DOI 10.1002/pmic.200800850 *Proteomics* 2009, *9*, 2788–2797

RESEARCH ARTICLE

Distinct contractile and cytoskeletal protein patterns in the Antarctic midge are elicited by desiccation and rehydration

Aiqing Li[1,2], Joshua B. Benoit[1], Giancarlo Lopez-Martinez[1], Michael A. Elnitsky[3], Richard E. Lee Jr[3] and David L. Denlinger[1]

[1] Department of Entomology, The Ohio State University, Columbus, OH, USA
[2] Department of Neuroscience, The Ohio State University, Columbus, OH, USA
[3] Department of Zoology, Miami University, Oxford, OH, USA

Received: October 31, 2008
Revised: February 2, 2009
Accepted: February 3, 2009

Desiccation presents a major challenge for the Antarctic midge, *Belgica antarctica*. In this study, we use proteomic profiling to evaluate protein changes in the larvae elicited by dehydration and rehydration. Larvae were desiccated at 75% relative humidity (RH) for 12 h to achieve a body water loss of 35%, approximately half of the water that can be lost before the larvae succumb to dehydration. To evaluate the rehydration response, larvae were first desiccated, then rehydrated for 6 h at 100% RH and then in water for 6 h. Controls were held continuously at 100% RH. Protein analysis was performed using 2-DE and nanoscale capillary LC/MS/MS. Twenty-four identified proteins changed in abundance in response to desiccation: 16 were more abundant and 8 were less abundant; 84% of these proteins were contractile or cytoskeletal proteins. Thirteen rehydration-regulated proteins were identified: 8 were more abundant and 5 were less abundant, and 69% of these proteins were also contractile or cytoskeletal proteins. Additional proteins responsive to desiccation and rehydration were involved in functions including stress responses, energy metabolism, protein synthesis, glucogenesis and membrane transport. We conclude that the major protein responses elicited by both desiccation and rehydration are linked to body contraction and cytoskeleton rearrangements.

Keywords:
Actin / Desiccation / Muscle proteins / Myosin / Rehydration

1 Introduction

One of the most pronounced challenges for an organism living in Antarctica is desiccation stress. The abundant water present on the continent is frozen for much of the year, thus the water is physiologically unavailable and effectively creates a desert-like environment [1]. The midge *Belgica antarctica* is one of the few arthropods to live in this environment [2, 3]. The larvae of this species are encased in ice during most of their two-year life cycle and are active only during the brief austral summer [4].

The larvae are highly permeable and lose water at an extremely high rate [5, 6]. Several mechanisms are exploited to suppress water loss [6, 7]. Behaviorally, water conservation can be achieved by clustering and by seeking moist protected microhabitats. Physiologically, the midges significantly increase their overall polysaccharide levels and accumulate trehalose and glycerol in response to dehydration [5, 6]. Other desiccation responses noted in this species include a shift from short to long cuticular hydrocarbons, a decrease in oxygen consumption, elevated metabolites from pathways of central carbohydrate metabolism and a shift in the free amino acid pool [5, 8]. Water stores are replenished

Correspondence: Dr. David L. Denlinger, Department of Entomology, The Ohio State University, 400 Aronoff Laboratory, 318 West 12th Avenue, Columbus, OH 43210, USA
E-mail: denlinger.1@osu.edu
Fax: +1-614-292-2180

Abbreviation: RH, relative humidity

mainly by intake of liquid water [5]. A novel form of dehydration, cryoprotective dehydration, appears to be critical for winter survival [9, 10]. Thus, bouts of dehydration and rehydration appear to be normal components of the midge's seasonal cycle.

Thus far, only a few studies have used proteomics to examine desiccation and rehydration responses of animals (nematodes by Chen *et al.* [11]; rats by Gouraud *et al.* [12]). In this study, a comparative proteome analysis was performed on larvae of *B. antarctica* under hydrated conditions and in response to desiccation and subsequent rehydration to gain insights into the molecular mechanisms that respond to shifts in osmotic stress. Our results indicate that an abundance of contractile and cytoskeletal proteins change dramatically in response to both desiccation as well as rehydration, thus suggesting that elements involved in body contraction and cytoskeleton rearrangements are among the functions most significantly altered by desiccation and rehydration recovery.

2 Materials and methods

2.1 Insects

Third and fourth instar larvae of *B. antarctica* Jacobs used in these experiments were collected on Cormorant Island, Torgersen Island and Bonaparte Point, near Palmer Station on Anvers Island (64°46′S, 64°40′W) on the Antarctic Peninsula in January 2006 and 2007. Substrate containing the larvae was brought into the Palmer Station laboratory and held at 4°C, shipped frozen to our home laboratory in Ohio and then maintained at 4°C for several months. Larvae were hand picked from the substrate in ice-cold water and stored at 4°C for 1–2 days prior to experimental use.

The experimental procedures are illustrated as a flow chart presented in Fig. 1.

2.2 Desiccation and rehydration

Relative humidities were generated in glass desiccators as described by Benoit *et al.* [6]. Control larvae were held at 100% relative humidity (RH) for 12 h. For the desiccation treatment, larvae were maintained at 75% RH for 12 h. For the rehydration treatment, larvae were first desiccated at 75% RH for 12 h, transferred to 100% RH for 6 h and then to water for 6 h. All experiments were performed at 4°C.

2.3 Extraction of proteins

Whole larvae ($n = 20$) were homogenized in 20 mM Tris-HCl at pH 8.0, with complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The homogenates were sonicated using a microtip, centrifuged and fractionated into soluble (supernatants) and insoluble (pellets) proteins.

