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RESEARCH ARTICLE

Mechanistic underpinnings of dehydration stress in the American dog tick revealed through RNA-Seq and metabolomics

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ABSTRACT

Ticks are obligate blood feeders but spend the majority of their lifetime off-host where they must contend with a multitude of environmental stresses. Survival under desiccating conditions is a determinant for habitats where ticks can become established, and water-balance characteristics of ticks have been extensively studied. However, little is known about the underlying aspects associated with dehydration stress in ticks. In this study, we examined the response of male American dog ticks, *Dermacentor variabilis*, to dehydration using a combined transcriptomics and metabolomics approach. During dehydration, 497 genes were differentially expressed, including an up-regulation of stress-response and protein-catabolism genes and concurrent down-regulation of several energetically expensive biological processes. Accumulation of several metabolites, including specific amino acids, glycerol and gamma aminobutyric acid (GABA), and transcript shifts in the associated pathways for generating these metabolites indicated congruence between changes in the metabolome and gene expression. Ticks treated with exogenous glycerol and GABA demonstrated altered water-balance characteristics; specifically, increased water absorption at high relative humidity. Finally, we observed changes in locomotor activity in response to dehydration, but this change was not influenced by the accumulation of GABA. Overall, the responses to dehydration by these ticks were similar to those observed in other dehydration-tolerant arthropods, but several molecular and behavioral responses are distinct from those associated with other taxa.

KEY WORDS: *Dermacentor variabilis*, Gene expression, GABA, Glycerol, Activity

INTRODUCTION

As obligate hematophagous arthropods, ticks face a diverse set of challenges to maintain water balance. While feeding, they ingest a large quantity of hypotonic fluid and water in the blood meal that must be extracted and expelled (Campbell et al., 2010; Kaufman and Philips, 1973; Sauer and Hair, 1972; Sonenshine, 1991). Conversely, water conservation is required during off-host periods, which for most members of Ixodidae constitutes the vast majority (>98%) of the tick's life (Needham and Teel, 1991; Norval, 1977). Ticks are particularly at risk of dehydration because of their small body size and consequent high surface area to volume ratio (Benoit and Denlinger, 2010). Therefore, a species' ability to establish itself in a particular habitat depends in large part on its ability to survive various environmental stresses, including maintenance of water content under potentially desiccating conditions.

Ticks are able to prevent dehydration through behavioral mechanisms, physical and physiological adaptations for water conservation, and the acquisition of exogenous water (Benoit and Denlinger, 2010; Needham and Teel, 1986; Sonenshine, 1991; Sonenshine and Roe, 2013). Ticks can avoid desiccating conditions by restricting host seeking to periods of high relative humidity and retreating to sheltered microhabitats at other times (Crooks and Randolph, 2006; Short et al., 1989). Water loss can be limited through changes in the cuticle, reducing respiratory water loss and limiting losses through excretion of waste (reviewed in Benoit and Denlinger, 2010). Additionally, ticks are able to absorb water from the air orally in an active, solute-driven process (water vapor absorption; Gaëde and Knülle, 1997), allowing most ticks to use water vapor as their primary mechanism for hydration during off-host periods (Needham and Teel, 1991, 1986; Sonenshine, 1991).

When conditions drop below the relative humidity at which ticks can absorb water from the air (critical equilibrium humidity, CEH; 75–95%, depending on life stage), ticks cannot absorb water vapor and begin to dehydrate (Needham and Teel, 1991; Yoder et al., 2012). Arthropods have a variety of mechanisms for preventing and tolerating dehydration-associated damage, including changes in the expression of proteins such as heat shock proteins, antioxidant enzymes and aquaporins (Benoit and Denlinger, 2010; Cornette and Kikawada, 2011; King and MacRae, 2015). Additionally, accumulation of various molecules, including trehalose and glycerol, can have protective effects during dehydration by acting to prevent damage to cellular components (Michaud et al., 2008; Watanabe, 2006; Yoder et al., 2006a).

Although characteristics of water balance in ticks have been extensively studied (reviewed in Benoit and Denlinger, 2010; Bowman et al., 2008; Knülle and Rudolph, 1982; Needham and Teel, 1991, 1986; Sonenshine, 1991), little is known about the molecular mechanisms associated with their dehydration resistance. In this study, we examined the response of the American dog tick, *Dermacentor variabilis* (Say 1821), to desiccating conditions at the transcript and metabolite levels. *Dermacentor variabilis* is a primary vector for Rocky Mountain spotted fever and tularemia (de la Fuente et al., 2008; Petersen et al., 2009), and its wide geographic distribution across most of the USA contributes to its medical importance (James et al., 2015; Sonenshine, 1993). It has been suggested that the range of *D. variabilis* is restricted by dry environments (James et al., 2015; Sonenshine, 1993), although the relationship between water-balance characteristics and habitat suitability in ticks is unclear (Benoit and Denlinger, 2010; Yoder et al., 2012). Understanding the mechanisms of dehydration tolerance can provide insight into how environmental factors

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List of symbols and abbreviations

CEH	critical equilibrium humidity
DAVID	Database for Annotation, Visualization and Integrated Discovery
e-value	expectation value
FDR	false discovery rate
GABA	gamma aminobutyric acid
GO	gene ontology
GSEA	gene set enrichment analysis
Hsp	heat shock protein
m	water mass
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance
nr	non-redundant
qPCR	real-time quantitative PCR
RH	relative humidity

contribute to the distribution of ticks and their infectious agents and may uncover novel control methods.

To elucidate the mechanisms of dehydration stress in *D. variabilis*, we examined high-throughput gene expression and biochemical shifts. RNA-sequencing (RNA-Seq)-based transcriptomics allowed us to simultaneously measure the expression of >60,000 contigs, revealing 497 genes with differential expression. Additionally, nuclear magnetic resonance (NMR)-based metabolomics identified 21 metabolites, with six that increased significantly in abundance during dehydration. Using this approach, we found several critical biochemical pathways underlying dehydration resistance in ticks, and supported these findings with functional analyses.

MATERIALS AND METHODS

Ticks and experimental design

Unfed, male *D. variabilis* were obtained from laboratory colonies maintained at the Oklahoma State University (OSU) Tick Rearing Facility (Stillwater, OK, USA). Colonies are kept at OSU under 97% relative humidity (RH), 25±1°C and a 14 h:10 h light:dark (L:D) cycle. Upon arrival, groups of 20–30 ticks were transferred to 50 cm^3 mesh-covered vials and placed in closed chambers containing a supersaturated solution of potassium nitrate, providing 93% RH (Winston and Bates, 1960) to prevent dehydration. Ticks were kept in these chambers at 26±1°C and 15 h:9 h L:D until used in experiments.

For treatments, ticks were randomly chosen from multiple rearing batches for each replicate and individually transferred to 1.5 cm^3 mesh-covered vials. Ticks were weighed (to 0.01 mg) on an electrobalance (CAHN 25, Ventron Co., Cerritos, CA, USA) at the beginning of the trial and measured daily until the treatment was complete. Ticks were handled with featherweight forceps and the weighing process required less than 30 s per individual. Desiccating conditions of 0% RH were maintained in a 5000 ml glass desiccator containing fresh anhydrous calcium sulfate with a cobalt chloride indicator (Drierite, Xenia, OH, USA). Ticks were dehydrated until they lost ∼25% of their initial body water, a process that took ∼9 days. Control ticks were kept at 93% RH as described above for 14 days. Both control and dehydrated ticks were kept at 26±1°C and 15 h:9 h L:D throughout the experiment. At the end of the treatment, ticks were flash frozen and stored at −80°C.

