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ORIGINAL PAPER

Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, *Belgica antarctica*

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Abstract The Antarctic midge, *Belgica antarctica*, experiences sub-zero temperatures and desiccating conditions for much of the year, and in response to these environmental insults, larvae undergo rapid shifts in metabolism, mobilizing carbohydrate energy reserves to promote synthesis of low-molecular-mass osmoprotectants. In this study, we measured the expression of 11 metabolic genes in response to thermal and dehydration stress. During both heat and cold stress, we observed upregulation of phosphoenolpyruvate carboxykinase (*pepck*) and glycogen phosphorylase (*gp*) to support rapid glucose mobilization. In contrast, there was a general downregulation of pathways related to polyol, trehalose, and proline synthesis during both high- and low-temperature stress. *Pepck* was likewise upregulated in response to different types of dehydration stress; however, for many of the other genes, expression patterns depended on the nature of dehydration stress. Following fast dehydration, expression patterns were similar to those observed during thermal stress, i.e., upregulation of *gp* accompanied by downregulation of trehalose and proline synthetic genes. In contrast, gradual, prolonged dehydration (both at a constant temperature and in conjunction with chilling) promoted marked upregulation of genes responsible for trehalose and proline synthesis. On the whole, our data agree with known metabolic adaptations to stress in *B. antarctica*, although a few discrepancies between gene expression patterns and downstream metabolite contents point to fluxes that are not controlled at the level of transcription.

Keywords Antarctic midge · Gluconeogenesis · Trehalose · Freeze tolerance · Dehydration stress

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Introduction

The ability to survive extremes in temperature and water availability is critical for insects living in polar environments, such as the Antarctic midge, *Belgica antarctica*, which inhabits offshore islands and ice-free areas along the Antarctic Peninsula (Sugg et al. 1983). While the microhabitat of *B. antarctica* is buffered from extreme variations in temperature (Baust and Lee 1981), *B. antarctica* is nonetheless exposed annually to sub-freezing temperatures for more than 8 months (Elnitsky et al. 2008). Additionally, because liquid water is unavailable much of the year (Kennedy 1993), larvae face a significant risk of dehydration. As such, larvae are extremely tolerant of both cold and dehydration; larvae can survive freezing to −20 °C (Lee et al. 2006) and can tolerate water losses of up to 70 % (Benoit et al. 2007).

Our recent studies have begun to elucidate the physiological and molecular mechanisms used by *B. antarctica* to tolerate environmental extremes. Larvae constitutively express both heat shock proteins (Rinehart et al. 2006) and antioxidant enzymes (Lopez-Martinez et al. 2008) in anticipation of adverse conditions. In response to

dehydration, larvae rapidly upregulate a number of genes, including genes encoding several chaperone proteins, membrane restructuring enzymes, and structural components of the cytoskeleton (Lopez-Martinez et al. 2009). Recent evidence also demonstrates the importance of aquaporins in mediating dehydration stress (Goto et al. 2011; Yi et al. 2011).

For animals exposed to adverse environmental conditions, rapid changes in metabolism are essential to maintain energy balance and protect structural components of the cell (e.g. lipids and proteins). The accumulation of low-molecular-mass cryoprotectants, such as glycerol and glucose, has been documented in numerous organisms, including both freeze-tolerant insects and frogs (Storey 1997). Trehalose has also been implicated as a key protective solute in a range of environmental stress responses, including low temperature (Lee 2011) and hypoxia (Chen and Haddad 2004), presumably by stabilizing cell membranes and proteins during periods of osmotic imbalance (Elbein et al. 2003). Trehalose appears to be particularly important during periods of extreme dehydration; for example, most invertebrates that are capable of anhydrobiosis use trehalose as the primary osmoprotectant (Clegg 2001). Finally, the amino acid proline has been identified as a potent cryoprotectant in insects. Proline is accumulated during cold acclimation (Kostal et al. 2011a), and diet supplementation with proline can substantially enhance the cold tolerance, and even confer freezing tolerance, in two species of drosophilid flies (Kostal et al. 2011b, 2012).

Metabolic adaptations also appear to be essential components of the stress response in *B. antarctica*. Using metabolomics, Michaud et al. (2008) identified several metabolites, including two osmoprotective polyols, glycerol and erythritol, that are responsive to cold and dehydration. During both freezing and dehydration, larvae significantly deplete glycogen reserves, presumably converting them to glucose and other osmoprotectants (Teets et al. 2011, 2012a). Indeed, glucose levels increase during both recovery from cold stress (Teets et al. 2011) and in response to cryoprotective dehydration (Elnitsky et al. 2008), although it is unknown whether glucose serves an osmoprotective role or simply functions as a precursor for other metabolites. In addition, several studies have described the importance of trehalose as an osmoprotectant in *B. antarctica*, particularly during dehydration stress. In response to dehydration, larvae rapidly accumulate trehalose (Benoit et al. 2007), which in turn seems to facilitate cross-tolerance between dehydration and cold tolerance (Benoit et al. 2009). Larvae also synthesize large amounts of trehalose during cryoprotective dehydration, an overwintering strategy by which larvae remain unfrozen by allowing body fluids to remain at vapor pressure equilibrium with surrounding ice (Elnitsky et al. 2008).

Despite mounting evidence that larvae of *B. antarctica* undergo significant metabolic adjustments in response to stress, the molecular mechanisms of these biochemical changes have not been investigated. In this study, we measured larval expression of 11 metabolic gene transcripts in response to both thermal and dehydration stress. For the thermal stress experiment, we exposed larvae to heat shock, as well as cold shock in both the frozen and supercooled states. It appears to be beneficial for larvae to be supercooled during periods of sub-zero temperature (Teets et al. 2011); thus we measured whether the type of cold exposure (i.e. freezing or supercooling) influences metabolic gene expression. In the dehydration experiments, we exposed larvae to three types of dehydration regimens: fast dehydration, gradual, slow dehydration, and cryoprotective dehydration. All three resulted in similar levels of water loss, thus enabling us to test whether there is a common molecular response to dehydration, or whether the severity and duration of dehydration treatment impacts gene expression.

Specifically, we isolated genes involved in (1) the synthesis of glucose via glycogenolysis and gluconeogenesis, (2) the synthesis and recycling of polyol cryoprotectants, (3) the synthesis, breakdown, and transport of trehalose, and (4) the synthesis of proline. The biochemical pathways for which we monitored gene expression are illustrated in Fig. 1. We report that expression profiles of metabolic genes are highly labile during periods of stress and that the magnitude and direction of expression changes are highly dependent on the type and severity of the stress experienced.