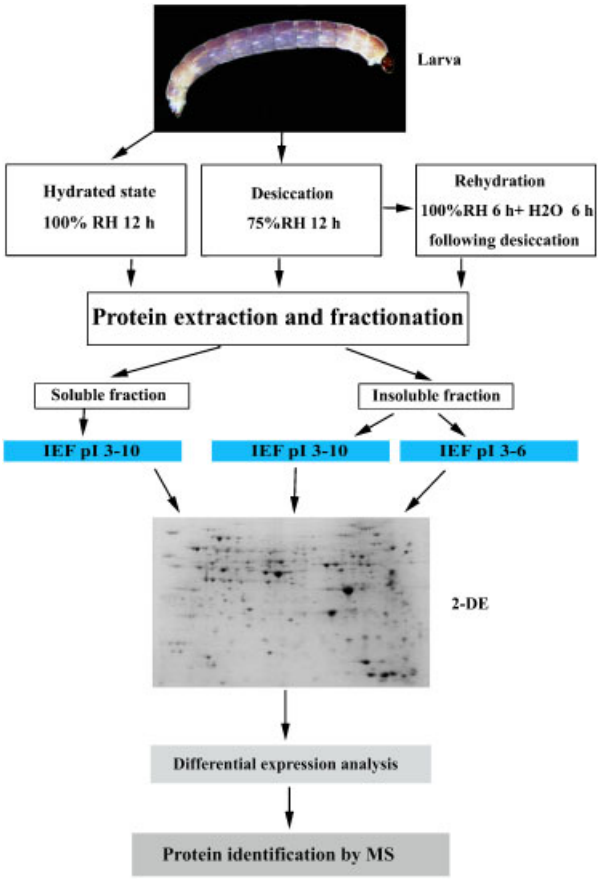

Figure 1. Flow chart of proteomic analysis of hydrated, desiccated and rehydrated *B. antarctica* larvae.

Proteins in the supernatants were precipitated using the ReadyPrep 2-D cleanup kit (BioRad) according to the manufacturer's instructions and resolved in general-purpose rehydration buffer [8 M urea, 2% w/v CHAPS. The protein solution in rehydration buffer was supplemented with 0.2% w/v Bio-Lyte 3/10 ampholyte, 0.002% w/v bromophenol blue, and 50 mM DTT], forming soluble fractions for 2-DE.

The protein pellets obtained from centrifugation of the homogenates were sequentially washed three times in Tris-HCl buffer (20 mM, pH 8.0) containing protease inhibitors, and centrifuged after each wash. The pellets were taken up in strongly chaotropic 2-DE rehydration buffer [7 M urea, 2 M thiourea, 4% w/v CHAPS. The protein solution in rehydration buffer was supplemented with 0.2% w/v Bio-Lyte 3/10 ampholyte, 0.002% w/v bromphenol blue, 2% w/v SB 3-10 (*N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) and 50 mM DTT] and kept for 1 h at room temperature. These samples were then centrifuged and supernatants containing the proteins were defined as the insoluble fractions used for 2-DE. Concentrations of soluble and insoluble proteins were determined using an RC/DC™ protein assay (BioRad) prior to 2-DE.

2.4 2-DE

The resulting protein samples were separated as described previously [13], with some modifications. Briefly, the IPG strips (BioRad) were rehydrated with approximately 200 μg proteins. Isoelectric focusing was carried out in a Protean BioRad IEF cell. The IEF running conditions were: 400 V for 20 min, 8000 V for 2.5 h and 8000 V for 35 000 V h for comparison of hydrated and desiccation soluble fractions with a pH range of 3–10; 250 V for 1 h, 500 V for 1 h, 8000 V for 2.5 h and 8000 V for 40 000 V h for comparison of hydrated and desiccation insoluble fractions with pH ranges of 3–10 and 3–6; 400 V for 20 min, 8000 V for 2.5 h and 8000 V for 35 000 V h for comparison of desiccation and rehydration soluble fractions with a pH range of 3–10; 250 V for 20 min, 8000 V for 1 h and 8000 V for 45 000 V h for separation of desiccation and rehydration insoluble fractions with pH ranges of 3–10 and 3–6 at 20°C, respectively. The strips were equilibrated and 2-DE was then performed at 200 V for 1 h on an 8–16% polyacrylamide SDS gel. For each condition, namely normal hydration, desiccation and rehydration, at least three biologically independent 2-D gels were run.

2.5 Staining of 2-D gels and image analysis

After fixation, proteins separated by 2-D gels were stained with Bio-safe Coomassie blue. The stained 2-D gels were scanned using a BioRad VersaDoc imaging system and images were processed by PDQuest. The relative change in protein abundance for each protein spot was calculated by quantitative comparisons of the averaged normalized spot quantity between two conditions. A two-tailed nonpaired Student's *t*-test was performed to determine if the relative change was statistically significant.

2.6 In-gel trypsin digestion

Proteins of interest were digested with trypsin from Promega (Madison WI) using the Multiscreen Solvinert Filter Plates from Millipore (Bedford, MA). Briefly, gel spots were washed in 50% methanol/5% acetic acid. The gel spots were dehydrated in ACN, then rehydrated and incubated with DTT solution (5 mg/mL in 100 mM ammonium bicarbonate) for 30 min prior to the addition of 15 mg/mL iodoacetamide in 100 mM ammonium bicarbonate. The gel spots were washed again with ACN and ammonium bicarbonate. After the gels were dried, the protease was driven into the gel spots by rehydrating them in 50 μL of trypsin at 20 μg/mL in 50 mM ammonium bicarbonate for 10 min. Twenty microliters of 50 mM ammonium bicarbonate was added. Peptides were extracted with 50% ACN and 5% formic acid and concentrated in a speed vac to 30 μL.