A subset of ticks was used to determine body water content of control and dehydrated groups. Ticks were exposed to either 93% RH or 0% RH and weighed as described above, frozen at −80°C, and then placed in a 75°C oven. Ticks were weighed until they reached a constant mass, and water content was determined by subtracting dry mass from the fresh mass. Body mass and water content were compared between groups using Student's *t*-tests.

Transcriptome generation and differential expression analysis

For each RNA sample, eight ticks were removed from −80°C and immediately homogenized together to reduce individual variation. Ticks were manually cut into quarters, placed in 1 ml of chilled TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and homogenized with a BeadBlaster 24 microtube homogenizer (Benchmark Scientific, Edison, NJ, USA). Total RNA was extracted following the manufacturer's protocol for the TRIzol reagent. RNA was treated with DNase I (Thermo Scientific, Pittsburgh, PA, USA) to eliminate potential genomic DNA contamination, and the RNA was concentrated using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific). RNA concentration and quality were determined with a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

Construction of the poly(A) library and sequencing were conducted by the DNA Sequencing and Genotyping Core at the Cincinnati Children's Hospital Medical Center (CCHMC). RNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and 150–500 ng of total RNA was poly(A) selected and reverse transcribed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, USA). Each sample was fitted with one of 96 adapters containing a different 8-base molecular barcode for high-level multiplexing. Following 15 cycles of PCR amplification, completed libraries were sequenced on a HiSeq 2500 sequencing system (Illumina) in Rapid Mode. Approximately 30 million high-quality, single-end reads that were 75 bases in length were generated per sample. Raw RNA-Seq data were uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive: Bio-project PRJNA305720.

Data generated by Illumina sequencing were trimmed for quality (using a Phred quality score limit of 0.05) and ambiguities (0 ambiguities were allowed). Sequences were trimmed to remove five and eight nucleotides from the 5′ and 3′ termini, respectively, and resulting sequences less than 40 nucleotides in length were removed entirely. The resulting cleaned sequences were analyzed with the FastQC package (S. Andrews 2010, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to verify quality. Transcripts were *de novo* assembled separately using Trinity (Grabherr et al., 2011), Velvet-Oases (Schulz et al., 2012) and CLC Genomics Workbench 7.5 (CLC Bio, Boston, MA, USA). Transcripts were assembled into contigs with Velvet (Zerbino and Birney, 2008) using a *k*-mer length of 31, and the resulting contigs were further assembled by Oases (Schulz et al., 2012) using a minimum contig length of 150. The Trinity assembly was run using the standard Inchworm, Chrysalis and Butterfly pipeline with a minimum contig length of 150. Assembly of the transcripts using CLC was performed using the default settings and a minimum contig length of 150. Coding regions were predicted for the resulting assemblies using TransDecoder (Haas et al., 2013) and transcripts predicted to encode at least 100 amino acids were retained. To remove redundant contigs, the assembly was mapped against itself and, when multiple contigs mapped together with 100% identity, only the longest contig was kept. The resulting sequences represented the full set of assembled transcripts from each assembly program.

Contigs were searched (BLASTx) against the NCBI arthropod, non-redundant (nr) protein database with an expectation value (e-value) of 0.001. Contigs were also searched against NCBI's SwissProt protein database and the reference protein sequences

(RefSeq) for fly (*Drosophila melanogaster*) and the *Ixodes* genus. For each search, the highest scoring BLAST hit was used to assign a gene ID to each contig. For contigs with a positive BLAST hit, gene ontology (GO) terms (Ashburner et al., 2000) were retrieved using the GO-mapping tool (e-value of 1×10^{-6}) and InterProScan tool in the Blast2GO program (BioBam; Conesa et al., 2005); GO annotations from these two tools were merged.

Reads were mapped to the *de novo* assembly with at least 70% of the read having at least 90% identity with the reference to be included in the mapping using CLC Genomics. A mismatch cost of 2 was used. A given read was allowed to align to no more than 20 different places on the reference assembly. Expression values were measured as total counts that were normalized by the total number of mapped reads in a sample and expressed as reads per 1 million. Fold changes were calculated by dividing the mean of the read values for the dehydrated group by the mean of the read values for the control group, and the Baggerly's test (a weighted *t*-type test statistic; Baggerly et al., 2003) was used to test the significance of the fold change. A multiple comparison correction was performed using a false discovery rate (FDR) (Benjamini and Hochberg, 1995). Transcripts were considered differentially expressed when the FDR *P*-value was ≤0.05 and the fold change was $\geq|1.5|$.

To identify pathways that were differentially regulated between control and dehydrated ticks, RNA-Seq results were analyzed with DAVID (Database for Annotation, Visualization and Integrated Discovery), Blast2GO enrichment analysis and the CLC gene set enrichment analysis (GSEA). Differentially expressed genes were blasted (BLASTx) against the *Ixodes scapularis* and *D. melanogaster* RefSeq protein sequences and the resulting hits (e-value <0.001) were submitted to the DAVID functional annotation database (http://david.abcc.ncifcrf.gov; Huang et al., 2009). GO terms, KEGG pathways (Ogata et al., 1999) and InterPro protein domains (McDowall and Hunter, 2011) were analyzed for over-representation, and functional annotations were considered significantly enriched when $P\leq0.05$. The Blast2GO enrichment analysis was also used to evaluate the differentially expressed genes to identify GO terms that were enriched as compared with the full transcriptome of *D. variabilis*. A two-tailed Fisher's exact test with a robust FDR was used, and over-represented terms were identified with a FDR ≤0.05. Finally, enriched GO terms were identified using the CLC's GSEA. Unlike the DAVID and Blast2GO analyses, which test for enrichment in a defined list of genes (those significantly up- or down-regulated), GSEA uses the expression values for all genes within a GO category when calculating enrichment scores. The GSEA was run using a standard *t*-test statistic as previously described (Tian et al., 2005), with 1000 permutations and a minimum category size of 10 genes. GO terms with $P\leq0.001$ were considered significantly perturbed between control and dehydrated ticks.

Quantitative real-time PCR

The same total RNA that was extracted and concentrated for the RNA-Seq analysis was used for quantitative real-time PCR (qPCR) analyses. Complementary DNA (cDNA) was reverse-transcribed from the concentrated RNA using DyNAmo cDNA Synthesis Kit (Thermo Scientific). Each reaction consisted of 300 ng RNA, 50 ng oligo$(dT)_{15}$ primers, reaction buffer containing dNTPs and 5 mmol l^{-1} $MgCl_2$, and M-MuLV RNase H^+ reverse transcriptase. qPCR reactions included KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich, St Louis, MO, USA), 300 nmol l^{-1} forward and reverse primers, cDNA diluted 1:50, and nuclease-free water for all reactions except superoxide dismutase. Reactions with superoxide dismutase contained 600 nmol l^{-1} primers. Primers were designed based on sequences obtained from the RNA-Seq analysis (Table S1). qPCR reactions were analyzed using an Eco Real-time PCR System (Illumina). Reactions consisted of polymerase activation for 3 min at 95°C followed by 40 cycles of denaturation for 10 s at 95°C, annealing/extension for 30 s at 55°C and denaturation for 10 s at 95°C. After amplification, a melt curve analysis was performed from 55 to 95°C with 0.5°C increments every 15 s. Each sample was run in triplicate and the average quantification cycle (C_q) value was determined. mRNA levels for the gene of interest were normalized to β-actin using the $\Delta\Delta C_q$ method as previously described (Schmittgen and Livak, 2008). The fold change in these genes for dehydrated ticks, as compared with control ticks, was determined and the logarithmic fold change was plotted against the corresponding value from the RNA-Seq analysis and a Pearson correlation coefficient (r) was determined.

