of these amino acids are constituents of biosynthetic pathways that off-shoot from glycolysis and the Krebs cycle, thus these alterations are likely a consequence of perturbation of central cellular respiration. Glycine and serine are synthesized in a complex enzymatic reaction that branches from the glycolytic intermediate, 3-phosphoglycerate. Phosphoglycerate, an intermediate of glycolysis, was elevated threefold in response to desiccation. This molecule may accumulate due to a reduction in the activity of a downstream glycolytic enzyme (e.g. phosphoglycerate mutase, enolase) or an increase in activity of an upstream enzyme. As determined in this study, desiccation increased phosphoglycerate concentration in *B. antarctica*, therefore one or more of the enzymes in the pathway of serine biosynthesis may be inhibited during desiccation, as well. To our knowledge, a reduction in serine has not been correlated previously with cellular survival to desiccation.

Unlike the multiple polyol upregulation seen in response to freezing, the only two detectable polyols that increased

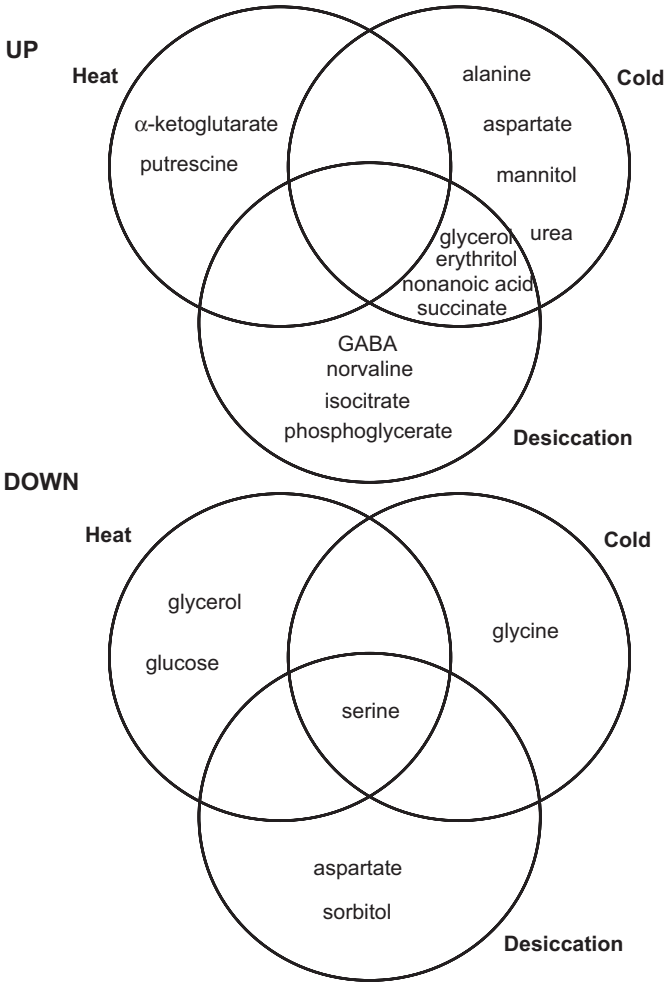

Fig. 5. Venn diagrams illustrating the overlap of elevated (above) and reduced (below) metabolites in response to three abiotic stressors in the Antarctic midge, *Belgica antarctica*.

due to desiccation in *B. antarctica* were glycerol and erythritol. Glycerol protects membranes and proteins during desiccation in the same manner as during low temperature stress (see the previous section). This important polyol protects plant cells against desiccation (Eastmond, 2004) and accumulates in response to desiccation in the flesh fly, *Sarcophaga crassipalpis* (Yoder et al., 2005). An increase in glycerol content due to desiccation in *B. antarctica* also has now been confirmed using an enzymatic assay (Benoit et al., 2007). Erythritol accumulation due to desiccation has not been previously reported, but its function in the Antarctic midge is assumed to be similar to glycerol. Sorbitol, another polyol, decreased in *B. antarctica* at the same amplitude as the increase of glycerol, thus suggesting that glycerol may replace sorbitol in this species during desiccation.

GABA was elevated in the Antarctic midge in response to desiccation. Even though GABA serves as a neurotransmitter in insects, it is unlikely that its osmotic stress upregulation is involved in neuronal functioning since the organism is non-motile. This non-protein amino acid may be linked to improved desiccation tolerance as seen in

plants, especially if such an upregulation is coupled with a proline increase (Bartyzel et al., 2003). For the Antarctic midge, desiccation did not increase proline levels, but there was considerable shifting in the amino acid pool (2 amino acids reduced and 2 amino acid derivatives increased).

Desiccation, like freezing, elicited accumulation of isocitric and succinic acid, indicating a general inhibition of aerobic metabolism. But, the fact that the 50% water loss used in this experiment does cause some mortality in the midge larvae makes it difficult to be certain whether inhibition of aerobic metabolism is an adaptive response or is indicative of a damaged physiological state. Other than the accumulation of phosphoglycerate, no changes in glycolytic intermediates were detected. The increase in glycerol may indicate that the gluconeogenic pathway remains active during desiccation, but other sources of glycerol are possible.

4.4. Summary

GC–MS based metabolomics successfully identified a number of polyols, sugars, amino acids, and intermediates of cellular metabolism that were altered in response to heat shock, freezing, and desiccation in larvae of the Antarctic midge, *B. antarctica*. A Venn diagram summarizing all metabolic changes (Fig. 5) shows that desiccation and freezing share the greatest number of elevated metabolites, but desiccation shares an equal amount of downregulated metabolites with heat treatment. PCA and hierarchical clustering determined that heat shock produces a result that is most similar to the untreated control. The greatest deviation from the untreated control occurred in desiccation, with freezing providing an intermediate overall response.

Heat shock induced the reduction of two cryoprotectants (glycerol and glucose) and a possible inhibition of the Krebs cycle at the level of α-ketoglutarate. Freezing induced the production of multiple polyols (glycerol, erythritol, mannitol), a possible inhibition of the Krebs cycle (succinate accumulation), and a perturbation of the amino acid pool (increased aspartate, alanine and decreased glycine and serine). Frozen larvae also display evidence of shifting nitrate equivalents (urea). Desiccating the larvae altered the activity of both the Krebs cycle (isocitrate, succinate accumulation) and glycolysis (phosphoglycerate and glycerol accumulation). All three stresses caused serine to decrease, making this metabolite an excellent marker for stress in the Antarctic midge. There appear to be no other generalized responses to abiotic stress in the Antarctic midge. Instead, most metabolic responses of the midge are highly controlled and tailored to each specific stress.

Acknowledgments

We are grateful to the staff at Palmer Station for facilitating our work in Antarctica and to Richard Sessler of the Campus Chemical Instrument Center at The Ohio State University for guidance in the analysis of samples. We also to thank the reviewers of this article for their thoughtful suggestions. This work was supported in part by Grants OPP-0337656 and OPP-0413786 from the Polar Program of the National Science Foundation.

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