2.7 LC/MS/MS

Nanoscale capillary LC/MS/MS was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ Plus system from LC-Packings A Dionex (Sunnyvale, CA) with a Famos autosampler and Switchos column switcher. Solvent A was water containing 50 mM acetic acid and solvent B was acetonitrile. Five microliters of each sample was first injected into the trapping column (LC-Packings A Dionex) and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off the trap onto the column. A 5 cm 75 μm id ProteoPep II C18 column (New Objective, Woburn, MA) was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2–80% B over 50 min, with a flow rate of 300 nL/min. The total run time was 60 min. The MS/MS was acquired according to standard conditions established in the laboratory. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C was used. The scan sequence of the mass spectrometer was based on the TopTen™ method, a Thermo Scientific pre-programmed method in Excalibur. The analysis was programmed for a full scan recorded between 350 and 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequences in consecutive instrument scans of the ten most abundant peaks in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 s, exclusion duration of 350 s and a low mass width of 0.5 Da and high mass width of 1.50 Da.

2.8 Protein identification

Sequence information from the MS/MS data was processed by converting the raw data files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script) with first scan number, last scan number, number of intermediate scans, minimum number of grouped scans and minimum number of ions set to blank, blank, 1, 0 and 8, respectively. The resulting mgf files were searched using Mascot Daemon by Matrix Science version 2.2.1 (Boston, MA) and the database searched against NCBInr 20060402 (3525863 sequences; 1211011241 residues). Data processing was performed following the guidelines of Carr *et al.* [14]. The mass accuracy of the precursor ions was set to 2.0 Da given that the data were acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. Peptides with a score less than 20 were filtered to eliminate low scoring, random peptide matches. Only proteins with a

Mascot score of 50 or higher (5% probability a protein identification is incorrect) were accepted. When different isoforms/homologies were identified, only the proteins from insect species were presented; two proteins that did not match insect proteins were also included. Identified peptides and protein were checked manually to confirm the identification.

3 Results

3.1 2-DE analysis of desiccated larvae

Desiccation-responsive proteins were fractionated into soluble and insoluble proteins. These proteins were run on replicate 2-D gels (8–16%, pH 3–10) and representative images are shown in Fig. 2A and B, respectively. When insoluble proteins, regardless of whether they were extracted from hydrated or desiccated larvae, were subjected to IEF in a 3–10 pH gradient, a nonrandom distribution was observed because many spots clustered in the 3–6 pH range. Therefore, in subsequent experiments, we fractionated insoluble proteins from the two conditions on a 3–6 pH gradient in the first dimension (Fig. 2C).

Quantitative analysis revealed that 107 proteins were regulated by at least 1.5-fold in response to desiccation, including 30 from the soluble fraction and 48 from the insoluble fraction on a 3–10 pH gradient and 29 from the insoluble fraction on a 3–6 pH gradient. Analysis by Student's *t*-test showed that the proteins with significant differences in abundance included 21 from the soluble fraction, 25 from the insoluble fraction on a 3–10 pH gradient and 6 from the insoluble fraction on a 3–6 pH gradient (Supporting Information Table S1). Relatively abundant protein spots with an average ratio value greater than 1.5-fold and a *t*-test p value < 0.05 were selected for mass spectrometric identification. A total of 27 selected proteins (13 soluble and 14 insoluble) are shown in Fig. 2, and their corresponding identities are summarized in Table 1.