Materials and methods

Experimental animals

Larvae of *B. antarctica* were collected on various off-shore islands in the vicinity of Palmer Station (64°46′S, 64°04′W) on the Antarctic Peninsula in January and February 2011. Larvae were returned to the station and extracted from their natural substrate into ice water using a modified Berlese apparatus. Concentrated samples of larvae were returned to natural substrate and stored at 2 °C until used for experiments. This temperature is near the average air temperature at Palmer Station during January and February. Prior to beginning an experiment, larvae were sorted from substrate in ice water and held on moist filter paper overnight to standardize body water content. Only fourth instar larvae were used for experiments.

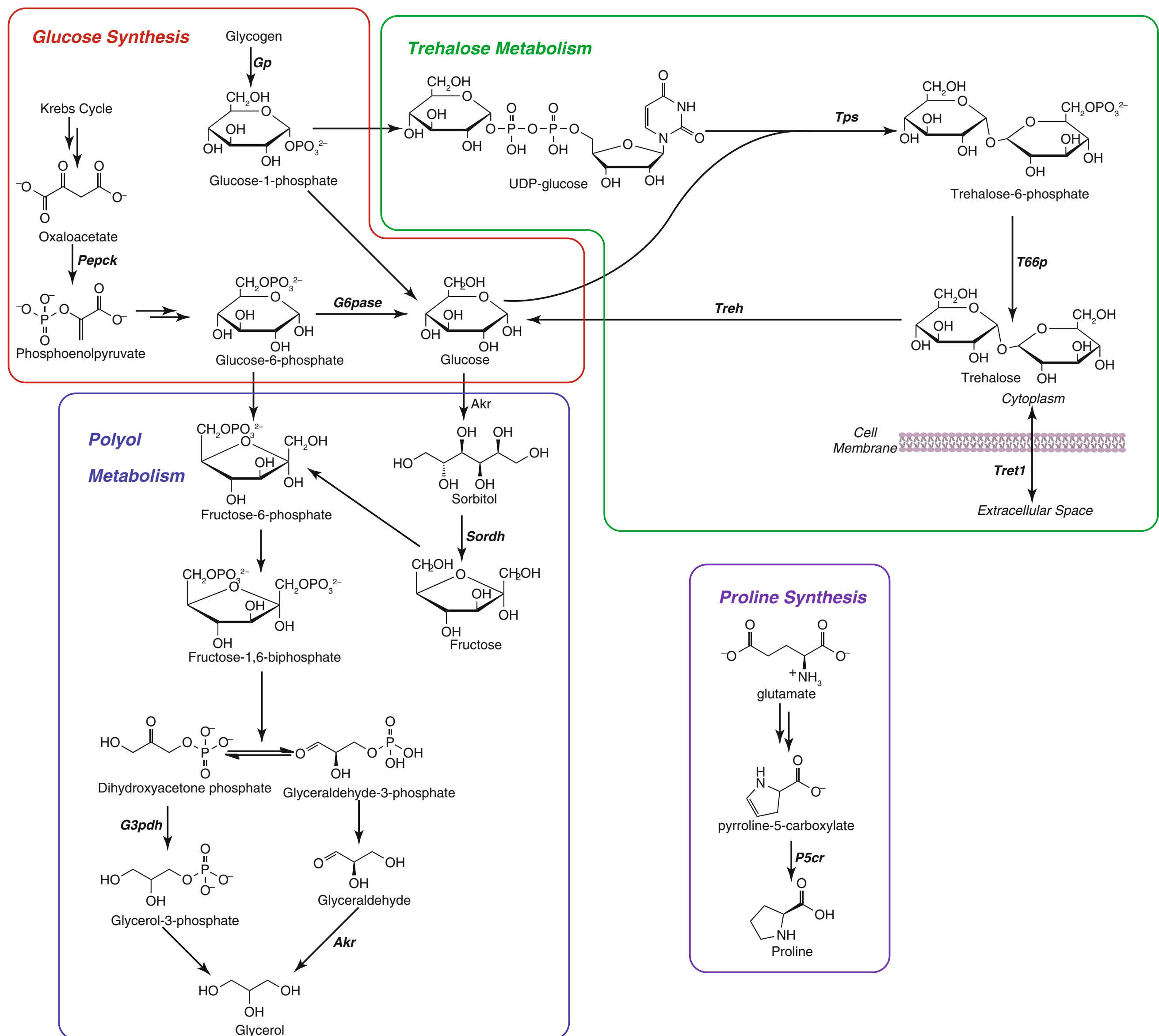

Fig. 1 Pathway diagram illustrating the biochemical reactions catalyzed by the genes examined in this study. For clarity, only the names of enzymes for which we measured expression are included in the diagram. *Consecutive arrows* indicate intermediate reactions that are not depicted in the diagram. *Akr* aldo–keto reductase, *G3pdh* glycerol-3-phosphate dehydrogenase, *G6pase* glucose-6-phosphatase, *GP* glycogen phosphorylase, *P5cr* pyrroline-5-carboxylate reductase, *Pepck* phosphoenolpyruvate carboxykinase, *Sordh* sorbitol dehydrogenase, *T66p* trehalose-6-phosphate phosphatase, *Tps* trehalose-6-phosphate synthase, *Treh* trehalase, *Tret1* trehalose transporter 1

Experimental conditions

For each treatment group described below, we collected 5 biological replicates, each replicate containing 20 larvae. The treatment conditions for the thermal stress experiments were as follows: Control (4 °C/24 h), heat shock (25 °C/24 h), supercooled (−5 °C/24 h in a dry centrifuge tube), supercooled with recovery (−5 °C/24 h in a dry centrifuge tube, followed by 12 h at 4 °C), frozen (−5 °C/24 h in a centrifuge tube with ~50 μl water), and frozen with recovery (−5 °C/24 h in a centrifuge tube with ~50 μl water, followed by 12 h at 4 °C). In the frozen with recovery treatment, water was removed immediately upon thawing.

For the dehydration experiments, treatments were as follows: Control (100 % RH/24 h at 4 °C), fast dehydration (75 % RH/24 h at 4 °C), fast dehydration + rehydration (75 % RH/24 h followed by 24 h rehydration on moist paper towel at 4 °C), slow dehydration (99 % RH/10 d at 4 °C), slow dehydration + rehydration (99 % RH/10 d followed by 24 h rehydration on moist paper towel at 4 °C), and cryoprotective dehydration (gradually chilled from −0.6 to