Metabolome analysis

An NMR-based metabolomics approach was used to identify metabolites that showed differential abundance between control and dehydrated ticks. NMR experiments were performed at the NMR-based Metabolomics Core at CCHMC. A total of 15 ticks per sample were lyophilized overnight. Dry samples were weighted into 2 ml standard tubes containing 2.8 mm metal beads (Bertin Corp, Rockville, MD, USA) then homogenized 3 times for 30 s at 4000 rpm with a Minilys homogenizer (Bertin Corp). Polar metabolites were extracted using a modified Bligh and Dyer extraction (Bligh and Dyer, 1959). Briefly, cold methanol and water were added to the samples in bead tubes and homogenized for 30 s. The samples were transferred into glass tubes containing cold chloroform and water. The final methanol:chloroform:water ratio was 2:2:1.8. The mixture was vortexed, incubated on ice for 10 min, and then centrifuged at 2000 ***g*** for 5 min. The polar phase was transferred into a 1.5 ml tube and dried by vacuum centrifuge for 2–3 h at room temperature. The dried metabolites were re-suspended in 0.6 ml of NMR buffer containing 100 mmol l^{-1} phosphate buffer (pH 7.3), 1 mmol l^{-1} TMSP (3-trimethylsilyl-2,2,3,3-d4 propionate), and 1 mg ml^{-1} sodium azide prepared in deuterium oxide. A final volume of 550 µl of each sample was transferred into a 5 mm NMR tube (Norell Inc., Marion, NC, USA) for NMR data acquisition.

A one-dimensional ^1H Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiment was used for data acquisition on a 600 MHz INOVA NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA). Two-dimensional ^1H-^{13}C heteronuclear single-quantum correlations (HSQC) and ^1H-^1H total correlation spectroscopy (TOCSY) were also collected on representative samples for metabolite annotation. Spectral data were phase corrected, baseline corrected, and aligned to the internal reference standard peak, TMSP, using TopSpin 3.1 (Bruker, Billerica, MA, USA). Data were analyzed using both univariate and multivariate statistical analysis approaches with AMIX (v3.9.11; Bruker) and MetaboAnalyst 3.0 (Xia et al., 2015).

The identity of each metabolite was assigned using Chenomx NMR Suite 7.8 (Chenomx Inc., Edmonton, AB, Canada), two-dimensional NMR experiments, and reference spectra found in databases such as the Human Metabolome Database (Wishart, 2007), the Madison Metabolomics Consortium Database (Cui et al., 2008) and the Biological Magnetic Resonance Data Bank (Markley et al., 2008). Concentrations of metabolites were determined using the Chenomx NMR Suite integration tool and normalized to the dry mass of the ticks. Normalized metabolite concentrations were compared using Student's *t*-tests.

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Glycerol and GABA loading

To determine whether levels of glycerol or gamma aminobutyric acid (GABA), metabolites found to be elevated during dehydration, impact water-balance characteristics, ticks were injected with glycerol and/or GABA solution and exposed to various RH, as follows. Ticks (N=6–8) were injected with either insect Ringer's solution (187 mmol l^{-1} NaCl, 21 mmol l^{-1} KCl, 7 mmol l^{-1} $CaCl_2$ and 1 mmol l^{-1} $MgCl_2$) or insect Ringer's solution with 1 mol l^{-1} glycerol, 10 mmol l^{-1} GABA, or 1 mol l^{-1} glycerol and 10 mmol l^{-1} GABA. The cuticle was pierced on the ventral idiosoma just posterior to the fourth coxa with a 28.5 gauge needle, a tapered polycarbonate tip (0.19 mm outer diameter) was inserted into the body cavity, and the solution (~0.5 µl) was dispensed by a manual microdispenser (Drummond, Broomall, PA, USA). After injection, ticks recovered for 4 h at 26°C and 100% RH and were then transferred to containers with distilled water (100% RH) or a supersaturated solution of sodium chloride (75% RH).

To assess the CEH, the humidity at which ticks can balance water loss and gain, ticks were weighed pre- and post-injection and daily until they died. Changes in water mass (m), expressed as a percentage $[100(m_t-m_0)/m_0]$, were monitored and the CEH was determined after 72 h exposure using Wharton's (1985) two-point method on a plot of percentage change in water mass versus RH corresponding to where $\%\Delta m=0$. For statistical analysis, percentage data was logit transformed (Warton and Hui, 2011) and a one-way ANOVA, followed by a Dunnett's test, was used to compare among groups.

Activity study

To measure the effect of dehydration on the activity of *D. variabilis*, the movement of control and dehydrated ticks was monitored using a Locomotor Activity Monitor (TriKinetics Inc., Waltham, MA, USA) and DAMSystem3 Data Collection Software (TriKinetics). Control ticks (N=10) were removed from 93% RH, weighed and placed into 21 cm^3 cylindrical tubes. These tubes were positioned horizontally in the activity monitor and the whole apparatus was placed into a clear plastic container with a supersaturated solution of potassium nitrate to maintain 93% RH. This setup was placed in an isolated room to prevent host cues and a temperature of 26±1°C and L:D cycle of 12 h:12 h was maintained throughout the study. The monitor counted every time a tick crossed the center of the tube and the number of counts was recorded onto a digital file every hour for 120 h. At the termination of each trial, ticks were weighed again. Dehydrated ticks (N=9) were treated identically, except they were placed at 0% RH for 2 days prior to the trial and anhydrous calcium sulfate was placed in the box with the activity monitor to maintain the desiccating conditions throughout the course of the trial. A two-factor ANOVA was used to examine the response of experimental variables as a function of photoperiod or treatment, or their interaction, with pairs of means distinguished using Bonferroni tests.

To examine the potential effects of elevated levels of GABA, a neurotransmitter that affects skeletal muscle activity (Buckingham et al., 2005), on tick activity, ticks were injected with an exogenous supply of GABA and their activity was measured as described for the control ticks. Ticks (N=5 ticks per group) were injected with either insect Ringer's solution or insect Ringer's solution with 1 or 10 mmol l^{-1} GABA, as described above. These treatments were compared with a one-way ANOVA.

RESULTS

Experimental dehydration

To examine the transcriptional and metabolic responses of *D. variabilis* to dehydration, ticks were exposed to either dehydrating (0% RH) or control (93% RH) conditions. Ticks in the experimental and control groups did not differ (t_{149}=0.06, P=0.955) in their starting mass (5.87±0.09 and 5.88±0.08 mg, respectively). The final body mass of the control ticks (5.90±0.10 mg) did not differ (t_{152}=0.2, P=0.827) from the starting mass; however, the mass of the dehydrated ticks (5.01±0.08 mg) decreased (t_{146}=7.7, P<0.0001) over the course of the treatment, resulting in a lower (t_{149}=7.1, P<0.0001) final mass as compared with that of control ticks. It was assumed that this loss of mass was due entirely to water loss as there was no difference (t_{24}=0.1, P=0.930) in dry mass between the two groups. Body water content of ticks exposed to dehydrating conditions was lower (t_{26}=12.4, P<0.0001) than that of fully hydrated controls (1.00±0.02 and 1.36±0.02 mg water mg^{-1} dry mass, respectively). Assuming that the dehydrated ticks started the treatment with a body water content similar to that of controls (57.6±0.3% water by fresh mass), they lost an average of 25.7% of their body water and attained a hydration state of 50.0±0.6% water by fresh mass by the end of the trial.