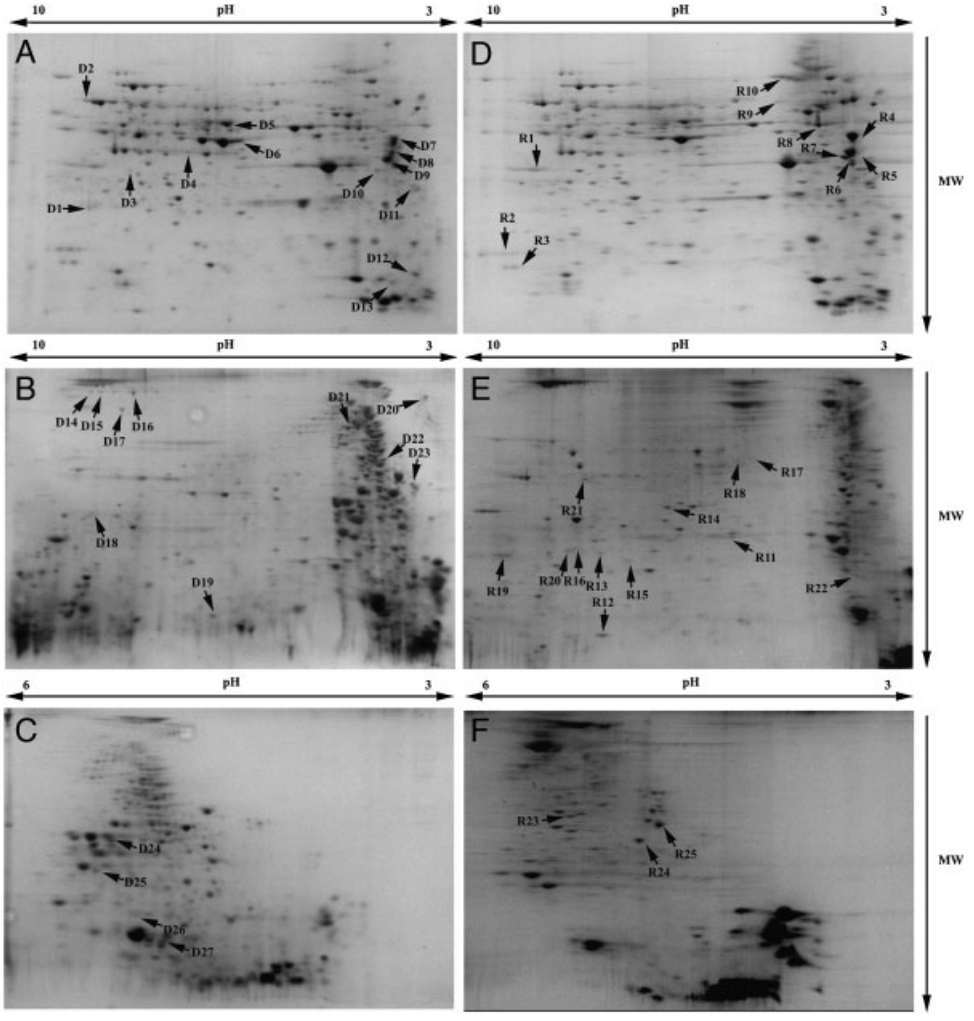

Figure 2. Representative 2-D maps of proteins from *B. antarctica* larvae after desiccation at 75% RH for 12 h, and after rehydration at 100% RH for 6 h and then in water for 6 h following desiccation at 75% RH for 12 h at 4°C. Left panels: desiccation-responsive proteins, (A) soluble proteins were resolved onto IPG3-10 strips; (B) insoluble proteins resolved onto IPG3-10 strips and (C) IPG3-6 strips in the first dimension. Right panels: rehydration-responsive proteins, (D) soluble proteins were resolved onto IPG3-10 strips; (E) insoluble proteins resolved onto IPG3-10 strips and (F) IPG3-6 strips in the first dimension. Proteins were separated by molecular weight in 8–16% polyacrylamide SDS-PAGE gels in the second dimension. Protein spots were then visualized by Bio-safe Coomassie staining. Maps were analyzed with PDQuest. The identified spots are annotated according to Table 1.

Table 1. Identification (LC/MS/MS) of larval proteins in *B. antarctica* that responded to desiccation and rehydration. Only relatively abundant proteins were selected for mass spectrometric analysis

Spot no.	Accession no.	Species	Protein ID	Sequence coverage (%)	Peptide count	Mowse score[a)]	Function
Proteins more abundant during desiccation							
D2	AAT06160	*Enallagma aspersum*	Catalase	13	3	184	Stress response
D3	XP_623725	*Apis mellifera*	PREDICTED: similar to porin	8	3	108	Membrane transport
D5	ABC96322	*Blattella germanica*	Enolase	9	4	245	Energy metabolism
D6	AAB31477	*Marsupenaeus japonicus*	Arginine kinase	18	5	306	Energy metabolism
D7	CAA09938	*Chironomus kiiensis*	Tropomyosin	46	18	1059	Contractile
D8	XP_391961[b)]	*Apis mellifera*	Predicted: similar to tropomyosin 1	29	11	674	Contractile
D9	XP_391961[b)]	*Apis mellifera*	Predicted: similar to tropomyosin 1	28	11	730	Contractile
D10	AAZ31061	*Aedes aegypti*	Actin 6	25	7	354	Contractile
D11	AAZ67334	*Culex pipiens pallens*	Putative myosin light chain 2	21	5	243	Contractile
D12	AAA51577	*Bos taurus*	Alpha-actin	10	3	178	Contractile
D13	ABC96325	*Clostera anastomosis*	Actin	26	2	179	Contractile
D18	AAA28686	*Drosophila melanogaster*	Myosin heavy chain	5	11	554	Contractile
D21	CAA37309	*Drosophila melanogaster*	Muscle myosin heavy chain	27	45	2022	Contractile
D22	CAA37309	*Drosophila melanogaster*	Muscle myosin heavy chain	18	29	1382	Contractile
D24	CAA37309	*Drosophila melanogaster*	Muscle myosin heavy chain	19	24	1185	Contractile
D25	CAA37309	*Drosophila melanogaster*	Muscle myosin heavy chain	14	17	810	Contractile
D27	AAA28318	*Drosophila melanogaster*	Actin	24	8	1022	Contractile
D4	–	–	Unknown	–	–	–	–
Proteins less abundant during desiccation							
D14	NP_524210	*Drosophila melanogaster*	Actin 79B CG7478-PA	13	4	236	Contractile
D15	CAA37308	*Drosophila melanogaster*	Muscle myosin heavy chain	1	2	92	Contractile
D17	AAA28686	*Drosophila melanogaster*	Myosin heavy chain	1	2	106	Contractile
D19	NP_524210	*Drosophila melanogaster*	Actin 79B CG7478-PA	14	4	134	Contractile
D20	CAA37308	*Drosophila melanogaster*	Muscle myosin heavy chain	9	11	361	Contractile
D23	CAA37308	*Drosophilan melanogaster*	Muscle myosin heavy chain	7	7	253	Contractile
D26	CAA37308	*Drosophila melanogaster*	Muscle myosin heavy chain	7	7	379	Contractile
D1,D16	–	–	Unknown	–	–	–	–
Proteins more abundant during rehydration							
R7	XP_001655948	*Aedes aegypti*	Tropomyosin invertebrate	13	4	300	Contractile
R10	AAN14526	*Chironomus yoshimatsui*	Heat-shock cognate 70	3	2	116	Stress response