Table 1 Bioinformatics analysis of the 11 metabolic genes profiled in this study

Gene	GenBank accession #	Predicted function	Species with closest homology	*E* value	% positive	Pfam ID(s)
gp	JX462658	Glycogen phosphorylase	*Anopheles gambiae*	0	91	PF00343
pepck	JX462659	Phosphoenolpyruvate carboxykinase	*Aedes aegypti*	0	85	PF00821
g6pase	JX462660	Glucose-6-phosphatase	*Aedes aegypti*	1.00E−45	59	PF01569
akr	JX462661	Aldehyde/ketone reductase	*Anopheles gambiae*	1.00E−129	76	PF00248
sordh	JX462662	Sorbitol dehydrogenase	*Drosophila yakuba*	0	86	PF08240, PF00107
g3pdh	JX462663	Glycerol-3-phosphate dehydrogenase	*Aedes aegypti*	0	93	PF01210, PF07479
tps	JX462664	Trehalose-6-phosphate synthase	*Anopheles gambiae*	0	87	PF00982, PF02358
t6pp	JX462665	Trehalose-6-phosphate phosphatase	*Culex quinquefasciatus*	9.00E−93	72	PF02358
treh	JX462666	Trehalase	*Aedes aegypti*	0	76	PF01204
tret1	JX462667	Trehalose transporter	*Aedes aegypti*	0	92	PF00083
p5cr	JX462668	Pyrroline-5-carboxylate reductase	*Aedes aegypti*	2.00E−113	85	PF03807

Coding sequences isolated from a *B. antarctica* draft genome were blasted against the RefSeq protein database using the blastx search algorithm. The species with closest homology, based on the expected value (*E* value), is included in the table, along with the % positive for that species. The % positive is the percentage of BLAST-aligned amino acids that are either identical or have similar chemical properties. The final column includes the predicted Pfam domain(s) based on a search of the predicted *B. antarctica* protein sequence against the Pfam database. The Pfam database ID is included in the table; for a full description see http://pfam.sanger.ac.uk/

−3 °C at vapor pressure equilibrium with ice, then held at −3 °C for 10 days; see Elnitsky et al. (2008)). For these experiments, 75 and 99 % RH were generated with saturated solutions of NaCl and K_2SO_4, respectively (Winston and Bates 1960). The % water loss was ∼35 % for each dehydration treatment, while 24 h of rehydration was sufficient to return water content to the control level (data not shown). For all the above conditions, survival was at or near 100 %. Immediately following exposure to the experimental conditions, larvae were frozen at −70 °C, and frozen samples were shipped back to Ohio State University on dry ice, where they were held at −70 °C until the time of RNA extraction.

Bioinformatics

Candidate genes for this study were identified from the following sources: (1) enzymes previously linked to cryoprotectant synthesis (Joanisse and Storey 1994), (2) genes involved in trehalose synthesis in the desiccation tolerant midge, *Polypedilum vanderplanki* (Mitsumasu et al. 2010), and (3) genes involved in proline synthesis in cold-selected *Drosophila melanogaster* lines (Misener et al. 2001). After assembling a candidate list of genes, homologs were identified from a *B. antarctica* genome and transcriptome that is in preparation. Protein sequences from *Aedes aegypti*, *Anopheles gambiae*, and *D. melanogaster* were obtained for the genes in Fig. 1. Using the tblastn algorithm, we searched the *B. antarctica* transcriptome for putative homologs. The *B. antarctica* homologs were reciprocally blasted against the Refseq protein database using blastx to provide further evidence of the transcripts' putative identity. Finally, the *B. antarctica* sequences were translated into protein sequences and searched against the Pfam database (http://pfam.sanger.ac.uk/) to confirm that the predicted protein had the expected domain structure. Accession numbers and results of the bioinformatics analysis are summarized in Table 1.

Isolating fragments for qPCR

PCR primers for the 11 metabolic genes plus 2 nuclear-encoded ribosomal protein reference genes, *rp49* [GenBank: JX462669] and *rpl19* [GenBank: JX462670], were manufactured by Integrated DNA Technologies (IDT, Coralville, IA). Using IDT's primer design software (http://www.idtdna.com), primers were designed to be 24 nt in length, have a product size between 100 and 180 bp, and an annealing temperature of 60 °C. Primers were tested against *B. antarctica* cDNA using conventional PCR, and products were visualized on an agarose gel for the presence of a single band at the expected size. Following PCR, products were purified using the Invitrogen PureLink PCR Purification Kit (Life Technologies, Grand Island, NY) and sequenced by Sanger sequencing at the Ohio State Plant Microbe Genomics Facility. Finally, primer linearity and efficiency were measured by running an eight-point standard curve, with purified PCR product as the template. For all primers tested, the R^2 value was >0.99 and the efficiencies were between 85.1 and 94.7 % (Table 2).

RNA extraction and cDNA synthesis

To measure gene expression in biological samples, we first extracted total RNA from frozen samples, each containing

Table 2 Primers used for qPCR gene expression assays

Gene	R^2	Efficiency (%)	Primers	T_m (°C)	Product size
gp	0.999	88.1	F: 5′-TGGATCGTAACTTGGCCGAGAACA-3′	60.2	125
			R: 5′-AACGATATCGGCGAGTGATGCAGA-3′	60.2	
pepck	0.998	90.6	F: 5′-AAATGCCTGCACTCAGTTGGAACC-3′	60.2	105
			R: 5′-GCTCAGTGCTGGTTTGTGCAAGAT-3′	60.3	
g6pase	0.999	85.1	F: 5′-AGTGCAGCTGACTGAGAAGTCGAA-3′	60.1	129
			R: 5′-TTGAAAGCCAGTTGAACAGACGCC-3′	60.1	
akr	0.999	88.2	F: 5′-GCCAACAACATTCTGATCACCGCA-3′	60.3	120
			R: 5′-AACGATGACCGAGTTCTCCAGCAA-3′	60.4	
sordh	0.998	94.7	F: 5′-TATCGTCGCGAAGCTCGGAAAGAA-3′	60.2	147
			R: 5′-GGTGTCGCACAGAAAGCCATTTCA-3′	60.2	
g3pdh	0.999	91.1	F: 5′-ATACTTGCCCGGACACAAATTGCC-3′	60.2	122
			R: 5′-CGCCCAAGCCTTTGATGAACTGAT-3′	60.0	
tps	0.999	86.3	F: 5′-GACTTTGCCGCTGGAAACCAAGAA-3′	60.2	148
			R: 5′-CAAACCGTGATTGCCGGCATAAGT-3′	60.3	
t6pp	0.998	94.5	F: 5′-TCGCACAACTTTGGCGAAGAATGG-3′	60.3	149
			R: 5′-GTTGCTTTGGTCGGCAGATTTGGA-3′	60.3	
treh	0.992	89.5	F: 5′-GTTGCAATCAGGCGAACAATGGGA-3′	60.3	118
			R: 5′-CCATTCTTGTGCAACAGCCTTCGT-3′	60.2	
tret1	0.997	88	F: 5′-TGCTGATCCCTGAAACACCGAGAT-3′	60.2	151
			R: 5′-GCATGTCGTTCAGCTTCGCAATGA-3′	60.3	
p5cr	0.999	91.7	F: 5′-ACAAATGATTGCGAGTGCCCATCC-3′	60.2	142
			R: 5′-AACGACGTTTGGCTTCACACACAC-3′	60.3	
rp49	0.996	90.6	F: 5′-TGGCAGTTCGACCAGCATTCAAAC-3′	60.2	142
			R: 5′-AAGCGACGTCTGACTCTGTTGTCA-3′	60.1	
rpl19	0.999	87	F: 5′-ACATCCACAAGCGTAAGGCTGAGA-3′	60.3	128
			R: 5′-TTCTTGTTTCTTGGTGGCGATGCG-3′	60.1	