Transcriptome analysis

Six cDNA libraries were generated from the total homogenates of control and dehydrated ticks (3 per group). These libraries were sequenced using Illumina HiSeq technology, resulting in 271,494,907 cleaned reads. Sequences from all samples were used in the *de novo* transcriptome assembly using the Velvet-Oases, Trinity and CLC Genomics Workbench assembly programs. The Velvet-Oases assembly was superior to the other assemblies in several aspects, including contig length and similarity of contigs to known genes (Table 1); however, because of the very low number of reads that mapped to this assembly, it was discarded. The Trinity and CLC assemblies were similar in most aspects, and the Trinity assembly was chosen for further analysis because of the higher number of reads that mapped to this assembly (Table 1). For the Trinity assembly, contig sizes ranged from 300 to ~10,000 bases, with the number of contigs decreasing with increasing contig length (Fig. S1). Contigs were annotated by searching NCBI's arthropod nr and SwissProt database as well as the *Ixodes* RefSeq sequences using BLASTx. Approximately 65% of the contigs matched to proteins in the arthropod nr database with an e-value ≤0.001, which is a value similar to other studies on tick transcriptomes (Xu et al., 2015). The majority were to the deer tick (*I. scapularis*), with 25,662 matches (Fig. 1). Most of the contigs showed more than 50% similarity with their respective top BLAST match (Fig. S2). Functional annotation based on GO terms is summarized in Fig. S3. For biological processes, the majority of contigs were categorized as metabolic, cellular and/or single-organism processes. Many contigs were assigned to binding and catalytic activity for the molecular functions, and cell part, membrane and organelle made up the majority of the cellular components.

Thirty-eight per cent (25–46%) of reads from control ticks and 42% (31–47%) of reads from dehydrated ticks mapped to the reference assembly. Read counts were normalized to their respective number of reads that mapped, and these normalized counts were used to compare the expression patterns of the annotated contigs (genes) between the control and dehydrated ticks. We found 497 genes with expression levels significantly different between control and dehydrated ticks. The majority of those genes (392) were up-regulated in dehydrated ticks. The full set of differentially regulated genes is presented in Table S2. Among the genes that were significantly up-regulated during dehydration were several associated with dehydration tolerance, including antioxidants,

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Table 1. Summary of the *de novo* assembly of the transcriptome of *Dermacentor variabilis* using three assembly programs

	Velvet-Oases	Trinity	CLC
Total number of contigs	55,057	61,800	40,231
N50	936	645	597
Average contig length (bp)	811	609	582
Maximum contig length (bp)	10,020	10,311	10,449
Total number of bases	44,678,132	37,659,819	23,423,616
[a]Percentage contigs that aligned to fruit fly, BLASTX (10^{-5})	51	42	39
[b]Percentage contigs that aligned to *Ixodes*, BLASTX (10^{-5})	72	60	60
[c]Percentage Arthropod BUSCO genes that aligned to assembly, TBLASTN (10^{-5})	92	94	91
[c]Percentage *Ixodes* BUSCO genes that aligned to assembly, TBLASTN (10^{-5})	93	95	92
[d]Percentage *D. melanogaster* CORE genes that aligned to assembly, TBLASTN (10^{-5})	66	68	66
[e]Percentage mapped back	25	40	31

N50, shortest sequence length at 50% of the transcriptome.
[a]Fruit fly, *Drosophila melanogaster*, proteins were from the NCBI's available reference sequences (RefSeq) as of February 2015.
[b]Proteins from *Ixodes* genus were from the NCBI's available RefSeq as of February 2015.
[c]The arthropod set of Benchmarking sets of Universal Single-Copy Orthologs (BUSCO) were downloaded from OrthoDB (Simão et al., 2015).
[d]Core eukaryotic genes dataset for *D. melanogaster* was acquired from the CEGMA database (Parra et al., 2007).
[e]Average percentage of reads from all six RNA-Seq samples that mapped onto the assembled transcriptome.

transporters and membrane-associated proteins (Table 2). To validate our RNA-Seq results, we used qPCR to measure the expression of three genes, normalized to β-actin. qPCR and RNA-Seq results were similar based on the Pearson correlation coefficient (r=0.971; Fig. S4).

Regulation of functional pathways was analyzed using several enrichment analysis methods, including DAVID, Blast2GO and CLC. The set of differentially regulated genes was searched (BLASTx, e-value ≤0.001) against the RefSeq proteins for *I. scapularis* and *D. melanogaster* and the resulting hits (339 and 254 genes, respectively) were analyzed with the DAVID functional annotation tool. Of the BLAST hits for *I. scapularis* and *D. melanogaster*, 239 and 187 of the genes, respectively, possessed functional annotation in the DAVID database. GO-FAT term, KEGG pathways and InterPro protein domains were tested for enrichment and the results of this analysis are shown in Table S3. Enriched GO terms in the differentially expressed genes were also identified using the Blast2GO enrichment analysis. This analysis compared the GO terms of the differentially expressed genes with those of the entire transcriptome of *D. variabilis* to determine enrichment. Of the up- and down-regulated genes, 213 and 36 were successfully annotated, respectively, and 6018 of the genes of the total transcriptome were assigned GO terms. Enrichment analysis with Blast2GO did not detect any GO terms that were significantly over-represented. Finally, CLC's GSEA was used to identify GO terms that were differentially expressed between control and dehydrated ticks. The GSEA tests for enrichment across an entire GO category, not just in individual genes; thus, it has the ability to detect differential expression of functional pathways. Results of the GSEA analysis are presented in Table S4. By using different enrichment analyses, several pathways that are well known to contribute to dehydration tolerance as well as other pathways, possibly tick specific, were found to be up-regulated following desiccation exposure (Table 3).

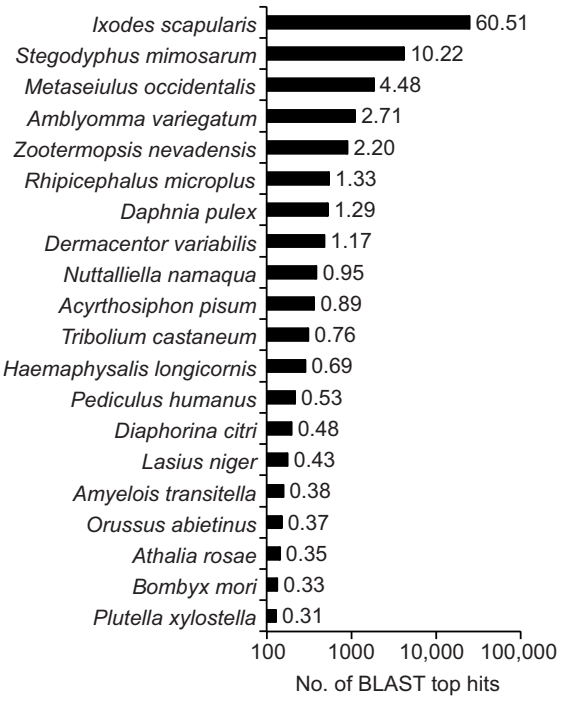

Fig. 1. Summary of the species distribution of the top BLAST hits for the contigs of the transcriptome of *Dermacentor variabilis*. Only the 20 species with the most BLAST hits are shown. A cut-off expectation value (e-value) of 0.001 was used. Numbers beside bars represent the percentage of contigs represented by each species out of the total number of contigs.