Table 1. Continued

Spot no.	Accession no.	Species	Protein ID	Sequence coverage (%)	Peptide count	Mowse score[a)]	Function
R19	XP_001651219	*Aedes aegypti*	Myosin heavy chain	1	4	132	Contractile
R20	ABC68667	*Orsotriaena medus*	Elongation factor 1 alpha	2	1	76	Protein synthesis
R21	XP_001651219	*Aedes aegypti*	Myosin heavy chain	2	5	215	Contractile
R22	BAB63441	*Chironomus yoshimatsui*	Actin	20	2	121	Contractile
R24	XP_391961[b]	*Apis mellifera*	Tropomyosin 2 CG4843-PB, isoform B	26	5	390	Contractile
R25	XP_392125	*Apis mellifera*	Tropomyosin 1 CG4898-PD, isoform D isoform 1	33	11	490	Contractile
R8,R9,R23	–	–	Unknown	–	–	–	–
Proteins less abundant during rehydration							
R1	XP_973533	*Tribolium castaneum*	Mitochondrial malate dehydrogenase precursor	16	5	319	Glucogenesis
R4	AAU20322	*Myzus persicae*	Tropomyosin	32	10	532	Contractile
R5	NP_732012	*Drosophila melanogaster*	Tropomyosin 2 CG4843-PB, isoform B	30	5	454	Contractile
R6	BAD52257	*Plutella xylostella*	Tropomyosin I	12	4	282	Contractile
R11	ABF72872	*Belgica antarctica*	Anterior fat body protein-like	41	3	210	Stress response
R2,R3, R12-18	–	–	Unknown	–	–	–	–

a) Mowse score >50 indicates identity or extensive homology ($p<0.05$); higher scores indicate higher confidence of identity.
b) This protein is upregulated in both desiccation and rehydration treatments.

3.2 Identification of proteins that changed in response to desiccation

The relative abundance of 27 differentially expressed proteins in control and desiccated larvae was determined (Fig. 3A): 18 were more abundant and 9 were less abundant upon desiccation. Quantities of the 27 proteins were normalized and divided by the quantity of the most abundant spot (D5). Changes in abundance of these proteins ranged from 1.5-fold (D19) to 2.2-fold (D13).

Twenty-four of the 27 proteins were successfully identified and could be functionally classified as cytoskeletal and contractile proteins (84%), proteins involved in energy metabolism (8%), stress responses (4%) and membrane transport (4%) (Fig. 4A). The majority of proteins that showed differences (20 proteins) were contractile and cytoskeletal proteins, including 6 actins, myosin light chain, 10 myosin heavy chains and 3 tropomyosins. Among these 20 cytoskeletal and contractile proteins, 2 isoforms of actin and 5 isoforms of myosin heavy chain were less abundant, but the remainder were more abundant following desiccation. The two proteins involved in energy metabolism (arginine kinase, enolase) were all more abundant following desiccation. The stress-related protein catalase was more abundant after desiccation. Porin, a protein functionally involved in membrane transport, increased in abundance when the larvae were desiccated.

3.3 2-DE analysis of larvae that were desiccated, then rehydrated

Similarly, proteins from larvae that were desiccated and then subsequently rehydrated were separated into soluble and insoluble fractions, and run on 2-D gels. Representative images for the soluble and insoluble fractions are shown in Fig. 2D and E, respectively. Insoluble proteins from both desiccation and rehydration treatments were also focused on a 3–6 pH gradient in the first dimension because clustered proteins were observed in the 3–6 pH range (Fig. 2F).

Based on quantitative analysis, 176 proteins were regulated by at least 1.5-fold due to rehydration, including 76 from the soluble fraction and 57 from the insoluble fraction on a 3–10 pH gradient and 43 from the insoluble fraction on a 3–6 pH gradient. Statistical analysis using Student's *t*-test showed that proteins with significant differences between the control state and rehydration state included 23 from the soluble fraction, 26 from the insoluble fraction on a 3–10 pH gradient and 6 from the insoluble fraction on a 3–6 pH

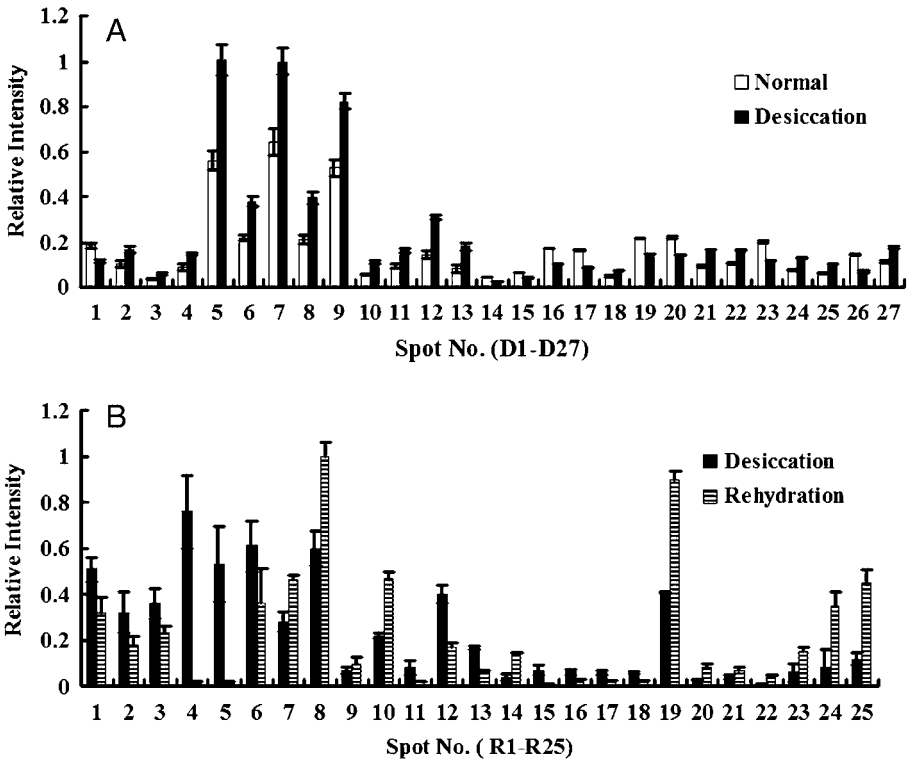

Figure 3. Quantification of proteins that changed in abundance after (A) desiccation at 75% RH for 12 h and (B) rehydration at 100% RH for 6 h and then in water for 6 h following desiccation at 75% RH for 12 h. All proteins were quantified by PDQuest. Error bars = standard deviation, $n = 3$–5. Desiccation and rehydration experiments were performed at different times. Thus, in B, to minimize variation, desiccation treatment was repeated so that it could be used as a comparison of rehydration; different parameters were set for image scanning and quantification in parts A and B.