The R^2 and efficiency values were calculated from an eight-point, tenfold dilution series of purified PCR product

20 larvae, using the Ambion RiboPure Kit (Life Technologies, Grand Island, NY). Samples were removed from −70 °C one at a time to prevent RNA degradation during processing. RNA quantity and purity were assessed on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) by measuring absorbance at 260, 280, and 230 nm. Starting with 1 µg total RNA for each sample, we synthesized cDNA using the Invitrogen SuperScript VILO cDNA Synthesis Kit (Life Technologies, Grand Island, NY). The resulting cDNA samples were diluted 10× in water prior to measuring gene expression and stored at −20 °C.

qPCR

For qPCR analysis, each 20 µl reaction contained 2 µl cDNA template, 2 µl of each primer at 250 nM concentration, 4 µl water, and 10 µl 2× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reactions were run on a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following parameters: 3 min at 94 °C followed by 40 cycles of 10 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. Following each reaction, a melt-curve analysis was conducted to verify that only one product was synthesized with no primer dimer.

Data analysis

Following qPCR reactions, background-corrected fluorescent intensities were obtained from the Bio-Rad analysis software. Background correction, amplitude normalization, and threshold cycles (C_t) for each reaction were calculated according to Larionov et al. (2005), using a custom MatLab script. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method. In short, for each sample, the C_t for the reference gene (either *rp49* or *rpl19*) was subtracted from the C_t of the gene of interest to obtain the ΔC_t value. The relative expression was then calculated using the formula $2^{-\Delta C_t}$, and the mean and SE were calculated for each treatment group ($N = 5$). Finally, relative fold change (FC) for each gene was calculated by dividing the mean relative expression value for each treatment group by the mean

expression value of the control group. Preliminary experiments determined that *rp49* was the most stable reference gene for the temperature series, while *rpl19* was more appropriate for the dehydration series.

To compare the relative mRNA abundance in each group, one-way ANOVA with a post hoc pooled t test was conducted on the ΔC_t values using JMP 9 (SAS Institute Inc., Cary, NC). To control the false discovery rate (FDR), P values were adjusted using the Benjamini and Hochberg method (Benjamini and Hochberg 1995). Statistical significance was set at FDR < 0.05. Since the thermal stress and dehydration stress samples were run on different qPCR plates (and were designed as separate experiments), separate hypothesis tests were conducted for the temperature and dehydration samples. Principal components analysis (PCA) was conducted for each experiment using the R package prcomp. The first two principal component scores for each sample were then plotted to determine whether the treatment groups formed distinct clusters.

Results

Bioinformatics

From the *B. antarctica* genome and transcriptome, we isolated transcripts for the following genes: glycogen phosphorylase (*gp*), phosphoenolpyruvate carboxykinase (*pepck*), glucose-6-phosphatase (*g6pase*), aldo–keto reductase (*akr*), sorbitol dehydrogenase (*sordh*), glycerol-3-phosphate dehydrogenase (*g3pdh*), trehalose-6-phosphate synthase (*tps*), trehalose-6-phosphate phosphatase (*t6pp*), trehalase (*treh*), trehalose transporter 1 (*tret1*), and pyrroline-5-carboxylate reductase (*p5cr*, Table 1; Fig. 1). With the exception of *g6pase*, full-length coding sequences were obtained for all transcripts. All 11 sequences showed high similarity to annotated *D. melanogaster* and mosquito sequences at the amino acid level, with blastx E values $\leq$1.00E$-$45 for all transcripts (Table 1). Furthermore, all translated protein sequences had the same predicted domain configuration as their closest homolog, as determined by a Pfam search (Table 1).

Gene expression during thermal stress

Using qPCR, we measured expression of the above 11 transcripts in response to both heat and cold. Following both heat and cold stress, there was significant upregulation of two genes involved in glucose synthesis, *gp* and *pepck* (Fig. 2). Of all the genes measured, *pepck* was the most labile, as mRNA levels were 2.22 ± 0.04-fold higher in supercooled individuals relative to control. Also, *pepck* was the only transcript that differed between supercooled and frozen individuals; while *pepck* was significantly elevated in frozen individuals relative to control (FC $= 1.72 \pm 0.06$), this level was significantly lower than that of supercooled individuals (ANOVA, pooled t, FDR < 0.05). During recovery from cold, the transcript levels of *gp* and *pepck* remained elevated relative to control. The only other transcript significantly elevated in response to thermal stress was *treh*, which was slightly elevated in supercooled individuals (Fig. 2).

There were also several transcripts that were downregulated in response to thermal stress. Two genes involved in polyol metabolism, *sordh* and *g3pdh*, were significantly downregulated in at least some of the temperature treatments (Fig. 2). Relative to control, *sordh* was downregulated in response to heat shock and recovery from supercooling, while *g3pdh* was significantly downregulated in all treatments, with transcript levels being 80–90 % of the control levels. The two genes involved in trehalose synthesis, *tps* and *t6pp*, were strongly down-regulated in response to thermal stress. Both *tps* and *t6pp* were downregulated with FC ≈ 0.55 in response to both heat and cold (Fig. 2) and remained lower than control values during recovery from cold. Likewise, *tret1*, a trehalose transport protein, was significantly downregulated during recovery from both supercooling and freezing. Finally, *p5cr*, a key enzyme in proline synthesis, was significantly downregulated in response to both supercooling and freezing and remained downregulated during recovery. A simplified summary of expression changes during thermal stress is presented in Fig. 3.

Gene expression during dehydration stress

In comparison with thermal stress, changes in gene expression were much more pronounced in response to dehydration stress. During fast dehydration, we observed upregulation of both *gp* and *pepck*, with levels returning to control levels during a 24-h recovery period (Fig. 4). In contrast, *gp* was significantly downregulated during both slow dehydration and cryoprotective dehydration, with levels at $\sim$80 % of control. However, *pepck* was strongly upregulated in response to both slow dehydration and cryoprotective dehydration, with levels peaking in the cryoprotective dehydration group (FC $= 5.21 \pm 0.25$). While *g6pase* levels did not differ from control in all five dehydration treatments, levels were significantly higher during fast dehydration than they were during slow dehydration (ANOVA, pooled t, FDR < 0.05).