Metabolome analysis

To identify differences in metabolite concentrations, the metabolomes of control and dehydrated ticks were analyzed using NMR-based metabolomics. A representative ^{1}H NMR spectrum of a whole-tick homogenate is shown in Fig. S5. These spectra were used to identify and determine the concentration of 21 metabolites, of which six were significantly more abundant in the dehydrated ticks as compared with controls (Table 4). No metabolites examined were more abundant in the control ticks. Both principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using the concentrations of the 21 metabolites, and the analyses indicated that 69.6%, 78.4%, 85.2%, 89.6% and 93.1% of the variance was cumulatively accounted for by the first five PCs, respectively (for PLS-DA: 68.8%, 77.4%, 82.6%, 86.5% and 90.5%). A scores plot analysis using the first two PCs indicated that the NMR spectra of the control and dehydrated ticks separated into two distinct clusters that were discriminated at the 95% confidence level, indicating that the metabolic profiles of these two groups differed (Fig. 2).

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Table 2. Expression of specific genes involved in dehydration stress in *D. variabilis*

Category	Gene	Contig	Description	Arthropod nr homolog	BLASTx e-value	Fold change	FDR *P*-value
Antioxidant/ Detoxification	CYP	m.39304	CYP, putative	XP_002404794	1.65E−161	3.08	1.07E−03
		m.65086	CYP, putative	XP_002435754	8.28E−121	6.98	7.36E−06
		m.65088	CYP, putative	XP_002435754	6.09E−143	10.84	1.31E−07
		m.65091	CYP, putative	XP_002435754	0	5.21	3.54E−02
		m.65092	CYP, putative	XP_002435754	8.28E−121	6.40	3.31E−05
		m.65093	CYP, putative	XP_002435754	8.28E−121	6.09	1.06E−08
		m.65095	CYP, putative	XP_002435754	3.93E−75	5.92	3.11E−05
	GST	m.19343	Putative GST	ACF35504	1.07E−163	2.61	1.14E−04
		m.19344	Putative GST	ACF35504	5.20E−74	4.12	5.33E−15
		m.19345	Putative GST	ACF35504	1.30E−53	2.88	2.37E−13
		m.20794	Putative GST	ACF35504	2.80E−156	2.90	2.73E−05
		m.38569	Putative GST	ACF35539	1.60E−147	3.41	1.92E−06
	Peroxidase	m.25724	Peroxinectin, putative	XP_002403898	0	4.08	2.66E−05
	SOD	m.7032	Cu–Zn SOD	AGE89778	3.44E−56	4.01	4.93E−02
Chaperone	Hsp70	m.43564	Heat shock 70 kDa protein cognate 4-like	XP_008471042	2.17E−55	2.18	1.95E−04
Structural	Tubulin	m.55806	α-Tubulin	AAG49533	2.26E−132	2.11	2.08E−02
Transport	Amino acid transporter	m.61508	Sodium/chloride-dependent transporter, putative	XP_002415934	0	7.08	1.91E−04
	Cl^- channel	m.55626	H^+/Cl^- exchange transporter 3-like	XP_003740736	0	1.66	1.92E−07
	Organic cation porter	m.57522	Organic cation/carnitine transporter, putative	XP_002414402	0	2.93	1.47E−04
		m.59196	Organic cation/carnitine transporter, putative	XP_002409745	1.88E−122	7.20	1.67E−02
Membrane restructuring and lipid synthesis	Fatty acid synthase	m.57559	Fatty acid synthase-like	XP_002403503	0	2.63	1.53E−02
	Fatty acid-CoA ligase	m.67524	Long-chain fatty acid CoA ligase, putative	XP_002407507	0	1.99	1.98E−05
		m.75395	Long-chain fatty acid CoA ligase, putative	XP_002407507	5.73E−106	2.37	3.37E−02

CYP, cytochrome P450; GST, glutathione *S*-transferase; SOD, superoxide dismutase; CoA, coenzyme A; nr, non-redundant; e-value, expectation value; FDR, false discovery rate.

Functional study

Glycerol and GABA were identified as potentially important molecules during tick dehydration, based on metabolomics and RNA-Seq data (Tables 3, 4; Table S2). To elucidate their potential roles, ticks were injected with glycerol, GABA, or a combination of the two and their effects on water characteristics and activity were examined. Seventy-two hours post-injection, there was a difference in water gain at 100% RH (Fig. 3A; $F_{3,25}$=3.7, P=0.025). Each of the experimental groups gained ∼2-fold more water (P<0.05 in each pairwise comparison) than ticks injected with insect Ringer's solution. Ticks at 75% RH showed no difference in water loss among the groups (Fig. 3B; $F_{3,24}$=1.4, P=0.258). Based on the two-point system (Fig. 3C), the CEH for ticks injected with glycerol, GABA, or both was slightly lower (92–93%) than for ticks injected with insect Ringer's solution (95%), although this was not analyzed statistically.

To evaluate the effects of dehydration on activity, ticks were placed in horizontally positioned chambers at 93% (control) or 0% RH and their activity (number of times they moved across the center of the tube) was monitored for 5 days. During the first several hours, both control and dehydrated ticks showed high activity, likely due to the effects of handling and residual host cues. Therefore, the first 24 h were removed from the statistical analyses. Control ticks showed little to no movement after 24 h whereas dehydrated ticks demonstrated cyclic activity, with the highest bouts of activity during the scotophase or early photophase (Fig. 4A). Overall, dehydrated ticks showed higher ($F_{1,34}$=15.5, P=0.0004) activity than control ticks and activity was further increased ($F_{1,34}$=8.2, P=0.0073) in the scotophase as compared with the photophase. Most of these differences were driven by the high activity of dehydrated ticks during the dark phase (Fig. 4B).

To determine whether GABA had any effects on activity, ticks were injected with 1 or 10 mmol l^{-1} GABA or insect Ringer's solution, and their activity was monitored at 93% RH. Ticks injected with GABA or insect Ringer's solution behaved similarly to control ticks at 93% RH in that there was very little activity during both the photophase and scotophase. Ticks injected with 1 mmol l^{-1} GABA, 10 mmol l^{-1} GABA, or insect Ringer's solution showed no difference ($F_{2,12}$=0.315, P=0.736) in total activity. As represented by arbitrary units of activity, values for these groups were 11.2±7.8, 19.5±10.7 and 27.8±23.2, respectively.

DISCUSSION

The American dog tick is a vector for a variety of diseases, including tularemia and Rocky Mountain spotted fever, and where tick species and their pathogens can establish themselves is dependent on the ticks' ability to survive in a particular habitat (Goethert and Telford, 2009; Klompen et al., 1996). In general, hard ticks (Ixodidae) are resistant to environmental stresses like dehydration, particularly when compared with other blood-feeding arthropods (Benoit and Denlinger, 2010). However, little is known about the molecular and biochemical mechanisms of dehydration tolerance in ticks. In the present study, we identified shifts in transcript levels and the metabolome that revealed several biological pathways that are likely important in the response to dehydration. Additionally, we demonstrated a role for glycerol and GABA in relation to water

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Table 3. Pathways enriched in dehydrated *D. variabilis*