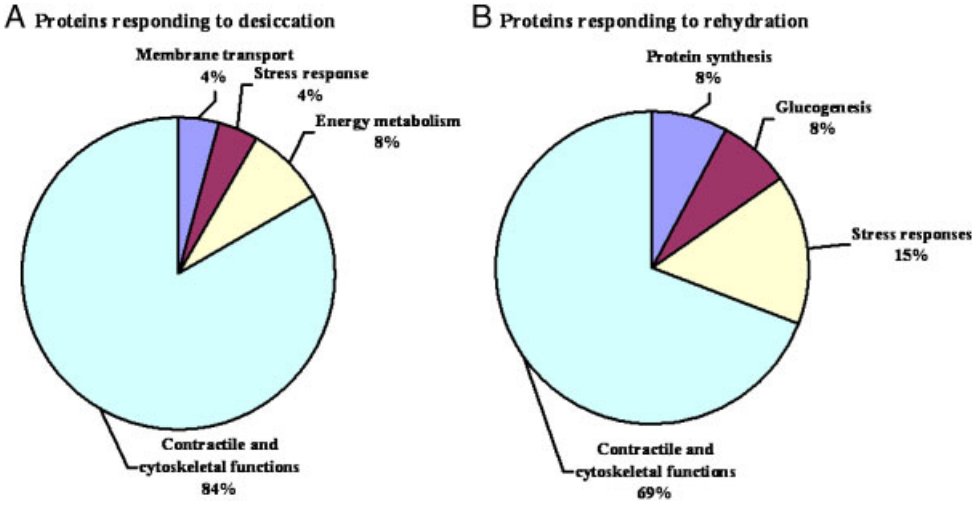

Figure 4. Functional classification of the identified proteins that changed in abundance in response to (A) desiccation and (B) rehydration.

gradient (Supporting Information Table S1). Relatively abundant protein spots with an average ratio value greater than 1.5-fold and a *t*-test p value < 0.05 were selected for identification (Fig. 2) and their corresponding identities are displayed in Table 1.

3.4 Identification of proteins that changed in response to rehydration

The quantities of all 25 desiccation- and rehydration-regulated proteins were normalized and divided by that of the most abundant spot (R8) (Fig. 3B). The extent of differential abundance of these proteins ranged from 1.5-fold (R9) to 4.4-fold (R24). Thirteen of the 25 proteins were successfully identified by LC-MS/MS (Table 1). These 13 proteins were classified according to their functions (Fig. 4B). Nine proteins (69% of the total) were contractile or cytoskeletal proteins: actin, 3 tropomyosins and 2 myosin heavy chains were more abundant and 3 tropomyosins were less abundant. Two proteins were stress related: heat-shock cognate 70 which increased in abundance, and anterior fat body protein-like, which was less abundant after rehydration. One protein that was more abundant, elongation factor 1 α, is

involved in the regulation of protein synthesis. Malate dehydrogenase, which is involved in glucogenesis, was less abundant following rehydration.

4 Discussion

We compared the proteomic profiles of larvae of *B. antarctica* during their normal hydrated state, following desiccation, and after rehydration. One might anticipate that rehydration would simply be the reverse of desiccation, and for some proteins, this may be the case. For example, one of the actins and two myosin heavy chains decreased in abundance during desiccation and increased again during rehydration. By contrast, three tropomyosins increased during desiccation and declined again after rehydration. But, such reversals were not consistently observed, indicating that rehydration is not simply the reverse of desiccation but elicits distinct changes in many contractile and cytoskeletal proteins.

Tropomoysin is well known to play a central role in regulating muscle contraction in skeletal muscle. In nonmuscle cells, it is associated with contractile actomyosin structures as well [15]. Actin, tropomyosin and myosin are functionally linked in the myofibrils [16]. Muscle contraction is driven by interactions of the actin–myosin cross-bridge, where the binding of myosin to actin is regulated by a complex of thin-filament associated proteins, tropomyosin and troponin [17]. Our results showing that 11 myosin proteins changed during desiccation and 2 myosins during recovery suggest that contraction caused by desiccation may modulate myosin synthesis, as suggested by previous research [18, 19]. Tropomyosin, a core component of the actin-linked regulatory system for contraction, stabilizes the actin filament and modulates muscle contraction [20]. Different isoforms of tropomyosin perform unique functions and are not redundant [21, 22]. Our results suggested that the same isoform of a protein, for example tropomyosin 2 isoform B (R24 and R5), may perform different functions. Animals contract when external water decreases. Thus, the contracted animals decrease their surface-to-volume ratio as they lose internal water during desiccation [23]. Changes of many different contractile proteins (actins, tropomyosins and myosins, including light chains and heavy chains) suggest that the midges actively contract to prevent mechanical damage during desiccation [23, 24].

Body contraction is also a key feature during rehydration, *i.e.* recovery from desiccation [23], and in moss the cytoskeleton also plays a significant role in both desiccation and rehydration [25]. During contraction, the actin skeleton is dynamic and actin remodelling occurs [26]. Tropomyosin regulates actin–myosin interactions in the cytoskeleton as well as in muscle, and the isoforms differ in their ability both to interact with myosin and to inhibit translocation of actin filaments by myosin [21]. Such interactions may account for the increased abundance of actin, myosin heavy chains and tropomyosins observed in the midge during rehydration.

Another group of proteins that increased in response to desiccation is involved in energy metabolism. Arginine kinase plays a role in maintenance of ATP levels by producing phosphoarginine, which can rapidly generate ATP. The other protein that increased in response to desiccation, enolase, is an enzyme involved in the energy-yielding phase of the glycolysis pathway. An increase in abundance of these enzymes suggests an increase in ATP production and enhanced ability of maintaining ATP levels during desiccation.