The three genes involved in polyol synthesis and breakdown, *akr*, *sordh*, and *g3pdh*, were all unresponsive to fast dehydration (Fig. 4). However, both *akr* and *sordh* were significantly downregulated in response to slow dehydration, while *akr* and *g3pdh* were upregulated during

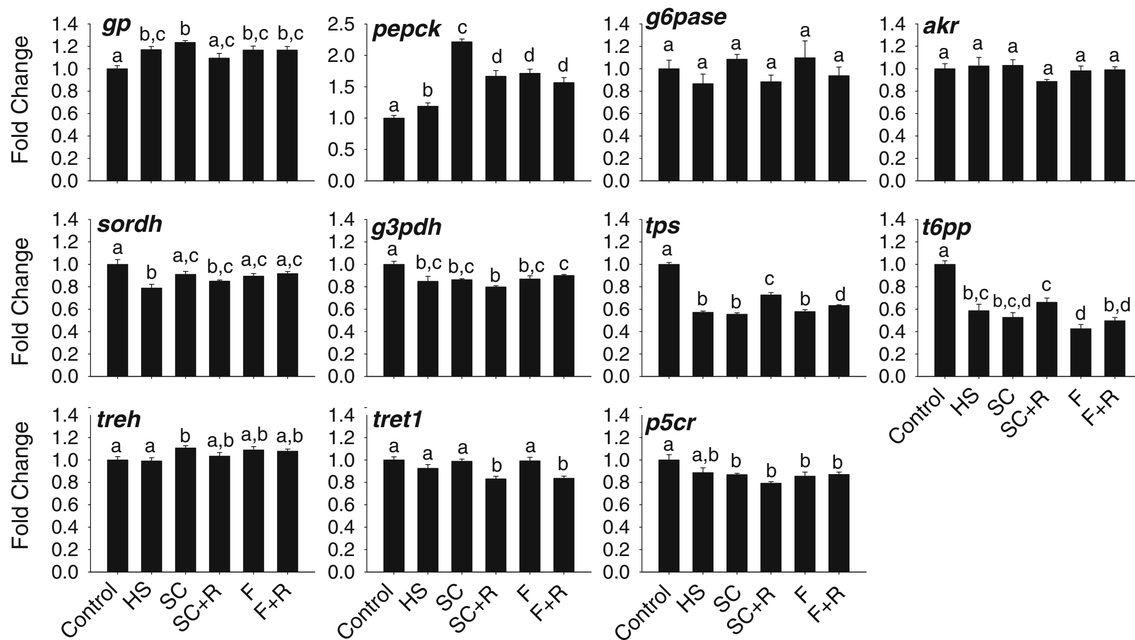

Fig. 2 Gene expression changes during thermal stress. *Bars* represent mean ± SE fold change of each group relative to control. *Different letters* represent significant differences in mRNA abundance for a particular gene (ANOVA, pooled *t*, FDR < 0.05). *HS* heat shock, *SC* supercooled, *SC + R* supercooled with recovery, *F* frozen, *F + R* frozen with recovery. See "Materials and methods" for full description of treatment conditions

Glucose synthesis

	HS	SC	SC+R	F	F+R
gp					
pepck					
g6pase					

Polyol metabolism

	HS	SC	SC+R	F	F+R
akr					
sordh					
g3pdh					

Trehalose metabolism

	HS	SC	SC+R	F	F+R
tps					
t66p					
treh					
tret1					

Proline synthesis

	HS	SC	SC+R	F	F+R
p5cr					

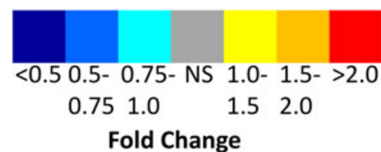

Fig. 3 Summary of gene expression patterns during thermal stress. *Each square* represents the relative fold change of a particular transcript relative to control. Expression changes that are not significant (NS) are denoted with *gray coloration*, while the *color scale* for significant fold changes is included in the figure. *HS* heat shock, *SC* supercooled, *SC + R* supercooled with recovery, *F* frozen, *F + R* frozen with recovery. See "Materials and methods" for full description of treatment conditions (color figure online)

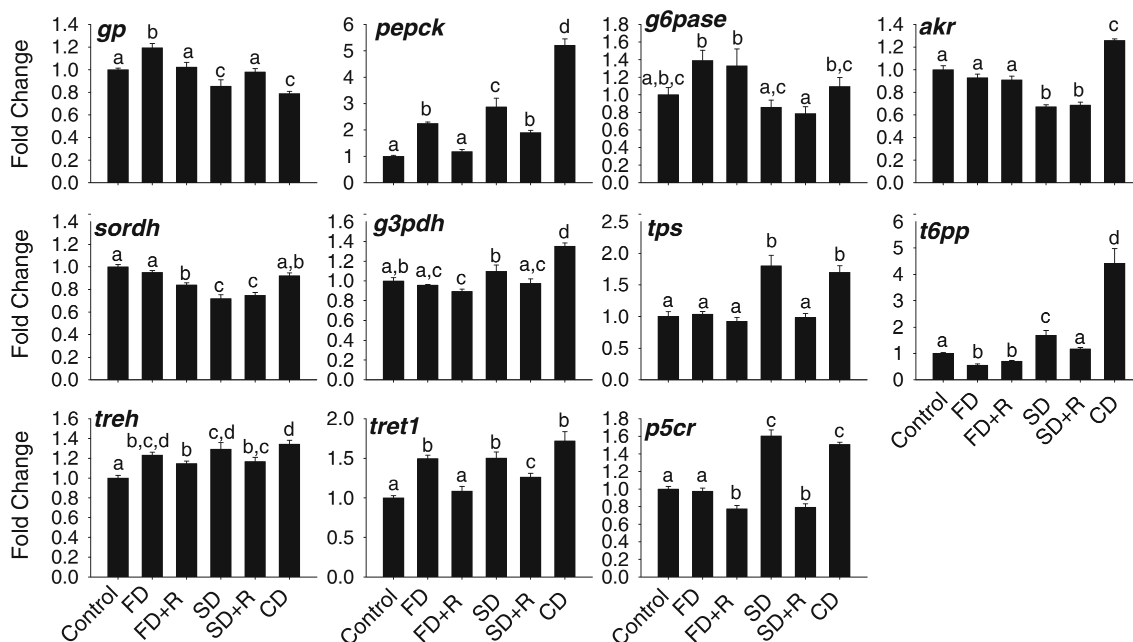

Fig. 4 Gene expression changes during dehydration stress. *Bars* represent mean ± SE fold change of each group relative to control. *Different letters* represent significant differences in mRNA abundance for a particular gene (ANOVA, pooled *t*, FDR < 0.05). *FD* fast dehydration, *FD + R* fast dehydration + rehydration, *SD* slow dehydration, *SD + R* slow dehydration + rehydration, *CD* cryoprotective dehydration. See "Materials and methods" for full description of treatment conditions

cryoprotective dehydration. In fact, *akr* was the only transcript that showed opposite expression patterns in response to slow and cryoprotective dehydration.