Category	GO type	GO category	Description	Gene count	*P*-value	GSEA/ DAVID
Structural	BP	007015	Actin filament organization	34	0.01	GSEA
	MF	051015	Actin filament binding	83	<0.001	GSEA
	MF	008092	Cytoskeletal protein binding	27	<0.001	GSEA
	MF	005200	Structural constituent of cytoskeleton	89	0.01	GSEA
	MF	005201	Extracellular matrix structural constituent	83	0.02	GSEA
Transport	BP	006811	Ion transport	40	<0.001	GSEA
	MF	005254	Cl^- channel activity	15	<0.001	GSEA
Membrane restructuring and lipid synthesis	BP	006869	Lipid transport	236	<0.001	GSEA
	BP	001676	Long-chain fatty acid metabolic process	23	<0.001	GSEA
	BP	006633	Fatty acid biosynthetic process	59	0.02	GSEA
	MF	004767	Sphingomyelin phosphodiesterase activity	68	<0.001	GSEA
	MF	004467	Long-chain fatty acid-CoA ligase activity	23	<0.001	GSEA
	MF	004312	Fatty acid synthase activity	12	<0.001	GSEA
	MF	005319	Lipid transporter activity	208	0.01	GSEA
Transcription/translation	BP	006352	DNA-dependent transcription, initiation	24	<0.001	GSEA
	BP	001731	Formation of translation preinitiation complex	48	0.01	GSEA
	BP	006446	Regulation of translational initiation	43	0.01	GSEA
	BP	006412	Translation	343	0.03	GSEA
	BP	006351	Transcription, DNA dependent	253	0.03	GSEA
	MF	003743	Translation initiation factor activity	257	<0.001	GSEA
	MF	003700	Sequence-specific DNA-binding transcription factor activity	310	0.04	GSEA
Protein catabolism	BP	006511	Ubiquitin-dependent protein catabolic process	186	0.01	GSEA
	BP	051603	Proteolysis involved in cellular protein catabolic process	15	0.01	GSEA
	BP	032268	Regulation of cellular protein metabolic process	6	0.03	DAVID
	BP	016567	Protein ubiquitination	230	0.04	GSEA
	MF	008233	Peptidase activity	168	<0.001	GSEA
	MF	004298	Threonine-type endopeptidase activity	32	<0.001	GSEA
	MF	034450	Ubiquitin-ubiquitin ligase activity	19	0.01	GSEA
	MF	016805	Dipeptidase activity	10	0.01	GSEA
	MF	004177	Aminopeptidase activity	87	0.04	GSEA
	MF	008237	Metallopeptidase activity	149	0.05	GSEA
Cell cycle	BP	007067	Mitosis	38	0.01	GSEA
	BP	016055	Wnt receptor signaling pathway	21	0.01	GSEA
	BP	001558	Regulation of cell growth	17	0.01	GSEA
	BP	010948	Negative regulation of cell cycle process	3	0.03	DAVID
	BP	045786	Negative regulation of cell cycle	3	0.04	DAVID
Chromatin organization	BP	034968	Histone lysine methylation	101	<0.001	GSEA
	BP	016568	Chromatin modification	6	0.02	DAVID
	BP	016571	Histone methylation	3	0.03	DAVID
	BP	016570	Histone modification	4	0.04	DAVID
	BP	016569	Covalent chromatin modification	4	0.04	DAVID
	BP	031060	Regulation of histone methylation	2	0.04	DAVID
	MF	018024	Histone-lysine N-methyltransferase activity	115	<0.001	GSEA
GABA metabolism	BP	006836	Neurotransmitter transport	81	<0.001	GSEA
	BP	009448	gamma-aminobutyric acid metabolic process	10	0.01	GSEA
	MF	005328	Neurotransmitter:sodium symporter activity	79	<0.001	GSEA
	MF	003867	4-aminobutyrate transaminase activity	10	0.02	GSEA

GO, gene ontology; BP, biological process; MF, molecular function; GSEA, gene set enrichment analysis; DAVID, Database for Annotation, Visualization and Integrated Discovery.

balance of *D. variabilis* and showed changes in locomotor activity during dehydration.

Experimental dehydration

During the dehydration treatment, ticks lost ~26% of their body water, an amount that approaches their limit of tolerance (~29%; Yoder et al., 2012). The rate of water loss, 0.12% h^{-1}, was lower than that previously reported for this species (Yoder et al., 2012) and may be the result of slightly different rearing conditions. In this study, ticks were maintained at 93% RH, whereas 97% RH was used in previous studies (Yoder et al., 2012). Although both conditions are above the CEH, this slight difference in RH can elicit effects on water characteristics of larvae (Yoder et al., 2006b). The possibility that this minor variation in RH can stimulate

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Table 4. Concentration of metabolites in whole-tick homogenates of *D. variabilis* held in control and dehydrating conditions

Metabolite	Control	Dehydrated	*P*-value
Alanine	20.94±1.00	23.70±0.36	0.021
Aspartate	1.70±0.11	2.17±0.10	0.012
GABA	4.30±0.19	5.21±0.14	0.004
Glycerol	13.68±0.69	15.81±0.49	0.030
Glycerophosphocholine	4.27±0.24	5.19±0.20	0.016
Leucine	4.31±0.20	4.88±0.11	0.025
Acetate	1.82±0.08	1.91±0.05	0.379
Acetone	1.35±0.04	1.54±0.07	0.053
Betaine	12.83±0.67	12.83±0.36	0.994
Choline	7.06±0.28	7.32±0.17	0.422
Glucose	27.72±1.13	27.77±0.77	0.970
Glutamate	18.46±1.21	20.76±0.63	0.111
Glycine	11.97±0.47	12.90±0.19	0.079
Isoleucine	2.31±0.11	2.54±0.09	0.131
Lactate	20.45±0.92	22.67±0.75	0.089
Methionine	1.33±0.06	1.47±0.06	0.123
Phenylalanine	1.77±0.07	1.93±0.06	0.128
Succinate	3.17±0.21	3.63±0.07	0.056
Threonine	4.83±0.40	5.71±0.20	0.068
Uracil	1.42±0.05	1.54±0.03	0.086
Valine	7.84±0.41	8.71±0.22	0.080

Groups of 15 ticks were pooled for analysis and values were determined from N=5 and 6 samples for control and dehydrated, respectively. Mean±s.e.m. values (nmol mg^{-1} dry mass) were compared with Student's t-tests and considered significant at P<0.05. Metabolites with significant differences are shown above the line within the table.

physiological responses that impact water balance warrants further investigation.

Dehydration-induced changes to the transcriptome

In response to dehydration, a suite of differentially regulated genes that likely contributes to the tolerance of desiccating conditions was identified. Several gene categories that are well known to be up-regulated in dehydrated arthropods were among the genes that showed increased expression (Table 2). Enzymes such as glutathione *S*-transferase and superoxide dismutase, which possess antioxidant and/or detoxification properties, were elevated, presumably to limit damage from oxygen radicals caused by desiccation-induced stress (Benoit and Denlinger, 2010; França et al., 2007; Lopez-Martinez et al., 2009). Several specific genes and multiple GO categories associated with membrane and cytoskeletal proteins were up-regulated (Tables 1 and 2) and may represent a common response to cellular water stress for most arthropods (Li et al., 2009; Sanders et al., 2003). Changes to these structural components could facilitate the restructuring of the membrane to regulate water movement and/or stabilize cells during osmotically induced changes to pressure and cell size (Benoit and Denlinger, 2010). Although aquaporins are also an important factor in regulating levels of cellular water (Benoit et al., 2014; Campbell et al., 2008), we did not observe any significant expression changes in aquaporin genes during dehydration (Table S2). Other transport proteins, which may contribute to water conservation or in the balance of ion concentrations, were up-regulated, including chloride transporters.

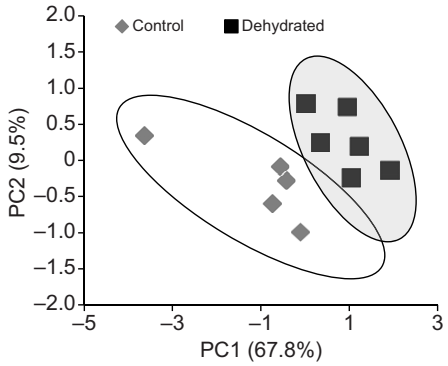

Fig. 2. A partial least squares discriminant analysis (PLS-DA) scores plot of control and dehydrated tick specimens. Each point represents the metabolic signature of one tick homogenate (15 ticks per homogenate). The directionality of the separation was observed in both the principal component (PC)1 and PC2 directions; taken together, these PCs account for 77.3% of the observed variance. The observed separation of the two groups into distinct clusters indicated that the metabolic profiles of the groups were significantly different.