Catalase also increased in response to desiccation stress. This protein response is consistent with the elevation of mRNA encoding catalase that was also noted in response to desiccation [27]. A similar elevation in catalase was observed in response to dehydration in yeast [28]. An increase in catalase is frequently linked to oxidative stress, and desiccation possibly contributes to oxidative stress in the midge larvae.

Porin is a nonspecific channel that is permeable to hydrophilic solutes and is involved in maintenance of cell surface structure in bacteria [29]. In *B. antarctica*, an increase in porin during desiccation may be involved in the efflux of water or other solutes during this time.

Heat shock proteins play an important chaperone role in a variety of cellular stress responses [30]. In this system we found that a member of the Hsp70 family was not present in increased abundance during desiccation, but it did increase during recovery from desiccation, *i.e.* rehydraton. This result is consistent with the observation that Hsp transcripts remained unchanged in response to desiccation stress [5]. Distinct Hsp responses were also noted in response to desiccation and rehydration in the flesh fly pupae [31], but in the fly pupae Hsp23 and Hsp70 were upregulated by desiccation and Hsp90 and Hsc70 were upregulated by rehydration. This suggests important roles for Hsps in these responses but also suggests that different species may respond differently.

Anterior fat body protein-like decreased during rehydration. This protein, previously associated with stress responses, is restricted to the anterior fat body [32] and is involved in the regulation of endocytosis of hexamerin by fat body cells [33]. It is not at all clear what role it may play in association with desiccation and rehydration.

Another protein that was less abundant during rehydration is malate dehydrogenase, an enzyme involved in carbohydrate metabolism. The decrease of this protein during rehydration may be associated with depression of the gluconeogenesis pathway, a pathway that is highly activated during osmotic stress [34] but switches off as the insect reacts to its normal hydrated state.

Elongation factor 1 α is a key factor in the elongation process of protein synthesis. Besides its role in translation, it is also implicated in cytoskeletal remodelling [35]. The increase of elongation factor 1 α during rehydration suggests

increased protein synthesis. The regulation of this protein together with the cytoskeletal proteins described earlier further illustrates that reorganization of the cytoskeleton is essential to desiccation stress tolerance and recovery. Our results clearly show that both desiccation and rehydration elicit dramatic changes in protein abundance within a short period of time. The majority of proteins that are altered in abundance are associated with contraction of the body and cytoskeleton rearrangements. It is also clear from this study that rehydration is not simply the reverse of dehydration, but instead generates a distinct protein profile. The approach we used focused on changes of relatively abundant proteins. Thus, less abundant proteins, not identified by this approach, are also likely contributors to desiccation and rehydration responses in the midge larvae, and such proteins of course may be of major importance. Some proteins known to be important in desiccation responses in other species, *e.g.* the LEA proteins from Collembola [36], the elongation factors EF-Tu and EF-G from *Enterobacter sakazakii* [37] and 2-Cys peroxiredoxin from the plant *Xerophyta viscose* [38] were not detected in this study, quite possibly because they were not among the most abundant proteins.

We thank the support staff at Palmer Station for their assistance in Antarctica, David Mandich of Plant-Microbe Genomics Facility at The Ohio State University for technical assistance with image scanning and Kari Green-Church, OSU Campus Chemical Instrument Center, for assistance with mass spectrometric identification of proteins. This research was supported by NSF grants OPP-0337656 and OPP-0413786.

The authors have declared no conflict of interest.

5 References

[1] Campbell, I. B., Claridge, G. G. C., *Antarctica: Soils, Weathering Processes and Environment*. Elsevier, Amsterdam 1987.

[2] Sugg, P., Edwards, J. S., Baust, J., Phenology and life history of *Belgica antarctica*, an Antarctic midge (Diptera: Chironomidae). *Ecol. Entomol.* 1983, *8*, 105–113.

[3] Usher, M. B., Edwards, M., A dipteran from south of the Antarctic Circle: *Belgica antarctica* (Chironomidae), with a description of its larva. *Biol. J. Linn. Soc.* 1984, *23*, 19–31.

[4] Convey, P., Block, W., Antarctic Diptera: ecology, physiology and distribution. *Eur. J Entomol.* 1996, *93*, 489–505.

[5] Hayward, S. A. L., Rinehart, J. P., Sandro, L. H., Lee R. E., Jr., Denlinger, D. L., Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*. *J. Exp. Biol.* 2007, *210*, 836–844.

[6] Benoit, J. B., Lopez-Martinez, G., Michaud, M. R., Elnitsky, M. A. *et al.*, Mechanisms to reduce dehydration stress in larvae of the Antarctic midge, *Belgica antarctica*. *J Insect Physiol.* 2007, *53*, 656–667.

[7] Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee R. E.,Jr., Denlinger, D. L., Moist habitats are essential for adults of the Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae), to avoid dehydration. *Eur. J Entomol.* 2007, *104*, 9–14.

[8] Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A. *et al.*, Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing, and desiccation in the Antarctic midge, *Belgica antarctica*. *J. Insect Physiol.* 2008, *54*, 645–655.

[9] Elnitsky, M. A., Hayward, S. A. L., Rinehart, J. P., Denlinger, D. L., Lee R. E.,Jr., Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*. *J. Exp. Biol.* 2008, *211*, 524–530.

[10] Holmstrup, M., Cold hardiness strategy in cocoons of the lumbricid earthworm *Dendrobaena octaedra* (Savigny). *Comp. Biochem. Physiol.* 1992, *102*, 49–54.