With regard to trehalose synthesis genes, we observed opposite results in response to fast versus slow dehydration. During fast dehydration, levels of *tps* were unchanged while *t6pp* was downregulated (FC = 0.56 ± 0.04); however, during slow dehydration, both *tps* and *t6pp* were significantly upregulated (FC = 1.80 ± 0.17 and 1.69 ± 0.18, respectively). Similarly, *tps* and *t6pp* were upregulated during cryoprotective dehydration. *Treh*, which codes for an enzyme that breaks down trehalose into glucose monomers, was upregulated in response to all three dehydration regimes, and remained elevated during rehydration (Fig. 4). Finally, *tret1* was likewise upregulated in response to all three dehydration treatments.

In our gene set, the lone gene involved in proline synthesis, *p5cr*, was upregulated in response to both slow and cryoprotective dehydration (FC = 1.61 ± 0.07 and 1.51 ± 0.03, respectively). During rehydration following both fast and slow dehydration, *p5cr* was downregulated relative to controls (Fig. 4). A simplified summary of expression changes during dehydration stress is presented in Fig. 5.

Multivariate statistics

To summarize expression patterns across all genes, we conducted PCA separately on each dataset (i.e. temperature and dehydration) to visualize which treatment groups are similar and distinct from one another. In the temperature series, we see three non-overlapping groups of samples along PC1: a cluster of control samples, a cluster containing heat shock, frozen, frozen + recovery, and supercooled + recovery samples, and a cluster of supercooled samples (Fig. 6a). For the dehydration experiment, a plot of PC2 versus PC1 shows that the control and fast dehydration + rehydration groups form an overlapping cluster of points (Fig. 6b). However, each of the remaining treatment groups forms a distinct cluster of points, with the cryoprotective dehydration group being the most distant from the control group.

Discussion

From a draft of the *B. antarctica* genome (in preparation), we isolated coding sequences for 11 genes related to energy mobilization and osmoprotectant synthesis. Using qPCR, we measured their expression in response to both thermal and dehydration stress. Environmental stress led to expression changes in a number of metabolic genes, with the magnitude and direction of change dependent on the type of stress experienced. On the whole, expression changes were well correlated with a priori knowledge of the metabolic changes that accompany thermal and dehydration stress.

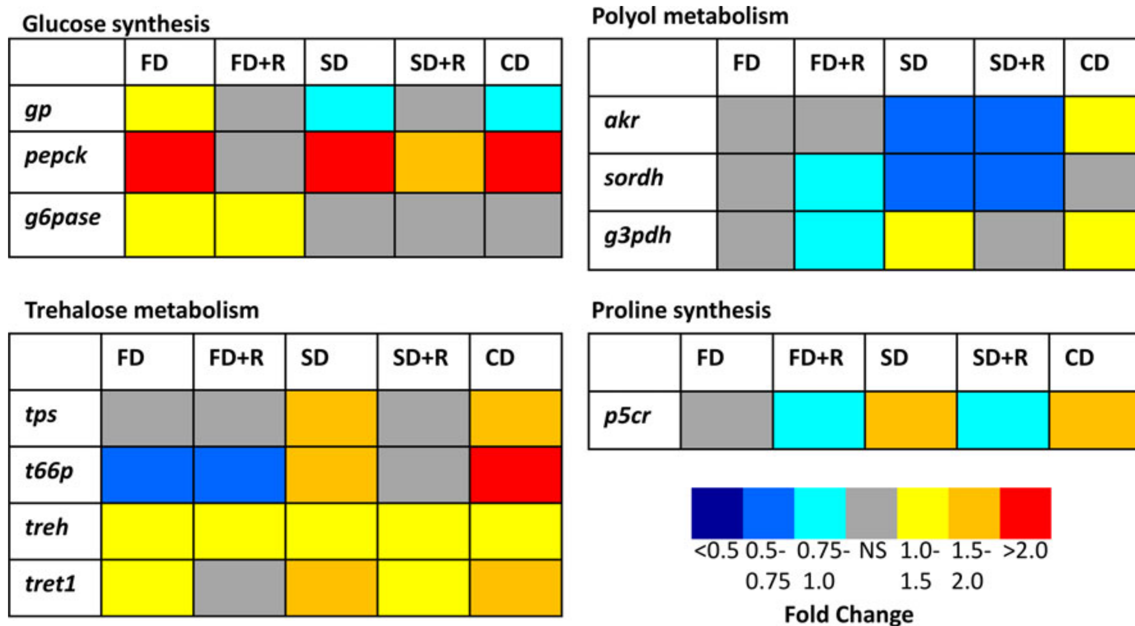

Fig. 5 Summary of gene expression patterns during dehydration stress. *Each square* represents the relative fold change of a particular transcript relative to control. Expression changes that are not significant (NS) are denoted with *gray coloration*, while the *color scale* for significant fold changes is included in the figure. *FD* fast dehydration, *FD* + *R* fast dehydration + rehydration, *SD* slow dehydration, *SD* + *R* slow dehydration + rehydration, *CD* cryoprotective dehydration. See "Materials and methods" for full description of treatment conditions (color figure online)

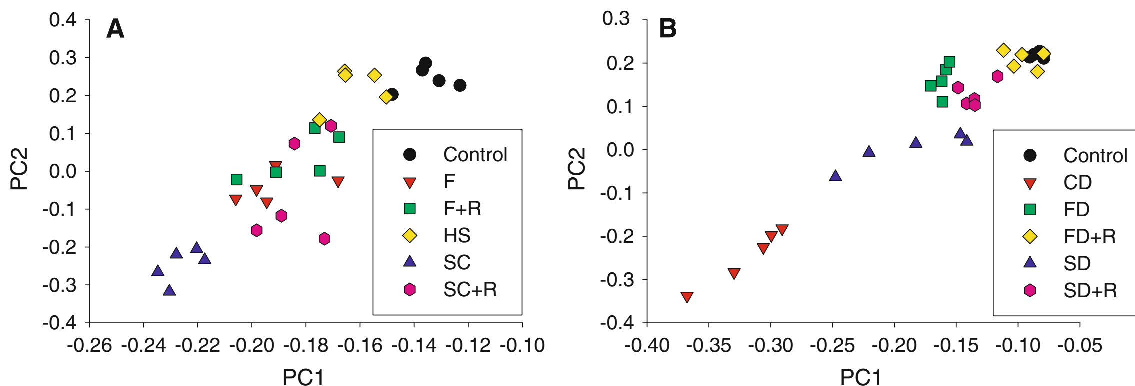

Fig. 6 Principal components analysis of thermal stress (**a**) and dehydration stress (**b**) gene expression datasets. The principal components were calculated on $2^{-\Delta C_t}$ values for each transcript in each sample (see "Materials and methods" for description of $2^{-\Delta C_t}$ calculations). Each individual sample is represented on the graph, with samples from the same treatment group having the *same symbol*. *F* frozen, *F* + *R* frozen with recovery, *HS* heat shock, *SC* supercooled, *SC* + *R* supercooled with recovery, *CD* cryoprotective dehydration, *FD* fast dehydration, *FD* + *R* fast dehydration + rehydration, *SD* slow dehydration, *SD* + *R* slow dehydration + rehydration. See "Materials and methods" for full description of treatment conditions