Another potentially important gene that was up-regulated was a homolog of heat shock protein (Hsp)70. Hsps have been well studied in arthropod dehydration (Benoit and Denlinger, 2010) and up-regulation of Hsp70 has been noted during dehydration in various species (Benoit et al., 2009; Hayward et al., 2004; Teets et al., 2012). Hsps can act to prevent protein damage through chaperone activity or target damaged proteins to recycle peptides and amino acids (Feder and Hofmann, 1999; Goldberg, 2003). Indeed, the GSEA and DAVID analyses showed an enrichment of ubiquitin-dependent proteolysis (Table 2). This coordinated up-regulation of proteolysis genes and Hsps occurs in other dehydration-tolerant arthropods (Teets et al., 2012) and may be a common response in arthropods to break down proteins damaged during dehydration.

Among dehydration-tolerant arthropods, metabolic depression is a common adaptation that helps to minimize water loss (Marron et al., 2003). Unlike other dehydrated arthropods, which reduce oxygen consumption rates (Benoit et al., 2007), ticks increase their metabolic rate during dehydration, presumably to produce metabolic water and defend against dehydration (Dautel, 1999; Fielden and Lighton, 1996). Contrary to this increased metabolic rate, we found several indicators of energy conservation in the RNA-Seq data, including cell cycle arrest and reduced DNA replication and repair (Table 2; Table S3). It is possible that ticks reduce non-essential processes for conservation of energy stores to ensure their availability for metabolic water production and energy for host questing. In fact, sufficient energy reserves are likely necessary to survive long-term dehydration (Dautel, 1999; Fielden and Lighton, 1996).

Dehydration can lead to changes in the regulation of transcription/translation (Rajpurohit et al., 2013; Wang et al., 2011), with some insects showing a general decrease in protein synthesis through a down-regulation of transcription-related genes (Rajpurohit et al., 2013). However, dehydrated *D. variabilis* showed an up-regulation of pathways associated with transcription and translation initiation (Table 2; Table S4). Additionally, transcription may have been regulated through changes in the chromatin organization, as there was an up-regulation of chromatin-related pathways, particularly histone lysine methylation. The methylation of lysine residues on histones can act in gene silencing or transcriptional activation, depending on the precise nature of the methylation (Martin and Zhang, 2005). This epigenetic regulation of transcription may contribute to the maternal effects on the characteristics of water balance observed in larvae of *D. variabilis* when their mothers are exposed to low humidities before oviposition (Yoder et al., 2006b).

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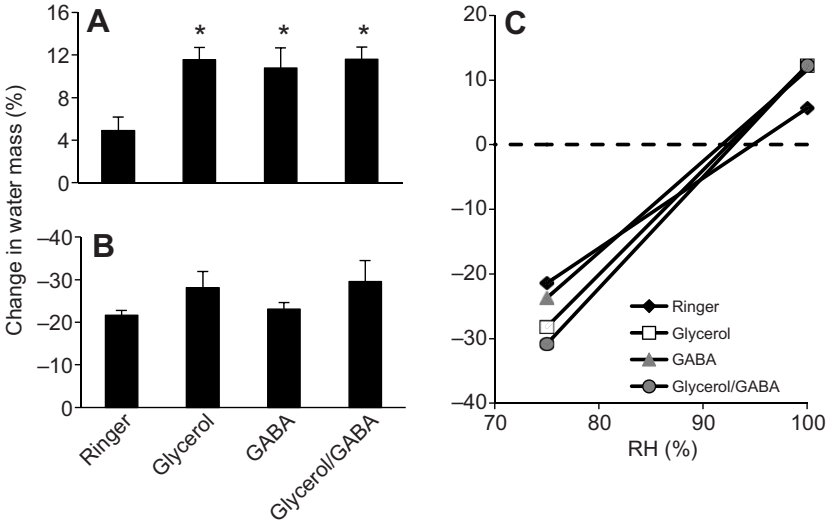

Fig. 3. Effect of exogenous glycerol and gamma aminobutyric acid (GABA) on water gain/loss in *D. variabilis*. Ticks were exposed to (A) 100% relative humidity (RH) and (B) 75% RH. (C) Percentage change in water mass of *D. variabilis* after 72 h at dehydrating and hydrating RH. The critical equilibrium humidity (CEH) is identified by a dashed line corresponding to 0% change in mass. Means±s.e.m., *N*=8 ticks per group. Asterisks indicate a significant difference from the corresponding value for Ringer solution-injected ticks (control; one-way ANOVA/Dunnett's test, $P<0.05$). The CEH was not analyzed statistically.

Dehydration-induced changes to the metabolome

Various molecules increase in concentration during desiccation to serve a protective role (Goyal et al., 2005), and *D. variabilis* showed several metabolic responses to dehydration that were supported by the transcriptome data. There was a significant increase in several amino acids during dehydration, including alanine, aspartate and leucine, and multiple other amino acids showed higher concentrations, although not significantly (Table 4). The higher level of amino acids was likely the result of an increase in protein catabolism, which was reflected in the up-regulation of proteolysis genes. These free amino acids may act in a colligative way to increase osmolality of body fluids and regulate osmotic levels, or they may be used as an energetic source and/or as substrate for the production of glycerol (discussed below). Alternatively, this increase may not be protective in nature but a consequence of degradation of proteins damaged during dehydration, a common consequence of excessive dehydration (França et al., 2007).

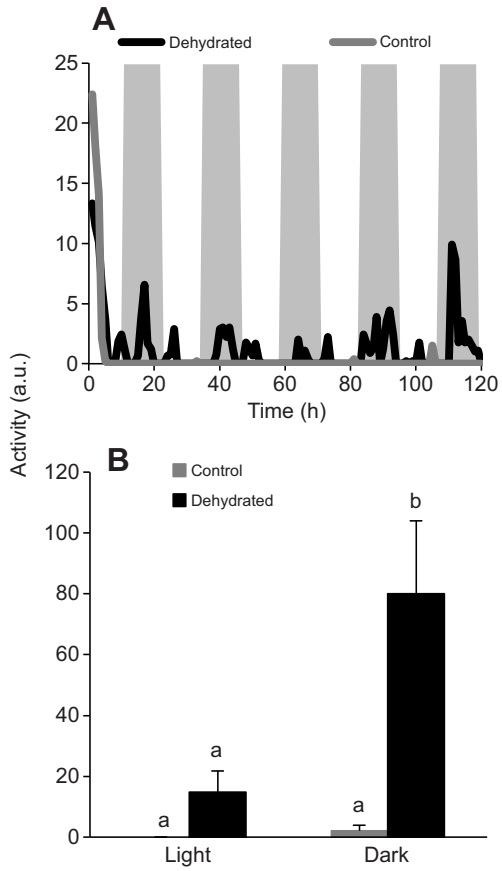

Fig. 4. Effect of dehydration on locomotor activity of *D. variabilis*. (A) Mean activity (assessed as the number of times the tick crossed the center of the tube per hour, and expressed as arbitrary units, a.u.) for dehydrated and control ticks over the course of 5 days. Light and dark (shaded areas) periods are shown. (B) Mean±s.e.m. activity for control and dehydrated ticks (*N*=9 and 10 ticks per group for dehydrated and control ticks, respectively) during the light and dark periods of the activity trial (first 24 h excluded). Different lowercase letters indicate a significant difference between groups.