[11] Chen, S., Glazer, I., Gollop, N., Cash, P. *et al.*, Proteomic analysis of the entomopathogenic nematode *Steinernema feltiae* IS-6 IJs under evaporative and osmotic stresses. *Mol. Biochem. Parasitol.* 2006, *145*, 195–204.

[12] Gouraud, S. S., Yao, S. T., Heesom, K. J., Paton, J. F. R., Murphy, D., 14-3-3 proteins within the hypothalamic-neurohypophyseal system of the osmotically stressed rat: transcriptomic and proteomic studies. *J. Neuroendocrinol.* 2007, *19*, 913–922.

[13] Li, A. Q., Popova-Butler, A., Dean, D. H., Denlinger, D. L., Proteomics of the flesh fly reveals an abundance of upregulated heat shock proteins during pupal diapause. *J. Insect Physiol.* 2007, *53*, 385–391.

[14] Carr, S., Aebersold, R., Baldwin, M., Burlingame, A. *et al.*, The need for guidelines in publication of peptide and protein identification data. *Mol. Cell. Proteomics* 2004, *3*, 531–533.

[15] Lin, J. J., Warren, K. S., Wamboldt, D. D., Wang, T., Lin, J. L., Tropomyosin isoforms in nonmuscle cells. *Int. Rev. Cytol.* 1997, *170*, 1–38.

[16] Corsi, A., Perry, S. V., Some observations on the localization of myosin, actin and tropomyosin in the rabbit myofibril. *Biochem. J.* 1958, *68*, 12–17.

[17] Sliwinska, M., Skorzewski, R., Moraczewska, J., Role of actin C-terminus in regulation of striated muscle thin filament. *Biophys. J.* 2008, *94*, 1341–1347.

[18] McDermott, P. J., Morgan, H. E., Contraction modulates the capacity for protein synthesis during growth of neonatal heart cells in culture. *Circ. Res.* 1989, *64*, 542–553.

[19] Qi, M., Puglisi, J. L., Byron, K. L., Ojamaa, K. *et al.*, Myosin heavy chain gene expression in neonatal rat heart cells: effects of [Ca2+]i and contractile activity. *Am. J. Physiol.* 1997, *273*, C394–C403.

[20] Yu, R., Ono, S., Dual roles of tropomyosin as an F-actin stabilizer and a regulator of muscle contraction in *Caenorhabditis elegans* body wall muscle. *Cell Motil. Cytoskeleton* 2006, *63*, 659–672.

[21] Gunning, P., O'Neill, G., Hardeman, E., Tropomyosin-based regulation of the actin cytoskeleton in time and space. *Physiol. Rev.* 2008, *88*, 1–35.

[22] Li, A., Denlinger, D. L., Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. *Insect Mol. Biol.* 2008, *17*, 565-572.

[23] Ricci, C., Melone, G., Santo, N., Caprioli., M., Morphological response of a bdelloid rotifer to desiccation. *J. Morphol.* 2003, *257*, 246–253.

[24] Alpert, P., The limits and frontiers of desiccation-tolerant life. *Interg. Comp. Biol.* 2005, *45*, 685–695.

[25] Proctor, M. C. F., Ligrone, R., Duckett, J. G., Desiccation tolerance in the moss *Polytrichum formosum*: physiological and fine-structural changes during desiccation and recovery. *Ann. Bot.* 2007, *99*, 75–93.

[26] Gerthoffer, W. T., Actin cytoskeletal dynamics in smooth muscle contraction. *Can. J. Physiol. Pharmacol.* 2005, *83*, 851–856.

[27] Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E., Denlinger, D. L., Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *J. Comp. Physiol. B* 2009, DOI 10.1007/s00360-008-0334-0.

[28] Franca, M. B., Panek, A. D., Eleutherio, E. C. A., The role of cytoplasmic catalase in dehydration tolerance of *Saccharomyces cerevisiae*. *Cell Stress Chaperones* 2005, *10*, 167–170.

[29] Nikaido, H., Vaara, M., Molecular basis of bacterial outer membrane permeability. *Microbiol Rev.* 1985, *49*, 1–32.

[30] Feder, M. E., Hofmann, G. E., Heat-shock proteins, molecular chapterones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 1999, *61*, 243–282.

[31] Hayward, S. A. L., Rinehart, J. P., Denlinger, D. L., Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. *J. Exp. Biol.* 2004, *207*, 963–971.

[32] Nakajima, Y., Natori, S., Identification and characterization of an anterior fat body protein in an insect. *J. Biochem.* 2000, *127*, 901–908.

[33] Hansen, I. A., Meyer, S. R., Schafer, I., Scheller, K., Interaction of the anterior fat body protein with the hexamerin receptor in the blowfly *Calliphora vicina*. *Eur. J. Biochem.* 2002, *269*, 954–960.

[34] Dihazi, H., Asif, A. R., Agarwal, N. K., Doncheva, Y., Muller, G. A., Proteomic analysis of cellular response to osmotic stress in thick ascending limb of Henle's loop (TALH) cells. *Mol. Cell. Proteomic* 2005, *4*, 1445–1458.

[35] Condeelis, J., Elongation factor 1a, translation and the cytoskeleton. *Trends Biochem. Sci.* 1995, *20*, 169–170.

[36] Bahrndorff, S., Tunacliffe, A., Wise, M. J., McGee, B., Holmstrup, M., Loeschcke, V., Bioinformatics and protein expression analyses implicate LEA proteins in the drought response of Collembola. *J. Insect Physiol.* 2009, *55*, 210–217.

[37] Riedel, K., Lehner, A., Identification of proteins involved in osmotic stress response in *Enterobacter sakazakii* by proteomics. *Proteomics* 2007, *7*, 1217–1231.

[38] Ingle, R. A., Schmidt, U. G., Farrant, J. M., Thomson, J. A., Mundree, S. G., Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscose*. *Plant Cell Environ.* 2007, *30*, 435–446.