Gene expression changes in response to thermal stress

In response to both heat and cold stress, we observed upregulation of *gp* and *pepck*, suggesting that glucose synthesis is upregulated via both glycogenolysis and gluconeogenesis (Figs. 2, 3). This is consistent with our previous study in which both freezing and supercooling resulted in significant glucose mobilization (Teets et al. 2011). Interestingly, despite the upregulation of *gp* in both the supercooled and frozen group, Teets et al. (2011) only observed significant glycogen depletion in frozen larvae. However, in that study, we only measured glycogen content following recovery from cold, so it is possible that supercooled larvae convert glycogen to glucose at low temperatures and then recycle glucose to glycogen during recovery. With regard to heat shock, our results do not mesh with previous biochemical studies, as Michaud et al. (2008) observed a significant decrease in glucose following

heat shock. This discrepancy could be the result of differences in treatment conditions (Michaud et al. used 30 °C for 1 h as their heat shock treatment vs. the 25 °C for 24 h used in this study), or it could be that elevated metabolism at higher temperatures outweighs glucose production, so that net glucose levels decrease even while glycogenolysis and gluconeogenesis increase. Also, it is possible that biochemical fluxes are not governed by changes at the level of transcription.

Indeed, upregulation of both glycogenolysis and gluconeogenesis appears to be a general feature of thermal stress responses. Glucose synthesis in response to low temperature has been observed in organisms ranging from plants (Sasaki et al. 1996), to insects (Overgaard et al. 2007), to frogs (Costanzo et al. 1993). At the molecular level, upregulation of *pepck* is a common response to environmental stress; recent studies have demonstrated strong induction of *pepck* in response to heat (Sorensen et al. 2005), cold (Teets et al. 2012b), hypoxia (Liu et al. 2006), and oxidative stress (Girardot et al. 2004). Furthermore, *pepck* is strongly upregulated during diapause in *D. melanogaster* (Baker and Russell 2009), *Sarcophaga crassipalpis* (Ragland et al. 2010), and *Rhagoletis pomonella* (Ragland et al. 2011), perhaps to upregulate glucose production in advance of adverse conditions. While the importance of glycogenolysis during thermal stress is well established (Storey and Storey 1991), to our knowledge this is the first study demonstrating stress-inducible upregulation of *gp* transcripts. In wood frogs, there is seasonal accumulation of GP protein during hibernation (Kiss et al. 2011), but expression at the mRNA level has not been examined. Thus, while glycogenolysis is regulated at many levels by post-translational and substrate-dependent events (Arrese and Soulages 2010), it appears in *B. antarctica* that glycogenolysis is also under transcriptional control.

Somewhat surprisingly, while there was clear upregulation of glucose production during thermal stress, we observed downregulation of genes involved in polyol, trehalose, and proline synthesis, despite their known function as cryoprotectants in other organisms. Larvae of *B. antarctica* synthesize glycerol in response to freezing (Michaud et al. 2008), but this was not reflected in the gene expression data. The two genes in our dataset involved in polyol synthesis, *akr* and *g3pdh*, were either unresponsive or downregulated during heat and cold exposure (Figs. 2, 4). It is worth noting that Michaud et al. used −10 °C for their cold treatment rather than the −5 °C used in this study, and this could explain the discrepancy. Perhaps even more puzzling, two genes involved in trehalose production, *tps* and *t6pp*, were strongly down-regulated in response to thermal stress. Thus, despite trehalose being a potent cryoprotectant (Duman et al. 1991), its synthesis appears to be shut down in response to low temperature, in favor of glucose production. Finally, while proline has not been established as a cryoprotectant in *B. antarctica*, it is a potent cryoprotectant in drosophilid flies (Kostal et al. 2011b, 2012). However, once again, *p5cr*, a key gene in proline synthesis, is downregulated during thermal stress in this species. Thus, despite the clear upregulation of glucose production during thermal stress, it appears that other cryoprotectant synthesis pathways are shut down, at least at the level of gene expression. One possible explanation for these observations would be that cryoprotectant levels are constitutively high in *B. antarctica*, as are other stress responses such as heat shock protein expression (Rinehart et al. 2006) and antioxidant defenses (Lopez-Martinez et al. 2009). However, previous metabolite studies do not support this, as baseline levels of glycerol and sorbitol are very low in *B. antarctica* (Elnitsky et al. 2008), while trehalose levels (Teets et al. 2012a) are on par with those observed in other larval insects (Storey et al. 1981; Kostal et al. 2011a).

One goal of this study was to determine whether any metabolic genes were differentially regulated between supercooled and frozen larvae. With supercooling points around −7 °C (Lee et al. 2006), larvae remain unfrozen at −5 °C, provided they avoid inoculative freezing from exogenous ice. In a previous study, we found that supercooled larvae fare much better than frozen larvae, with regard to whole animal survival, cell survival, and energy depletion (Teets et al. 2011). Thus, we hypothesized that necessary adaptations to low temperature, such as cellular metabolic restructuring, may be inhibited when larvae are in the frozen state. Looking at the PCA analysis (Fig. 6a), it is evident that frozen and supercooled larvae are transcriptionally distinct. The gene most responsible for this separation is *pepck*, which was the only gene significantly different between supercooled and frozen larvae; expression was 30 % higher in supercooled larvae (Fig. 2). As the rate-limiting enzyme of gluconeogenesis, perhaps elevated expression of *pepck* in supercooled larvae gives them a “head-start” in glucose production at low temperature, thus permitting better survival down the line. However, this idea is speculative and needs to be verified with enzyme and substrate-level data.