The neurotransmitter GABA increased in concentration during dehydration, and this accumulation was coordinated with an up-regulation of GABA-related genes, including genes encoding a GABA transporter and a GABA receptor-associated protein (Table 3; Table S2). In arthropods, GABA and its receptor serve as inhibitors of synaptic transmission (Buckingham et al., 2005), but GABA has also been demonstrated to alter secretion by the salivary glands of ticks (Lindsay and Kaufman, 1986). Because of the tissue-specific effects of GABA, it is difficult to pinpoint whether there are one or more functions of GABA during dehydration. However, the accumulation of GABA by other desiccation-tolerant arthropods (Teets et al., 2012) does suggest an important role for this molecule during dehydration and our functional assessment of GABA (discussed below) supports this possibility.

Glycerol is a commonly accumulated molecule in stressed arthropods and is capable of suppressing water loss and improving dehydration tolerance (Benoit and Denlinger, 2010; Benoit et al., 2007; Michaud et al., 2008; Yoder et al., 2006a). Glycerol accumulation likely relies on biosynthetic pathways that utilize glycogen stores, although lipids may also serve as a source of glycerol (Li et al., 2002; Zachariassen, 1985). The source of glycerol in dehydrated *D. variabilis* is unclear; however, the up-regulation of pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK) and enolase suggests that pyruvate or other Krebs cycle-associated substrates, including amino acids from the increased proteolysis, may contribute to the accumulation of glycerol. This glycerogenesis pathway contributes to glycerol synthesis in mammals, with PEPCK acting as the rate-limiting step (Kalhan et al., 2001). Alternatively, glycerol could be released

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from triglycerides through lipolysis, with the resulting free fatty acids being a good potential source of metabolic water (Arrese and Soulages, 2010). In fact, several lipid metabolism pathways were upregulated with dehydration (Tables S2 and S4), supporting an increase in lipolysis during dehydration. Further study is needed to fully elucidate glycerol synthesis in ticks.

Functional analysis of glycerol and GABA during dehydration

The dehydration-induced accumulation of glycerol and GABA suggests an osmoprotectant role for these molecules. The effect of exogenously supplied glycerol and GABA on the water characteristics of *D. variabilis* suggests that these molecules at least play a role in the recovery from dehydration. These injections did not reduce water loss at 75% RH (Fig. 3B), nor did they affect time to mortality when ticks were placed at 0%, 70% or 75% RH (Table S5). This is likely because even a 3-fold increase in osmolality only slightly reduces water loss rates (Benoit and Denlinger, 2010). Instead of reducing water loss, glycerol and GABA seemed to improve water vapor absorption when ticks were placed above their CEH (Fig. 3A). By increasing water vapor uptake, glycerol and GABA conceivably would facilitate recovery from dehydration, reduce the amount of time that ticks need to spend in sheltered, humid microhabitats, and increase opportunities for host-seeking behavior.

Water uptake occurs at the mouthparts, with the salivary glands as the likely source of the hygroscopic material involved in water vapor absorption (Bowman et al., 2008; Sonenshine, 1991). Although the components of the rehydration saliva are not completely known, it has been speculated that glycerol, a highly hygroscopic polyol, might be a significant component (Needham and Teel, 1986). The increase of glycerol may promote the production of hyperosmotic saliva, thereby facilitating water absorption when ticks return to more humid conditions. The increase in glycerol we observed in the whole tick was relatively modest; however, if the accumulation was predominately in the salivary glands, changes in glycerol levels could be substantial enough to contribute to saliva production and increase the rate of water vapor absorption. GABA potentiates the effects of dopamine on stimulating fluid secretion by the salivary glands (Lindsay and Kaufman, 1986), and levels of GABA increase during feeding and spike immediately following feeding (Lucien et al., 1995). The role of GABA in modulating fluid secretion may extend into periods of dehydration and contribute to generating hygroscopic saliva for water vapor absorption. The lack of an additive effect of glycerol and GABA on water gain (Fig. 3A) suggests that these molecules may be working through the same mechanism to facilitate water gain, potentially through the production of glycerol-containing saliva necessary for water vapor absorption.

There was a distinct difference in the activity of fully hydrated and dehydrated ticks. The fully hydrated ticks showed little to no movement, consistent with other studies (Crooks and Randolph, 2006; Perret et al., 2003), and this was likely due to the absence of host cues and the horizontal position of the testing tube, as locomotion is conventionally thought to be predominately in the vertical plane (Crooks and Randolph, 2006). Conversely, dehydrated ticks showed relatively high activity levels, predominately during or immediately following periods of darkness. The increase in movement during dehydration and the preference for nocturnal walking is consistent with the findings in nymphs of *I. ricinus* (Perret et al., 2003). GABA alone does not seem to play a role in this dehydration-induced behavior, as there was no increase in activity when exogenous GABA was supplied.

Under dehydrating conditions, ticks will retreat to microhabitats with higher relative humidity (Crooks and Randolph, 2006; Short et al., 1989). The increase in activity was likely an attempt by dehydrated ticks to move to areas above their CEH for rehydration. This is supported by Crooks and Randolph (2006), who found that dehydrated ticks are more likely to move towards areas of fully saturated air than drier air. Greater activity would cause an increase in respiration and thus spiracle opening (Lighton et al., 1993), resulting in increased water loss (Knülle and Rudolph, 1982; Rudolph and Knülle, 1979). Therefore, ticks must balance the cost of increased movement with the need to find more suitable microhabitats, and restricting movement to dark periods, when desiccation risk is lower under natural conditions, would limit the negative effects of activity.

Conclusions

Ticks spend nearly all of their lives off-host, and maintaining body water homeostasis is extremely important to their survival (Needham and Teel, 1991). We found that *D. variabilis* exhibit a suite of biochemical and molecular responses that likely improves their survival during dehydration. Although we studied a single tick species, many of the responses, including up-regulation of stress-response and proteolysis-related genes, are common across a diverse range of arthropods (Benoit and Denlinger, 2010; Cornette and Kikawada, 2011; King and MacRae, 2015; Teets et al., 2012), suggesting that our findings could be applicable to ticks in general; however, further study is needed to verify that all ticks respond similarly to dehydration. Despite these efforts to reduce stress, the ability of ticks to cope with desiccation is not particularly extraordinary, and their tolerance of ∼30% body water loss (Yoder et al., 2012) is similar to that of many terrestrial arthropods (Hadley, 1994). Ticks instead rely on their relatively high resistance to water loss (Benoit and Denlinger, 2010) during periods of desiccation and their ability to reabsorb water vapor when conditions are favorable. Water reabsorption is likely facilitated by the accumulation of glycerol and GABA, which we observed in dehydrated ticks, and potential utilization of these molecules by the salivary glands to facilitate recovery from dehydration may be novel to acarines. Overall, ticks seem to respond to dehydration by activating means to reduce stress from water loss while concomitantly priming mechanisms to recover water stores when the relative humidity increases above the CEH.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.J.R. and J.B.B. conceived the study; A.J.R., L.E.R.-R., M.W. and M.E.D. designed the experiments and collected the data; A.J.R., M.W. and L.E.R.-R. analyzed the data; A.J.R. wrote the paper; A.J.R., L.E.R.-R., M.W., M.E.D. and J.B.B. contributed substantially to interpreting the data and developing the manuscript, and take full responsibility for the content of the paper.

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Data availability

RNA-Seq data are available in the NCBI Sequence Read Archive: Bio-project accession number PRJNA305720.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.137315.supplemental

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