Gene expression changes in response to dehydration stress

As was the case for thermal stress, dehydration stress resulted in a clear upregulation of gluconeogenesis via upregulation of *pepck* (Figs. 4, 5). These results are supported by previous physiological studies of dehydration tolerance in *B. antarctica*, as glucose is accumulated both in response to repeated dehydration at a constant temperature (Teets et al. 2012a) and in response to cryoprotective

dehydration (Elnitsky et al. 2008). However, whereas thermal stress also caused upregulation of *gp* to support glycogenolysis, this was not always the case during dehydration. During fast dehydration, we observed a modest increase in *gp* levels, similar to that observed during thermal stress (Figs. 4, 5). In contrast, *gp* expression was down-regulated during slow dehydration and cryoprotective dehydration, suggesting larvae are not relying heavily on glycogen for glucose production. While slow dehydration does cause some glycogen depletion, the rate of breakdown is much more rapid during fast dehydration (Teets et al. 2012a). In addition to serving as a carbon pool, glycogen also binds three to five times its weight in water (Gibbs et al. 1997), so perhaps slowing down glycogen breakdown is a means of conserving water during slow, prolonged dehydration.

In addition to their role as cryoprotectants, polyols are also important components in the response of *B. antarctica* to dehydration. Glycerol levels increase during both fast and slow dehydration (Benoit et al. 2007; Michaud et al. 2008), while sorbitol levels decrease (Michaud et al. 2008). In our dataset, we measured three genes involved in polyol metabolism, *akr*, *sordh*, and *g3pdh*. While none of these genes were responsive to fast dehydration, we observed downregulation of *akr* and *sordh* during slow dehydration (Figs. 4, 5). These changes are opposite of what we would expect based on Michaud et al.'s data; thus it appears polyol synthesis during dehydration is not controlled at the transcriptional level. However, in the cryoprotective dehydration group, we did see upregulation of polyol synthesis at the transcript level. Both *akr* and *g3pdh* are significantly upregulated, which would seemingly push sugars towards their respective sugar alcohols (Fig. 1).

Trehalose, the blood sugar of insects, is accumulated in a wide-range of organisms in response to dehydration (Clegg 2001). Larvae of *B. antarctica* synthesize trehalose in response to fast, slow, and cryoprotective dehydration (Benoit et al. 2007; Elnitsky et al. 2008; Benoit et al. 2009), presumably to help conserve water and protect cellular structures. These observations were well supported in the gene expression data for the slow dehydration and cryoprotective dehydration groups; in response to both treatments, there was strong upregulation of the two genes that synthesize trehalose from glucose, *tps* and *t6pp* (Figs. 4, 5). Furthermore, there was upregulation of *tret1*, a high-affinity trehalose transporter responsible for bringing trehalose into cells (Kikawada et al. 2007), as well as *treh*, an enzyme that breaks down trehalose into glucose monomers at the target tissue (Mitsumasu et al. 2010). Indeed, the expression patterns we observed for these genes were similar to those observed during dehydration in the anhydrobiotic sleeping midge, *P. vanderplanki* (Mitsumasu et al. 2010), and the Arctic collembolan, *Megaphorura arctica* (Clark et al. 2009). Thus, it appears that upregulation of genes along the trehalose biosynthesis axis is a critical adaption for dehydration-tolerant arthropods.

While the role of proline as a cryoprotectant is well established (Kostal et al. 2011b), its potential role as an osmoprotectant during dehydration has not been examined extensively (Benoit 2010). We observed upregulation of *p5cr*, the final enzyme required for proline biosynthesis, during both slow and cryoprotective dehydration (Figs. 4, 5). Proline plays an important role during drought stress in plants, although stress-induced accumulation is primarily regulated by expression of pyrroline-5-carboxylate synthetase, an enzyme upstream of P5CR (Verbruggen and Hermans 2008). Nonetheless, our expression data provide evidence that proline synthesis is a component of the dehydration response in *B. antarctica*. Additional substrate-level experiments are needed to determine whether proline is accumulated during dehydration or serves as a precursor for other metabolites required for dehydration.

Similar to our aforementioned supercooled versus frozen comparison, a secondary objective of the dehydration experiment was to compare transcriptional signatures in response to fast, slow, and cryoprotective dehydration. While all three treatments resulted in $\sim$35 % water loss, the transcriptional responses were clearly distinct (Fig. 6b). Most notably, in several cases, expression patterns were opposite between fast and slow dehydration. For example, as discussed earlier, *gp* was upregulated during fast dehydration but downregulated during slow dehydration (Figs. 4, 5). Also, whereas there was clear upregulation of both *tps* and *t6pp* during slow dehydration, *tps* was unaffected by fast dehydration while *t6pp* was downregulated. Larvae do accumulate modest amounts of trehalose during fast dehydration, but amounts are much higher during slow dehydration (Benoit et al. 2007). While some of this could be due to time differences, our data show that trehalose synthesis is actually shut down at the gene expression level during fast dehydration. Instead, it appears that larvae are relying primarily on glucose mobilization, via both gluconeogenesis and glycogenolysis, during fast dehydration.

Comparison of thermal stress and dehydration stress

While the thermal stress and dehydration stress treatments were conducted and analyzed as separate experiments, we are able to makes some general comparisons between the two. Most notably, fast dehydration yielded expression changes that were more similar to those observed during thermal stress than during other types of dehydration (Figs. 3, 5). Fast dehydration and thermal stress both resulted in upregulation of glucose synthesis via *gp* and *pepck*, downregulation of trehalose synthesis, and no increase in *p5cr*. In both cases, larvae were rapidly transferred to stressful conditions (i.e. temperature extremes or

low humidity), and it appears that the metabolic demands of acute thermal and water stress are very similar. It would be interesting to investigate whether gradual, prolonged chilling (in the absence of dehydration) leads to a shift towards trehalose and proline synthesis, as was observed during slow and cryoprotective dehydration. On the whole, it was clear that while thermal stress and dehydration stress shared some of the same molecular responses (most notably induction of gluconeogenesis via *pepck*), the transcriptional responses were largely different. Thus, even though adaptations for cold and dehydration are thought to have a common origin (Ring and Danks 1994), our data suggest notable metabolic differences between the two. However, since it is difficult to make thermal and dehydration stress bouts "equally stressful," this idea warrants further investigation.

Conclusions

Our gene expression results show that larvae of *B. antarctica* undergo rapid metabolic restructuring during periods of environmental stress. Previous work has illustrated some of the metabolic consequences of changes in temperature and water balance (Baust and Edwards 1979; Baust and Lee 1983; Benoit et al. 2007; Elnitsky et al. 2008; Michaud et al. 2008; Benoit et al. 2009; Teets et al. 2011, 2012a), and now we provide molecular mechanisms that govern some of these changes. While gene expression data do not always reflect protein levels or protein activities (Feder and Walser 2005), for the most part observed changes in metabolic gene expression meshed with previous phenotypic studies. Also, in some cases, such as the central role of *pepck* gene expression in regulating gluconeogenesis (Hanson and Reshef 1997) and the transcriptional regulation of trehalose synthesis (Mitsumasu et al. 2010), there is a clear link between mRNA levels and metabolic endpoints. In the case of *B. antarctica*, our results suggest that coordinated changes in metabolic gene expression are a critical survival mechanism for Antarctica's southernmost free-living insect.

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